Controlling Tobacco Mosaic Virus in Tobacco through Resistance

by

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Thesis submitted to the Faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Master of Science

In

Crop and Soil Environmental Sciences

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December, 2001

Blacksburg, Virginia

Key Words: N gene, virus movement, germplasm

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ABSTRACT

Tobacco mosaic virus (TMV) infects all classes of tobacco (*Nicotiana tabacum* L.) and causes losses worldwide. The N gene is the most effective means of controlling TMV; however, this gene is associated with reduced yield and quality in flue-cured tobacco. The mode of inheritance of TMV resistance was determined in two tobacco introductions (TI) from *N. tabacum* germplasm, both of which produced a hypersensitive response when inoculated with TMV. Inheritance studies with TI 1504 and TI 1473 indicate that a single dominant gene controls resistance. The gene governing resistance in TI 1504 is allelic to the N gene in NC 567. The gene providing resistance in TI 1473 is not allelic to the N gene, providing a potentially new source of resistance. Currently, plant breeders must rely on the N gene. The N gene is used in the heterozygous state to help overcome poor agronomic effects associated with homozygous resistance; however, systemic movement of TMV is occasionally seen in resistant plants. A TMV susceptible inbred (K 326), a resistant inbred (NC 567), and three resistant hybrids (NC 297, RGH4, and Speight H2O) were inoculated with TMV at transplanting, layby, and topping using different inoculation methods. Plant parts were tested for viral presence and biological activity. Viral movement into all plant parts was observed in K 326. No systemic movement was evident in the plant parts of NC 567, while virus did move into the corollas, pistils, late season sucker growth, and roots of the resistant hybrids showing systemic necrosis.

ACKNOWLEDGEMENTS

I give my sincere thanks and appreciation to many individuals who provided guidance throughout this project. Thanks go to Dr. Carol Wilkinson, a wonderful advisor who guided me in the right directions so that I may become a better researcher. I thank Dr. Charles Johnson, who always took time to answer my questions. Dr. Sue Tolin, who taught me more about TMV than I will ever know and who made me use my mind to its limits, I give great thanks. And thanks go to Dr. Glenn Buss for offering his guidance toward this project.

The faculty, staff, and technicians at Southern Piedmont Agricultural Research and Extension Center provided invaluable help and support. Special thanks go to Mr. Danny Powers, who managed the greenhouse project in my absence, and Margaret Kenny who provided answers to the many computer questions. The following individuals worked closely with this project, offering invaluable assistance: Dr. James Jones, Joanne Jenkins, Katherine Jones, Brent Forrester, Jenny Crowder, Margaret Coates, Jennifer Bricker, Stephen Flippen, and Mac Tilson. In addition, thanks go to RJ Reynolds for funding this project.

I would like to thank my family for their loving support. Sincere gratitude go to my mom, Nancy Bagley, and my dad, Grayson Bagley, who encouraged and supported me every step of the way. Thanks go to my sisters, Beth and Ashley Bagley, for offering their interest and friendship. My fiancée, Katherine Jones, provided my source of strength through this project. She gave unselfishly of herself many hours of encouragement and labor towards my dream. I could not be blessed with a more wonderful family, the same family who listened to all my ups and downs, and because of them I never faltered.

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Introduction

Tobacco mosaic virus (TMV) infects commercially grown tobacco (*Nicotiana tabacum* L.) worldwide, lowering both yield and quality. The RNA plant virus is mechanically spread and causes a mottling pattern of light and dark green areas to develop on the infected leaves. Good sanitation and cultural practices are the best means of preventing the disease. Resistance provides the best means of TMV management once the disease infects a crop.

The N gene is the most widely used gene conferring resistance to TMV. The N gene was transferred into *N. tabacum* from *N. glutinosa*. Once a plant containing the N gene is inoculated with TMV, a hypersensitive reaction occurs at the viral points of entry. The interaction of the N gene product with the TMV virus causes the plant to kill its own cells at any point where virus entered. Subsequently, lesions form on any inoculated leaf, restricting the movement of the virus to other plant parts and more importantly, to other plants.

The N gene is effective in burley tobacco and keeps the incidence of TMV very low. The N gene has also been transferred to flue-cured tobacco, but plants containing the gene generally have lowered yield and quality. There may be negative linkage factors associated with the N gene that are very hard to break in flue-cured tobacco.

The N gene has been incorporated into flue-cured tobacco in the homozygous as well as the heterozygous state. Cultivars deriving resistance from the N gene in the heterozygous state also have reduced yield and quality, but the effect is not as great as in plants containing the N gene in the homozygous state. Plant breeders have begun to develop more tobacco hybrids that incorporate the N gene in the heterozygous state so that TMV may still be controlled, but with fewer deleterious agronomic effects.

The N gene in the heterozygous state may be the answer for farmers who need to plant tobacco in fields where high incidences of TMV are known to occur.

The N gene induces a very reliable and satisfactory defense mechanism against TMV; however, the disadvantages to using the N gene have not been completely overcome. Identification of TMV resistance in *N. tabacum* germplasm may be less likely to have negative linkage traits associated with resistance. Chaplin and Gooding (1969) screened tobacco introductions from the *N. tabacum* germplasm and found some accessions that induce a hypersensitive response when inoculated with TMV. The N gene may be responsible for controlling the resistance found in these tobacco introductions, or a different resistance source altogether may be responsible.

Chapter 2

Literature Review

Tobacco mosaic virus

Tobacco mosaic virus (TMV) is an economically important disease infecting tobacco (*Nicotiana tabacum* L.) and other *Solanaceous* crops worldwide. TMV infects 199 different species from 30 families; however *Solanaceous* crops incur the most dramatic losses from the disease. Commercially grown tobacco in Virginia includes flue-cured, burley, dark fire-cured, and sun-cured tobacco. Flue-cured and burley are the most widely grown with losses incurred from TMV most devastating in flue-cured tobacco; all currently grown burley tobacco is resistant.

Tobacco mosaic virus is a very difficult disease to control because it is spread so easily. The disease is mechanically transmitted, resulting in quick and effective infection. Once a susceptible plant is infected, symptoms show up in 7 to14 days past infection (dpi). The first symptom to occur in newly infected plants is vein clearing. A plant exhibiting vein clearing allows the veins in a leaf to be more clearly seen. The vein clearing is seen in the new upper leaves and can be seen more clearly at 40°C than 25°C (Dawson, 1999). No known mechanisms for vein clearing have been reported.

Soon after vein clearing, mosaic symptoms occur in the newer leaves. This mottling consists of irregularly shaped dark green areas of tissue surrounded by light green areas of tissue. The dark green areas are called green islands and contain no virus, while the lighter green areas have virus (Dawson, 1999). The young leaves infected by TMV often are deformed and wrinkled. TMV infection may also cause the production of nonviable seed.

Even though mosaic symptoms are only seen in the new growth of a plant, virus movement is not restricted to symptomatic leaves. Virus has been found in the roots, leaves, and corollas of susceptible plants even when no visible symptoms are seen on the

plant part sampled (Allard, 1914). Likewise tobacco mosaic virus is also known to move into the stamens and pistils of plants infected with the disease (Allard, 1915).

The TMV particle

Tobacco mosaic virus is a 300 nm rod shaped virus belonging to the Tobamovirus genus of viruses. The virus contains a single positive sense strand of RNA, 6395 nucleotides long (Goelet et al., 1982). The 2165 coat protein units that make up the TMV rod protect this RNA strand.

The RNA genome encodes up to four proteins that enable the virus to infect and replicate. The first two proteins are the 126 K protein and the 183 K protein. Both of these are replicase proteins that allow the virus to replicate itself. The 126 K and the 183 K protein are translated straight from the RNA genome. The 183 K protein is an extension of the 126 K protein and is translated by a read-through of the 126 K protein's stop codon. Viral constructs containing only the 126 K replicase gene do not infect plants while the 180 K protein by itself will infect plants; although to a lesser extent than wildtype virus (Ishikawa et al., 1986). Therefore, the 180 K replicase protein is required, but both proteins are needed at regulated ratios for full infectivity.

The virus also encodes a 30 K movement protein that is translated from a subgenomic messenger. This protein is responsible for allowing cell to cell movement of the TMV virus, also called local movement. When the intact TMV particle enters a cell, it disassembles by cotranslational disassembly (Wilson, 1984) and begins to replicate. Translation of the movement protein is also taking place. The translated movement protein binds a disassembled TMV RNA genome and moves it through the plant's plasmodesmata to neighboring cells. The movement protein is then phosphorylated, which allows the TMV RNA to be translated so that a new infection may occur in the cell (Karpova et al., 1999).

TMV encodes a fourth protein, the 17.5 K coat protein. Each virus particle is made up of 2165 coat proteins. The virus translates thousands and thousands of these

coat proteins from a subgenomic message (Hunter et al., 1976) so that it can form new virus particles. During virus assembly, the coat proteins form aggregates called 20S disks (Butler, 1999). Each 20S aggregate is a two layer disk consisting of 17 coat proteins that looks like a washer, and enables faster assembly. RNA is associated with these disks and the disks stack one upon another to form a new particle and encompass the viral RNA. The coat protein is also necessary for long distance movement. While the movement protein moves the RNA from cell to cell, a fully assembled virus is needed for movement through the vascular system of infected plants.

TMV effects on tobacco

Due to the easy spread of TMV, infection can be established quite quickly. Reductions in yield and quality are attributed to TMV infection. Valleau and Johnson (1927b) reported a 60% loss in crop value due to TMV infection. TMV was estimated to cause a loss of one million dollars each year between 1960 and 1965 in North Carolina's flue-cured tobacco (Gooding, 1969). In 2000, TMV caused an estimated 1.4% loss in North Carolina's tobacco yield resulting in a 10.7 million dollar loss (Melton et al., 2000).

Several experimenters have shown relationships between the time of infection and the severity of TMV infection. Chaplin (1964) and Wolf and Moss (1933) concluded virus infection of younger plants results in a greater yield loss than infection of older plants. Chaplin reported up to a 20% loss when infection occurred at transplanting, but only 17% loss of yield when older plants were infected. Wolf and Moss observed a 31% loss in yield at transplant infection and only a 17% loss of yield when infected at topping.

Later experiments observed reductions in yield, grade index, and value averaging 13%, 16%, and 16%, respectively (Johnson et al., 1983). However, even though TMV caused yield reductions, no differences in yield were observed between different times of inoculation. Johnson et al. (1983) reported that more emphasis should be put on the disease incidence as a means of determining yield and quality reductions as opposed to time of inoculation. A field that has a higher incidence of TMV will result in a tobacco

crop with a greater reduction in yield. The reduction in yield and quality associated with resistant cultivars may not be as high as the reduction in yield observed from a field of susceptible cultivars with a great TMV incidence. In fields where TMV incidence levels exceed 37%, even after proper sanitation practices, resistant cultivars may be the best method of control for farmers (Johnson and Main, 1983).

TMV Control

Primary infection control

In order to help prevent TMV infection, it is necessary to know all sources of potential inoculum. Any overwintering source of TMV infection is referred to as 'primary' inoculum. Certain weeds can harbor TMV and thus serve as overwintering sources of primary inoculation. Horsenettle (*Solanum carolinense* L.) is one of the weeds found in the southeastern United States known to harbor TMV(Gooding, 1969). Controlling these weeds around tobacco fields helps to minimize potential TMV infections.

TMV can survive within infected plant debris located in and on the soil. Any infected soil debris from previous years that comes into contact with healthy plants can transmit the disease resulting in primary infection (Gooding, 1969). This infected soil debris consists mainly of root material, but may also include old stalks and leaves. Once the debris completely degrades, then the virus does not live very long in the soil, probably due to desiccation. Fields should be plowed and disked to promote decay of debris. Crop rotation is also very important. Crop rotation allows plant debris more time to break down and thus rid the soil of any remaining TMV. However, the rotational crop should not belong to the *Solanaceous* family, such as pepper or tomato. These crops are also vulnerable to infection.

Secondary infection control

Most TMV infection occurs from primary infected plants causing other TMV infections (Gooding, 1969). Any spread other than overwintering spread is referred to as 'secondary' spread. The most effective means of controlling secondary spread is through

proper sanitation. Sanitation can be applied at three levels consisting of greenhouse sanitation, equipment sanitation, and worker sanitation.

Commercial tobacco production involves growing tobacco transplants in styrofoam trays that are floated on a nutrient solution in a greenhouse. A lawn mower is used to clip the plants in order to improve plant uniformity and to stiffen up the transplant stalks so that the plants can withstand transplanting. However, if one plant is infected and then clipped, the infected plant serves as a source of inoculum for the entire greenhouse. The mower should therefore always be washed with a 1:1 bleach:water solution before use to minimize the spread of TMV among transplants (Reed et. al., 2000).

Sanitation in the field can also help minimize TMV infection. Farm equipment constantly brushes against tobacco plants throughout the growing season. An infected plant can thus serve as a source of inoculum across an entire field when contaminated farm equipment comes into contact with healthy plants. Therefore, all farm equipment should always be cleaned thoroughly before going into another field to minimize TMV infection.

One of the most important sanitation practices is from the worker themselves. Workers who have TMV particles on their clothing or hands may transmit the disease to tobacco plants. A common source of inoculum comes from commercial tobacco products such as snuff, chewing tobacco, and cigarettes. Any commercial tobacco product made from TMV infected plants can still transmit the disease (Valleau and Johnson, 1927a). TMV is very stable and can survive the curing process. Workers should not use any commercial tobacco products during transplanting when TMV infection can be most detrimental and should wash their hands well before handling any tobacco.

Plants infected from secondary sources may also be rogued out. However, rogueing is not always feasible. Gooding (1969) devised the general guideline establishing rogueing as feasible if fewer that 41 plants/ha show symptoms within 3 to 5

wk after transplanting. Rogueing should be carefully executed so as not to touch an infected plant against a healthy plant.

There is no currently effective chemical control. Milk can provide some protection against TMV infection (Apple et al., 1963). Milk is applied to susceptible plants before inoculation as a preventative measure. However, the use of milk is not very feasible in a farm setting.

Resistance to TMV

Good managerial practices as well as proper sanitation can help prevent the primary and secondary spread of TMV. However, once a plant becomes infected, it will always harbor the virus. No measures exist to eliminate TMV once symptoms are present besides rogueing. An effective means of control, once TMV comes into contact with a plant can only be accomplished through resistance. Plant breeders and scientists have worked for years to develop resistant plants for various diseases. Through the years, various genes and forms of resistance have been found and applied to TMV.

Ambalema type resistance

Nicotiana tabacum is grown all over the world for commercial tobacco production. Yet due to TMV infections, yield and quality can be dramatically reduced in infected fields. However, when touring Columbia, South America in 1929, J. Nolla noticed two *N. tabacum* cultivars that did not show symptoms when inoculated with TMV. These cultivars were taken to Puerto Rico, grown, and inoculated with TMV where the cultivar Ambalema showed no symptoms (Nolla and Roque, 1933). Tentative reports concluded that two recessive genes controlled resistance (Kentucky Agr. Exp. Station, 1937); a finding later verified by Clayton et al. (1938). Additional studies also reported possible modifying genes involved in this form of resistance, which further contributes to the difficulty in developing resistant cultivars (Clayton et al., 1938). The first source of resistance to TMV had been discovered, giving plant breeders the resources necessary to begin developing resistant cultivars.

Fifteen years of using Ambalema as a resistance source to TMV gave very unsatisfactory results. Plants which received the resistance genes would wilt and scald very severely on hot days (Valleau, 1952). The severity of wilting was bad enough that plants would never fully recover from the scalding.

The Ambalema resistance also has other drawbacks. In the initial screening of the Ambalema plants, four of the initial 600 Ambalema plants expressed mosaic symptoms and some of the other plants also showed vein-clearing (Nolla and Roque, 1933). Even though the Ambalema plants are symptomless, virus is still present. The Ambalema variety does not give a true resistance because the virus is still replicating, although the resistance genes do seem to inhibit the rate of multiplication. The inhibition of the rate of replication results in no TMV symptoms (Valleau and Diachen, 1941). Even though the infection process is much slower in plants with the two recessive genes, virus does invade the plants' tissue.

Chaplin and Gooding (1969) have screened other symptomless lines of tobacco. These lines still contain some virus in the leaves. Some of the symptomless lines evaluated by Chaplin and Gooding (1969) showed sunscald like the Ambalema plants. These other symptomless lines may have the same resistance genes as Ambalema or may contain different genes. Regardless, the leaves still contain virus and can serve as an inoculum source for susceptible cultivars. Due to severe wilting and the fact that symptomless plants are not virus free, Ambalema and symptomless plants are undesirable as good sources of resistance.

Nicotiana glutinosa derived resistance

Nicotiana tabacum was screened for resistance since this species comprises all commercially grown tobacco. However, other species of *Nicotiana* were also evaluated in the early 1900s. Allard (1914) showed that *N. glutinosa* did not develop mottling symptoms when inoculated with TMV, but instead developed a 'rot' that stemmed from the point of infection.

Holmes (1929) also concluded that other species of tobacco did not give the same response as *N. tabacum* to TMV infection. Holmes (1929) selected seventeen different *Nicotiana* species and inoculated the plants with TMV in order to observe the symptoms. All species showed mosaic symptoms except for *N. rustica, N. langsdorffi, N. acuminata, N. sandera,* and *N. glutinosa*. These five species all gave a hypersensitive response (HR) on the leaves that were inoculated.

A hypersensitive reaction is characteristic of resistance to viruses. The plant tissue around the virus point of entry dies, causing a small lesion of dead tissue, which prevents the virus from spreading systemically through the plant. The hypersensitive response may be a two step process in which a necrotic lesion is quickly formed, killing the majority of the virus, and then surviving virus at the edge of the lesion is eventually also killed (Wright et al., 2000). When a necrotic lesion is formed due to the presence of infected plant cells, the plant's xylem is also greatly restricted, which may be a primary factor in keeping virus from spreading to the rest of a resistant plant.

Holmes (1929) identified a different variation of the hypersensitive response when he inoculated the five different species of *Nicotiana*. *Nicotiana glutinosa* gave the smallest, most uniform, and quickest appearing local lesion while the other species gave larger and less uniform lesions. *Nicotiana glutinosa* was used as the new standard in quantifying tobacco mosaic virus resistance through the local lesion assay. Holmes (1929) was also the first to demonstrate that with the local lesion assay, the number of lesions, is directly correlated to virus concentration.

Holmes (1938) concluded that the resistance found in *N. glutinosa* was controlled by one dominant gene designated as **N**. One dominant gene, such as the N gene, is much easier to work with than the two recessive genes found in the *N. tabacum* cultivar Ambalema. The problem of transferring the N gene into *N. tabacum* remained, since a natural cross between two different species is not possible unless the two species have the same chromosomal number. *Nicotiana tabacum* contains 48 chromosomes while *N. glutinosa* contains 24 chromosomes. Therefore, Clausen and Goodspeed (1925)

performed an interspecific bridge cross, successfully creating a *N. glutinosa-tabacum* hybrid. Goodspeed named the new hybrid *N. digluta;* a new tetraploid species created through science.

Holmes (1938) crossed the new *N. digluta* species with *N. tabacum* and finally developed a *N. tabacum* line, Samsoun, that was homozygous resistant for the N gene. The new line Samsoun, showed a hypersensitive reaction in response to TMV inoculation, resulting in the transfer of the N gene from *N. glutinosa* to a cultivar of flue-cured tobacco. Gerstel (1943) concluded that an entire *N. glutinosa* chromosome containing the N gene had been exchanged for a *N. tabacum* chromosome.

Development of Samsoun demonstrated that the N gene had been successfully transferred to *N. tabacum* and these plants gave a hypersensitive reaction when inoculated with TMV. The Kentucky Agricultural Research Station also used *N. digluta* to help develop burley and dark fire-cured homozygous resistant tobacco cultivars (Valleau, 1952).

The N gene proved to be a sufficient means of resistance in controlling TMV; the resistance was much better than the Ambalema type resistance. The virus was sufficiently contained so that the virus could not spread from one plant to another. Many breeding programs completely dropped working with Ambalema and started working with the N gene.

Many trials were conducted to determine the effects of the N gene on tobacco. The N gene was transferred by a chromosome substitution resulting in possible negative linkage associated with the N gene. Burley was evaluated using Burley 21, homozygous resistant for the N gene, and eight other susceptible cultivars (Legg et al., 1979). These eight susceptible cultivars were crossed to Burley 21 and then backcrossed seven times. Resistant and susceptible plants from each of the eight burley cultivars were evaluated along with the recurrent parent for numerous agronomic and chemical traits. Legg et al. (1979) found that no consistent negative results could be found for any agronomic traits

studied due to the transferred N gene. Quality of tobacco was also not affected. The N gene had been successfully transferred to burley tobacco without any negative effects and complete resistance had been observed. All current commercially grown burley cultivars contain TMV resistance derived from *N. glutinosa*.

Flue-cured tobacco was also evaluated to understand any effects potentially associated with the introduction of the N gene. Virginia 45 was used as the TMV resistant flue-cured cultivar (Chaplin et al., 1961). Virginia 45 was developed by the Virginia Agricultural Research Station (Keller, 1958), deriving the N gene from Holmes' Samsoun. Nine susceptible cultivars were used as recurrent parents; each cultivar was crossed with Virginia 45. Backcrosses were conducted and the resulting resistant and susceptible cultivars were compared to the recurrent parent. Various agronomic as well as quality traits were characterized. Chaplin et al. (1961) showed that when Virginia 45 was used as a resistant source, yields in all nine susceptible cultivars were reduced by an average of 148 kg/ha. This yield reduction suggested negative linkage was either associated with the N gene when incorporated into flue-cured tobacco or was a result of the N gene itself.

TMV resistant burley tobacco deriving its resistance from the N gene has no negative attributes, while TMV resistant flue-cured tobacco is associated with yield depression and quality loss. One possible method of overcoming the negative attributes associated with flue-cured tobacco is to transfer the N gene from burley tobacco to flue-cured tobacco. It seems probable that the N gene used in the initial transfer of flue-cured tobacco was linked to negative attributes, while the N gene used in burley was not. Therefore, using the N gene from burley to transfer resistance into flue-cured tobacco may prove beneficial in alleviating the negative effects seen in flue-cured tobacco. Burley 21 was used as a resistant source for crosses to six flue-cured TMV susceptible cultivars (Chaplin and Mann, 1978). Backcrosses were conducted to each of the six recurrent parents. Resistant selections, susceptible selections, and the recurrent parent were all compared for chemical and agronomic traits. The results were very similar to

the earlier study using Virginia 45 as the flue-cured resistant source showing the six TMV resistant families had reduced yield and quality losses (Chaplin and Mann, 1978).

The N gene gives very satisfactory resistance, but not satisfactory agronomic and quality traits in flue-cured tobacco. In breeding programs, the N gene was being used in the homozygous state. However, Chaplin et al. (1966) evaluated the N gene in the heterozygous state. Six susceptible flue-cured cultivars were crossed to a resistant parent. After backcrossing, resistant families from each recurrent parent were developed. These resistant plants from the backcross generation were selfed and then crossed back to the recurrent parent for a resistant F_1 hybrid. Backcrossed selfed plants were homozygous (NN) while the hybrids were heterozygous (Nn) for TMV resistance. The homozygous, heterozygous, and recurrent parents were once again evaluated for agronomic and chemical performance. Many of the families showed a reduction in yield when the N gene was incorporated. The heterozygous state showed intermediate variation from the recurrent parent and the homozygous state. The heterozygous state contains only one copy of the partial *N. glutinosa* arm resulting in a less deleterious effect than the double copy of the *N. glutinosa* gene.

In order to determine whether the N gene itself was the cause of poor agronomic characteristics in flue-cured tobacco, Wernsman (personal communication) transformed K 326 plants with the cloned N gene. The plants that were transformed with the N gene were compared to interspecific K 326 lines homozygous dominant, heterozygous, and homozygous recessive for the N gene. The K 326 lines deriving resistance interspecifically arose from crossing K 326 with Coker 51 and repeatedly backcrossing. The interspecific lines containing the N gene showed reduced yield compared to the control, with the homozygous dominant plants showing more reduction than the heterozygous plants. These results confirm Chaplin et al. (1961) results. In contrast, the transformed K 326 plants containing only the cloned N gene yielded the same as non-transformed K 326. Some of the transformed lines also received more than one copy of the N gene and still did not significantly differ in yield from non-transformed K 326. When the N gene is transferred interspecifically, reductions in yield and quality are

observed. In contrast, there is no observational reduction in yield and quality when flue-cured tobacco is transformed with the N gene. This suggests that the N gene itself does not cause poor agronomic effects, but something that is linked to the N gene in its transfer into flue-cured tobacco is causing the reduced yield and quality.

The N gene in the heterozygous state gives plant breeders a new tool in plant breeding. F_1 hybrids have started to become standard for many crops and tobacco is no exception. Many hybrids offer the advantage of hybrid vigor. F_1 hybrids also are male sterile so that at the end of the growing season, no viable seed is produced. Several TMV resistant hybrids have been developed in recent years. These hybrids include RGH4, NC 297, Speight H20; all of which contain the N gene in the heterozygous state. In preliminary tests, yields seem to be very good, however, farmers have not had access to the cultivars very long. Only time will tell if these new hybrids will be used year after year by the farmer.

There is one potential problem with using the N gene in the heterozygous state. A single dominant gene, such as the N gene, can potentially be overcome by the pathogen. Many single genes throughout the years have been defeated, resulting in disease even in the presence of a resistance gene. The N gene has seen selection pressure in *N. tabacum* since being first introduced by Holmes in 1938. The gene is still holding up and gives full resistance through a hypersensitive reaction, strongly supporting the fact that the N gene is a very stable resistance gene. As the new hybrids introduce the N gene in the heterozygous state, it becomes much easier for the pathogen to overcome resistance. In the case of single resistance genes, it is imperative in a breeding program to find other genes conferring resistance. When these genes are pyramided, resistance becomes much harder to break.

The N gene

The N gene induces a hypersensitive response (HR), a very dependable form of resistance against viral pathogens. The HR has been studied in great detail and many details about this gene and its response have been investigated. The N gene is

temperature sensitive, providing a hypersensitive response at temperatures lower than 32°C and allowing the virus to move systemically at temperatures higher than 32°C (Richael and Gilchrist, 1999). A giant leap into understanding the molecular role of the N gene came when it became the first plant disease resistance gene ever cloned. The Activator (Ac) transposon (McClintock, 1948) in maize was used to isolate the N gene. Whitham et al. (1994) isolated the N gene by inserting the transposon into heterozygous N gene tobacco and isolating mutants caused by the insertion. Once mutants were found by a positive selection scheme, genomic DNA sequences flanking the transposon were used to create cDNAs containing the N gene sequence.

The isolation of the N gene enabled genomic analysis of the N gene. The N gene contains three domains. These domains are a leucine rich region (LRR), a nucleotide binding site (NBS), and a Toll/interleukin-1 receptor homology region (TIR) (Whitham et al.; 1994, Dinesh-Kumar et al., 2000). Other resistance genes also share the TIR-NBS-LRR motif, including genes for rust resistance, downy mildew resistance, and bacterial resistance (Dinesh-Kumar et al., 2000). Induced mutations in each of these motifs of the N gene have shown that the TIR, NBS, and LRR are all needed for the N gene to properly induce a HR (Dinesh-Kumar et al., 2000). Whitham et al. (1994) showed comparisons of the N gene using Genbank. The NBS region consists of three motifs including a P-loop, a kinase 2, and a kinase 3a. These motifs are known to bind ATP and GTP. The LRR region of the N gene consists of three imperfect repeats of approximately 26 amino acids. LRR regions found in other proteins are known to be involved in such functions as signal transduction and cell adhesion. The third domain identified is the TIR, which has 55% similarity to the Toll protein and 49% similarity to interleukin-1 (Whitham et al., 1994).

The N gene contains five exons, however, these exons can be alternatively spliced to give different variations of the N gene (Whitham et al., 1994; Dinesh-Kumar and Baker, 1995). The five exons of the N gene are spliced together resulting in a Ns transcript. The Ns transcript is translated into the N protein of 131.4 kD. An additional exon (AE) (Dinesh-Kumar and Baker, 2000) can also be spliced, resulting in a Nl

transcript. Since the AE results in a different reading frame, the newly translated protein is terminated earlier than the N protein resulting in a Ntr protein of 75.3 kDa.

Plants containing the N gene are always transcribing and translating the full N protein, whether virus is present or not. The purpose this protein may play, if any, in *N. tabacum* or *N. glutinosa*, is not known. The introduction of TMV particles into the plant however, changes the way the N protein is made. Dinesh-Kumar and Baker (2000) studied the ratio of N protein to Ntr protein. The first four hours after infection, the full length N protein is translated. The next three hours seem to be the most crucial in activating a defense response as the N protein switches from full length to the truncated N protein transcribed from the Nl transcript. The truncated protein is produced in higher numbers than the normal N protein three hours after inoculation. After the Ntr protein concentration plateaus at three hours, a switch again occurs favoring the N protein. This suggests that the N gene's mechanism of inducing a hypersensitive response after contact with TMV involves alternative splicing of the transcript.

The elicitor of the N gene was also a question leading to many investigations in order to determine what actually signals the different size N gene transcripts and proteins. Padgett et al. (1997) and Paggett and Beachy (1993) conducted initial experiments pointing towards the helicase region in the 126 K/180 K replicase protein as the elicitor. Confirmation of these results was found using transgenic methods. TMV cDNA's were used to produce transgenic tobacco using the *Agrobacterium* method. Some of these plants contained the N gene while others did not. The transgenic plants that expressed the helicase region of the 126 K protein in the absences of virus particles showed a hypersensitive response on the plants also expressing the N gene (Erickson et al.,1999). These data confirms previous work showing that the helicase region of the 126 K/180 K TMV replicase is the elicitor of the hypersensitive response.

The alternate splicing of the N gene provides the means for the plant to mount a hypersensitive response against TMV. The N gene product interacts with the TMV

helicase region, either directly or indirectly, and signals the alternate splicing of the N gene. The N gene then sets off a signal cascade resulting in regulated cell death.

N' gene

When susceptible *N. tabacum* plants are inoculated with TMV, a mottling effect is seen, while plants carrying the N gene give a hypersensitive response. When the 'ordinary' strain of TMV is inoculated on *N. sylvestris*, a different species of tobacco, the classic mottling symptoms are again seen. However when a 'mild' strain of TMV is inoculated onto *N. sylvestris*, a necrotic response was seen (Weber, 1951) and yet *N. sylvestris* does not have the *N. glutinosa* derived N gene. The gene in *N. sylvestris* was named the N' gene.

While the common strain of TMV does not induce a hypersensitive reaction, TMV mutants have been found to still show a necrotic response on *N. sylvestris* (Funatsu and Fraenkel-Conrat, 1964). Further mutational studies have shown that directed amino acid changes in the coat protein of TMV will cause a hypersensitive reaction on *N. sylvestris* demonstrating that the TMV coat protein is the elicitor of the N' hypersensitive reaction (Culver and Dawson, 1991).

The N' gene is a possible allelomorph of the N gene where the N gene is dominant over N' and the N' gene is dominant over n (Dunigan et al., 1987). The N' gene provides resistance in *N. tabacum* only to TMV mutants and other closely related TMV strains. The use of the N' gene in breeding for resistance is of little practical value.

Systemic Acquired Resistance

Many plants have a natural defense response when introduced to a disease that causes necrotic type infections. This systemic acquired resistance (SAR) uses salicylic acid as a signal to alert the plant to the pressure of a pathogen and to signal a defense response (Shulaev et al., 1995). Therefore any subsequent infections will be much milder than the initial infection. Inoculation of Xanthi tobacco, which gives a necrotic reaction when inoculated with TMV, showed a higher level of salicylic acid production than non-

inoculated plants (Shulaev et al., 1995). When resistant Xanthi plants are inoculated with TMV a second time, the lesions are fewer and smaller when compared to the first inoculation. When the leaves containing lesions are reinoculated, an area of no lesion appearance will be seen around the initial lesions.

However, plants must first be infected before the SAR will be induced. There is a potential for new chemical development using this resistance mechanism. Any chemical that could actually jumpstart SAR could protect a plant from infection. The purpose of the chemical is so that the plant is already expecting the disease. A plant will be resistant to the first exposure to a disease once the chemical has been applied. Chemicals such as Actigard are now commercially available that strengthen the plant defense system to a broad range of diseases, such as blue mold. Experiments have shown that SAR does occur with TMV, so there is a chance for chemical development protecting against TMV.

Transgenically Induced Resistance

Coat-protein mediated resistance

Transgenic techniques have also been applied to try and develop TMV resistant cultivars. The *Agrobacterium* method was used to construct transgenic tobacco plants that produce TMV coat protein (Powell et al., 1986). When these plants were inoculated with TMV, symptom development was delayed, with some of the transgenic plants never displaying symptoms. Transgenic tomato plants encoding the TMV coat protein were also constructed and were found resistant to TMV while still giving high yields (Nelson et al., 1987). This resistance was named coat-protein-mediated-resistance (CP-MR).

Plants already containing the N gene from traditional breeding were also transformed, as well as plants not containing the N gene. The coat protein positive transgenic plants also containing the N gene produced fewer lesions than plants not transgenic for the coat protein but still carrying the N gene (Nelson et al., 1987). The coat protein made transgenically is somehow interfering with TMV infection in both susceptible and resistant plants. This resistance is either induced from the coat protein transcript or from the actual coat protein itself.

Several experiments were conducted to determine if the transcript or the coat protein were the resistance factor in CP-MR. One experiment involved the removal of the intiation codon from the coat protein transcript (Powell et al., 1990). Since the initiation codon was removed in the transgenic plants, the transcript was produced, but never translated. No coat protein was produced and no resistance was observed. Another experiment involved the observation that at higher temperatures CP transcript was made, but the coat protein was not translated (Nejidat and Beachy, 1989). The transgenic plants were inoculated at a higher temperature and no resistance was observed. When the temperature was lowered so that coat protein was produced, the resistance was once again seen. These experiments concluded that the presence of coat protein within plant cells is the responsible factor in CP-MR.

The coat-protein-mediated resistance involves the transgenic coat protein interacting with the challenge virus in a way that delays infection. The transgenic coat protein interactions with each other also govern the strength of resistance, as seen through mutational studies. When the transgenic coat protein is mutated in such a way that its CP-CP interactions are strengthened, then resistance is also stronger (Bendahmane et al., 1997). Whereas in the same experiment, mutants were also constructed which had weaker CP-CP interactions and the resistance was poor. In order for resistance to take place, the transgenic CP must interact in certain ways.

Another interesting aspect of CP-MR involves the type of inoculum. CP positive transgenic plants are resistant when inoculated with TMV particles, but when TMV-RNA is used as the source of inoculum, susceptible and resistant plants transformed with the CP gene show only minimal resistance to TMV infection. Once infection occurs, CP-MR provides less resistance to cell-to-cell local movement, which requires only TMV RNA, than to systemic movement, which requires the entire particle (Wisniewski et al., 1990). Since CP-MR is ineffective against TMV-RNA inoculation, cell-to-cell movement is not stopped.

The fact that transgenic plants containing coat protein that are inoculated with TMV-RNA are not resistant helps explain the mechanism of CP-MR. Since inoculation with TMV-RNA does not provide resistance, but an intact virus inoculum does, the resistance mechanism is initiated prior to disassembly of the TMV virus. One model predicts that the transgenic coat proteins are interacting with the challenge virus coat proteins to prevent disassembly of the TMV virion (Bendahmane et al., 1997). As TMV disassembles, the transgenic coat protein reencapsidates the virus and prevents further disassembly. The stronger CP-CP interactions of the transgenic CP result in stronger encapsidation, and thus the stronger the resistance. Transgenic CP which do not form strong CP-CP interactions with each other, cannot form strong interactions when reencapsidating the TMV particle and thus have weaker resistance. Since the transgenic coat protein must interact with the challenge virus, similarity between the transgenic coat protein and virus coat protein result in stronger resistance (Nejidat and Beachy, 1989). Therefore, CP-MR is effective against TMV and other tobamovirues, but the effectiveness of resistance is lost as homology between the transgenic coat protein and challenge virus coat protein is reduced.

Replicase mediated resistance

Tobacco mosaic virus encodes four known proteins. A fifth open reading frame encoding the 54 K protein has also been found within the frame containing the 183 K replicase protein (Sulzinski et al., 1985). The protein has several polymerase motifs; however this putative protein has never been found in vivo.

The question of the function of this protein was sought after by many. With the introduction of transgenic methods, a transgenic plant encoding the 54 K protein could be constructed. Golemboski et al. (1990) transformed tobacco plants with the 54 K protein to determine the role of the elusive 54-K protein. The tobacco plants were analyzed and a protein transcript was found, however, still no protein could be detected. The transgenic plants were successful in producing 54 K transcript, but 54 K protein still remained elusive. The transformed plants were inoculated with TMV for observation and the inoculated plants were resistant!

The resistance from TMV inoculation with the 54 K protein may result from the protein itself or from the actual transcript. CP-MR results from the protein and not the transcript, yet no 54 K protein was detected in the transgenic plants. The fact that no 54 K protein was detected implies that the 54 K transcript is responsible for resistance unlike the CP-MR. The 54 K protein may be produced at undetectable levels, in which case the 54 K protein may compete with the 183 K replicase in providing resistance.

Replicase mediated resistance has several differences from CP-MR. While CP-MR is only effective against intact virions, replicase mediated resistance is effective against intact virions and TMV-RNA (Carr and Zaitlin, 1993). Also, replicase mediated resistance does not lose its efficacy as does CP-MR when high concentrations of inoculum are used (Carr and Zaitlin, 1993). However, replicase mediated resistance is strain specific so that protection is only given against the same strain from which the 54-K transgene was originally derived (Carr and Zaitlin, 1993).

New sources of resistance

For the past 100 yr, controlling TMV has proved rewarding and frustrating at the same time. Many forms of control have been found, yet all seem to have drawbacks, which result in yield and quality losses worldwide from TMV infections. The only proven resistance gene to date is the N gene derived from *N. glutinosa*. However due to poor agronomic characteristics in flue-cured tobacco, cultivars containing the gene have not received farmer acceptance. The N gene in the heterozygous form is being used to produce hybrids that yield well; however, the one dominant gene has the potential for breakdown.

The transgenic means of resistance seem promising, however, they still have many trials before commercial cultivars are released. The CP-MR and the replicase mediated form of resistance also only delay symptoms against TMV infection, so that complete resistance is not achieved. The consumer must also accept these transgenically produced resistance plants as ethical, since many people harbor reservations towards transgenic plants.

Other forms of resistance to TMV could greatly help improve TMV control. Chaplin and Gooding (1969) screened tobacco introductions (TI) within the *N. tabacum* germplasm collection. Many entries were found to be susceptible, some were found symptomless, and others gave a hypersensitive response. The authors concluded that the hypersensitive response was due to the presence of the N gene in the plant introductions.

Beekwilder (1999) studied TI 1500, which gives a hypersensitive reaction to TMV inoculation. An inheritance study and allelism study found that resistance in TI 1500 was due to a single dominant gene, but the gene was not allelic to the N gene found in NC 567. Other TI lines potentially contain new and desirable TMV resistance genes.

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Figure 1. Mosaic symptoms from TMV inoculation of a susceptible leaf (top) and a TMV induced hypersensitive response on a resistant leaf (bottom).

Chapter 3

Inheritance of Tobacco Mosaic Virus Resistance in Tobacco Introductions 1504 and 1473

Abstract

Tobacco mosaic virus (TMV) is a helical, positive sense RNA virus that reduces yield and quality in all classes of commercially grown tobacco (*Nicotiana tabacum* L.). Resistant genes provide the best means of controlling the disease. The objectives of this study were to determine the inheritance of resistance in two tobacco introductions (TI) and to determine allelism of these sources to the N gene. Two TI lines (1504 and 1473) were each crossed to a susceptible parent (K 326) and to a TMV resistant parent (NC 567). The susceptible by resistant cross was used to determine how many genes control resistance, while the resistant by resistant cross was used to determine allelism with the N gene. The F_1, F_2, and $F_{2:3}$ generations were inoculated with TMV and visually examined for susceptibility or resistance. Chi-square analysis was used to determine genetic fit. Resistance in TI 1504 was controlled by a single dominant gene and this gene was allelic to the N gene. Resistance in TI 1473 was also controlled by a single dominant gene, but the gene was not allelic to the N gene, providing a potential new source of TMV resistance.

Introduction

Tobacco mosaic virus (TMV) is a RNA plant virus that affects *Solanaceous* crops including tobacco. This virus causes a mottling effect of light and dark green areas on the plant and may also result in stunting as well as aborted seed. The virus is mechanically spread and may quickly spread to an entire field of tobacco resulting in a loss of tobacco yield and quality.

Resistance remains one of the most effective means of controlling TMV. The first source of resistance came from the *N. tabacum* cultivar Ambalema (Nolla and Roque, 1933). Two recessive genes with modifying factors controlled the resistance

(Clayton et al., 1938) and the use of these genes resulted in plants that wilted severely on hot days.

A different source of TMV resistance came from *N. glutinosa*. Holmes (1929) inoculated different *Nicotiana* species with TMV and found that inoculation induced a hypersensitive reaction in several of the species instead of the usual mosaic symptoms. Among the species showing a hypersensitive response, *N. glutinosa* produced the quickest, smallest, and most uniform local lesions. The source of this resistance is a single dominant gene designated **N** (Holmes, 1938). The N gene was transferred into *N. tabacum,* but while the N gene provided effective control in burley tobacco (Legg et al., 1979), the N gene was associated with poor agronomic characteristics in flue-cured tobacco (Chaplin, 1961). Incorporation of the N gene into flue-cured tobacco cultivar K 326 by interspecific transfer resulted in reduced yield and quality, while K 326 plants receiving a cloned N gene transgenically do not show reductions in yield and quality (Wernsman, personal communication). These data suggest negative yield and quality observed in flue-cured tobacco is likely due to negative linkage associated with the N gene and not the N gene itself.

The N gene is currently the most used resistance gene for controlling TMV. However, due to the loss in yield and quality associated with cultivars incorporating the N gene, it is very important to find new sources of TMV resistance. Chaplin and Gooding (1969) screened the entire *N. tabacum* germplasm. These tobacco introductions (TI) were inoculated with TMV and some were susceptible, some were symptomless, and some gave a hypersensitive response. The objective of this study was to determine the inheritance of TMV resistance in two TI lines that were identified by Chaplin and Gooding (1969) to have a hypersensitive response when inoculated with TMV. These lines may be allelic to the N gene or may prove to be a completely different source of resistance.

Materials and Methods

Greenhouse experiments were conducted at the Southern Piedmont Agricultural Research and Extension Center (SPAREC) in Blackstone, Virginia. TI 1504 and TI 1473 were initially evaluated by Chaplin and Gooding (1969) and exhibited a hypersensitive reaction when inoculated with TMV. According to GRIN (Germplasm Resource Information Network), TI 1504 is a flue-cured type of tobacco donated by Taiwan. TI 1473 is a primitive class of tobacco donated by Venezuela. TI 1473 produced only 6.1% nicotine compared to 37.9% nicotine in TI 1504. The leaf green weight yield for TI 1504 is 848 kg/plant while TI 1473 only yields 147 kg/plant.

TI 1473 and TI 1504 were each crossed to the TMV susceptible cultivar K 326 to determine the mode of inheritance of resistance. To determine whether the gene(s) conferring resistance in either TI line is allelic to the N gene, a second cross was made between NC 567 and each TI line. NC 567 derives its TMV resistance from the N gene in *N. glutinosa* (Chaplin, 1969).

F_1 seed from TI 1504 X NC 567, K326 X TI 1504, K326 X TI 1473, and TI 1473 X NC 567 were sown in 10 cm clay pots filled with vermiculite. Seed of the parents were also sown in clay pots. Ten F_1 seedlings along with 40 seedlings of each parent were transplanted approximately two weeks after sowing into 11 cm Jiffy Pots (Jiffy Products N.B.I. LTD, Canada) filled with Carolina's Choice Tobacco Mix (Carolina Soil Company, North Carolina). Parents were also grown along with plants from each generation to serve as a control. Plants were fertilized twice a week with 125 mg/L N of 16-5-16 fertilizer. Once F_1 and parent plants reached the 4 to 6 leaf stage, a leaf on each plant was marked with a marking pen and inoculated with TMV. TMV inoculum was prepared by grinding 0.5 g of infected TMV tissue in 1% K_2PO_4 buffer with 1% Celite at a pH of 7 and diluting to 1:100. Inoculation was carried out by gently rubbing the marked leaf with a foam-tipped swab (Fisher, Suwanee, GA). Plants were visually identified as either giving a susceptible mottling symptom or a local lesion resistant symptom. Plants showing no symptoms were re-inoculated three weeks after initial inoculation and characterized.

F_2 seed was collected from seven of the F_1 plants for each of the four crosses. Seventy-five F_2 seedlings from each F_1 plant were grown in separate 10 cm clay pots for each of the four crosses. F_2 seedlings were transplanted into jiffy pots 2 to 3 wk after seeding and characterized as indicated above. After each F_2 plant was scored, the plant was transferred to a clay pot. The plants were maintained using an automatic watering system until seed was harvested from each F_2 plant.

$F_{2:3}$ seed acquired from the F_2 plants were randomly drawn from six of the F_2 families for each of the four crosses. Approximately 15 $F_{2:3}$ seed sets were drawn randomly from each F_2 family. There were 25, 50, 50, and 75 $F_{2:3}$ seedlings from TI 1504 X NC 567, K326 X TI 1504, K326 X TI 1473, and TI 1473 X NC 567, respectively, transplanted and inoculated as stated above. The number of seedlings grown in each $F_{2:3}$ family was based on the population size needed to successfully evaluate the expected ratios. Each $F_{2:3}$ plant was characterized as showing a hypersensitive response or a susceptible response and discarded. Data from the segregating F_2 and $F_{2:3}$ progeny families were analyzed using a chi-square analysis for a 3:1 and a 15:1 ratio.

Results and Discussion

Chaplin and Gooding's (1969) initial tobacco mosaic virus inoculation of TI 1504 and TI 1473 resulted in a hypersensitive resistant response (Table 1) and was confirmed in greenhouse tests at SPAREC. K 326 was susceptible to TMV infection and exhibited mosaic symptoms, whereas NC 567 exhibited local lesions. The same distribution of symptoms was seen in all cases where parents were inoculated as a control. The F_1 progeny of K 326 x TI 1504 and K 326 x TI 1473 each resulted in all resistant progeny (Table 2) which is expected if the resistance gene in TI 1504 and TI 1473 is dominant. The F_2 progeny segregated and were subjected to a chi-square analysis for a 3 local lesion:1 mosaic fit (Table 3). The individual F_2 families as well as the combination of all F_2 plants from all families fit a 3:1 resistant:susceptible ratio. This indicates that a single dominant gene controls resistance in both TI lines.

In order to support the conclusions drawn from the F_1 and F_2 data, $F_{2:3}$ progeny families were evaluated. $F_{2:3}$ progeny families were expected to fit into one of three different classes (Table 4). The F_2 plants that were susceptible were expected to remain susceptible in the $F_{2:3}$ families since susceptibility is present only in the homozygous condition. The F_2 plants carrying the resistant gene could either be in the homozygous or heterozygous state. Homozygous resistant F_2 plants did not segregate when selfed progeny were inoculated; all resistant plants were observed in the $F_{2:3}$ families. Plants which were resistant in the F_2 generation and in the heterozygous state gave segregating families fitting a 3 local lesion: 1 mosaic ratio in the $F_{2:3}$ generation. The resistant families that segregated were analyzed with a chi-square analysis and did fit the 3:1 ratio. The $F_{2:3}$ progeny families categorized into the three classes were also fit for a 1 (all resistant):2 (3:1):1 (all susceptible) ratio. However, the families did not fit this ratio based upon chi-square analysis. Many of the resistant F_2 plants in the greenhouse died from systemic necrosis after inoculation with TMV. Thus, a greater proportion of susceptible plants were carried forward into the $F_{2:3}$ generation and a greater number of all susceptible $F_{2:3}$ families were observed. A heterogeneity test was used to support the fact that no significant difference was observed among segregation ratios for the individual $F_{2:3}$ families segregating 3:1 (Tables 5 and 6). Therefore, the $F_{2:3}$ generation supports the claim that resistance in TI 1504 and TI 1473 is controlled by a single dominant gene.

TI 1504 was crossed with NC 567 in order to determine whether the single dominant resistance gene is allelic to the N gene in NC 567. The F_1 progeny of the allelism cross resulted in all resistant plants (Table 2). The F_2 progeny were also all resistant (Table 3). Since no segregation occurred in the F_2 plants, it can be concluded that the resistance gene in TI 1504 is allelic to the N gene in NC 567. The $F_{2:3}$ families derived from individual F_2 plants would not be expected to segregate and did not upon inoculation (Table 4). The $F_{2:3}$ data supports the conclusion that the N gene in NC 567 is the same gene responsible for governing TMV resistance in TI 1504.

A single dominant gene controls resistance in TI 1473 and an allelism study was also conducted to determine if the resistance gene in TI 1473 is the same as the N gene in NC 567. The F_1 progeny of the TI 1473 x NC 567 cross failed to segregate, showing a hypersensitive reaction to TMV (Table 2). The F_1 plants were carried forward to the F_2 generation and segregation was observed (Table 3). The ratios were tested with the chi-square method for a 3 local lesion : 1 mosaic fit and for a 15 local lesion : 1 mosaic fit. The 15:1 ratio fit gave the lowest value. From this segregation pattern, it was concluded that the resistance gene in TI 1473 and the N gene found in NC 567 are segregating independently. Thus either it is not the same gene or, if the same gene, it resides at a different chromosomal location.

To support this conclusion, F_2 plants were advanced to the $F_{2:3}$ generation. Since the F_2 plants segregated 15 resistant:1 mosaic (Table 3), the $F_{2:3}$ progeny families were categorized into one of four different categories (Table 4). The gene controlling resistance in TI 1473 is not allelic to the N gene in NC 567, so that more than one resistance gene should segregate in the F_2 and $F_{2:3}$ generations. One resistance gene is from TI 1473 while the other comes from NC 567. The $F_{2:3}$ progeny families will fall into four categories depending on the genotype of each selfed F_2 plant. One genotype involves both resistant genes in the homozygous recessive condition. Any selfed F_2 plant having this genotype does not segregate and all $F_{2:3}$ families will have mosaic symptoms. F_2 plants may also have a genotype in which both of the resistance genes are homozygous dominant. Likewise only one of the resistant genes could be in the homozygous dominant state while the other gene is either in the heterozygous or homozygous recessive state. F_2 plants which have one of these genotypes will result in all resistant plants in the $F_{2:3}$ generation and no segregation will occur. When the resistant F_2 plants have a genotype consisting of one resistance gene in the homozygous recessive condition and the other gene in the heterozygous condition, then the selfed progeny will segregate into a 3 resistant: 1 mosaic. The 15 resistant: 1 susceptible ratio seen in the $F_{2:3}$ generation is observed when the resistant F_2 plants exhibit a genotype consisting of both resistance genes in the heterozygous state. Chi-square analysis was used to determine the correct fit for $F_{2:3}$ plants from families that segregated 15:1 and 3:1. The $F_{2:3}$ families

categorized into the four categories were also analyzed for a 7 (all resistant) :4 (3:1): 4 (15:1): 1 (all susceptible) ratio. The $F_{2:3}$ progeny families did not fit this ratio. A heterogenity test was conducted on the $F_{2:3}$ families segregating 15:1 and 3:1. The heterogenity test demonstrated that all individual families fit the respective ratio and that no significant difference among families segregating for a ratio was observed. Therefore, the $F_{2:3}$ generation supports the conclusion that the single dominant gene controlling resistance in TI 1473 is not allelic to the N gene found in NC 567.

Conclusions

Tobacco mosaic virus continues to cause tobacco losses worldwide. Resistance has become the most effective defense mechanism for control of the disease. The N gene has been successfully transferred from *N. glutinosa* into *N. tabacum* cultivars. Historically, the commercially grown flue-cured tobacco cultivars deriving resistance from the N gene have poorer agronomic characters than do those cultivars not containing the N gene. These resistant cultivars have been predominantly inbred lines homozygous for the N gene. Currently, hybrids heterozygous for the N gene have been developed which still have a reduction in yield, but it is not as great as the traditional homozygous inbred lines. These inbred lines may work well for farmers who have a high incidence of TMV in a particular field, where the reduction in yield due to the virus may have outweighed the reduction in yield due to the N gene.

Due to the poor quality effects still associated with the N gene, it is important to discover new sources of resistance to TMV. In the hopes of finding new sources of resistance which do not have poor characters associated, the tobacco introductions from the *N. tabacum* gene pool have been screened for TMV resistance (Chaplin and Gooding, 1969). TI 1504 derives its resistance from a single dominant gene. However, this gene is allelic to the N gene contained in NC 567. Since NC 567 has poor agronomic characters associated with the inclusion of the N gene, it may be possible that TI 1504 also has negative attributes associated with resistance. TI 1504 may not be a good choice of breeding material to use in developing new cultivars resistant to TMV.

TI 1473 also derives its resistance from a single dominant resistance gene. However, the allelism study confirms that resistance conditioned by TI 1473 is not allelic to the N gene in NC 567. Since the resistance is not allelic, TI 1473 may thus be a new source of resistance to TMV. The gene responsible for resistance in TI 1473 may also still be the same gene as the N gene, just on a different chromosomal location. Nonetheless, TI 1473 offers a potential resistance gene that may not be associated with negative agronomic characteristics. TI 1473 may be further studied as a potential new source of resistance and to determine whether the single dominant gene is the N gene at a different chromosomal position. Additional research needs to be conducted to determine whether the gene from TI 1500 and 1473 are allelic.

Acknowledgements

The authors thank Dr. Verne Sisson, North Carolina State University, for contributing seed of the tobacco introductions used in this study. Many thanks go to the Carolina Soil Company who donated all of the Carolina mix soil used in this project.

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Table 1. Reaction of a susceptible cultivar (K326), a resistant cultivar (NC 567), and tobacco introductions inoculated with tobacco mosaic virus.

Parent	Number of plants	
	Local Lesions	Mosaic
K 326	0	40
NC 567	40	0
TI 1504	40	0
TI1473	40	0

Table 2. Reaction of F_1 progeny from crosses between tobacco introductions and a susceptible cultivar (K 326) or a resistant cultivar (NC 567) inoculated with tobacco mosaic virus.

Cross	Number of F_1 plants	
	Local Lesion	Mosaic
K 326 X TI 1504	10	0
K 326 X TI 1473	10	0
TI 1504 X NC 567	10	0
TI1473 X NC 567	10	0

Table 3. Segregation analysis of F_2 progenies from crosses between tobacco introductions (TI) and a susceptible cultivar (K 326) or a resistant cultivar (NC 567) inoculated with tobacco mosaic virus.

Cross	No. of plants		Chi-Square			
	LL	Mosaic	3:1	P-value	15:1	P-value
K 326 x TI 1504 F_2	336	89	3.73	0.05-0.20	156.55	<0.01
K 326 x TI 1473 F_2	331	124	1.23	0.2-0.3	342.54	<0.01
TI 1504 x NC 567 F_2	453	0				
TI 1473 x NC 567 F_2	405	19	95.21	<0.01	2.26	0.05-0.20

Table 4. Segregation analysis of $F_{2:3}$ progeny families inoculated with tobacco mosaic virus.

Cross	Number of $F_{2:3}$ progeny families			
	All resistant	3:1	15:1	All susceptible
K 326 x TI 1504 $F_{2:3}$	16	28	0	44
K 326 x TI 1473 $F_{2:3}$	10	30	0	59
TI 1504 x NC 567 $F_{2:3}$	150	0	0	0
TI 1473 x NC 567 $F_{2:3}$	54	10	14	11

Table 5. Segregation analysis and heterogeneity test of $F_{2:3}$ families from individual F_2 plants segregating 3:1 from K 326 x TI 1504 after inoculation with tobacco mosaic virus.

Cross	No. of plants		Chi-Square		df
	LL	Mosaic	3:1	P-value	
K 326 x TI 1504 1 16	33	14	0.57	0.30-0.50	1
K 326 x TI 1504 1 37	40	10	0.67	0.30-0.50	1
K 326 x TI 1504 1 45	39	11	0.24	0.50-0.70	1
K 326 x TI 1504 1 55	37	13	0.03	0.80-0.95	1
K 326 x TI 1504 1 68	37	12	0.01	0.80-0.95	1
K 326 x TI 1504 1 9	35	14	0.33	0.50-0.70	1
K 326 x TI 1504 2 45	38	12	0.02	0.80-0.95	1
K 326 x TI 1504 2 52	36	11	0.06	0.80-0.95	1
K 326 x TI 1504 2 56	38	11	0.17	0.50-0.70	1
K 326 x TI 1504 2 67	33	14	0.57	0.30-0.50	1
K 326 x TI 1504 3 11	42	8	2.16	0.05-0.20	1
K 326 x TI 1504 3 15	34	14	0.44	0.50-0.70	1
K 326 x TI 1504 3 31	34	13	0.17	0.50-0.70	1
K 326 x TI 1504 3 33	34	9	0.37	0.50-0.70	1
K 326 x TI 1504 3 41	31	8	0.41	0.50-0.70	1
K 326 x TI 1504 3 57	33	9	0.28	0.50-0.70	1
K 326 x TI 1504 4 34	39	11	0.24	0.50-0.70	1
K 326 x TI 1504 4 73	33	9	0.28	0.50-0.70	1
K 326 x TI 1504 4 74	35	10	0.18	0.50-0.70	1
K 326 x TI 1504 5 23	40	9	1.15	0.20-0.30	1
K 326 x TI 1504 5 36	39	11	0.24	0.50-0.70	1
K 326 x TI 1504 5 37	41	8	1.97	0.05-0.20	1
K 326 x TI 1504 5 60	43	7	3.22	0.05-0.20	1
K 326 x TI 1504 6 23	35	15	0.67	0.30-0.50	1
K 326 x TI 1504 6 25	35	11	0.03	0.80-0.95	1
K 326 x TI 1504 6 35	38	12	0.03	0.80-0.95	1
K 326 x TI 1504 6 63	41	8	1.97	0.05-0.20	1
K 326 x TI 1504 6 66	39	10	0.55	0.30-0.50	1
Total	1032	304	17.03		28
Heterogeneity			13.44	0.95-0.99	27

Table 6. Segregation analysis and heterogeneity test of $F_{2:3}$ families from individual F_2 plants segregating 3:1 for K 326 x TI 1473 after inoculation with tobacco mosaic virus.

Cross	No. of plants		Chi-Square		df
	LL	Mosaic	3:1	P-value	
K 326 x TI 1473 3 14	34	13	0.18	0.50-0.70	1
K 326 x TI 1473 3 54	36	12	0	0.99	1
K 326 x TI 1473 3 74	34	16	1.30	0.20-0.30	1
K 326 x TI 1473 5 13	39	10	0.55	0.30-0.50	1
K 326 x TI 1473 5 28	32	15	1.20	0.20-0.30	1
K 326 x TI 1473 5 46	31	12	0.19	0.50-0.70	1
K 326 x TI 1473 5 53	37	12	0.01	0.80-0.95	1
K 326 x TI 1473 6 35	36	11	0.06	0.80-0.95	1
K 326 x TI 1473 6 50	33	13	0.26	0.50-0.70	1
K 326 x TI 1473 6 51	33	16	1.53	0.20-0.30	1
K 326 x TI 1473 6 70	31	17	2.78	0.05-0.20	1
K 326 x TI 1473 7 60	34	12	0.03	0.80-0.95	1
K 326 x TI 1473 7 68	34	16	1.31	0.20-0.30	1
K 326 x TI 1473 8 12	36	12	0	0.99	1
K 326 x TI 1473 8 29	36	10	0.26	0.50-0.70	1
K 326 x TI 1473 8 39	34	13	0.18	0.50-0.70	1
K 326 x TI 1473 8 47	32	14	0.72	0.30-0.50	1
K 326 x TI 1473 8 55	31	16	2.04	0.05-0.20	1
K 326 x TI 1473 8 63	39	11	0.24	0.50-0.70	1
K 326 x TI 1473 8 64	38	13	0.01	0.80-0.95	1
K 326 x TI 1473 8 75	40	10	0.67	0.30-0.50	1
K 326 x TI 1473 8 8	35	13	0.11	0.70-0.80	1
K 326 x TI 1473 9 22	32	12	0.12	0.70-0.80	1
K 326 x TI 1473 9 30	32	12	0.12	0.70-0.80	1
K 326 x TI 1473 9 32	30	16	2.35	0.05-0.20	1
K 326 x TI 1473 9 35	32	15	1.19	0.20-0.30	1
K 326 x TI 1473 9 39	34	15	0.82	0.30-0.50	1
K 326 x TI 1473 9 40	38	12	0.03	0.80-0.95	1
K 326 x TI 1473 9 42	38	11	0.17	0.50-0.70	1
K 326 x TI 1473 9 66	36	14	0.24	0.50-0.70	1
Total	1037	394	18.55		30
Heterogeneity			13.65	0.95-0.99	29

Table 7. Segregation analysis and heterogeneity test of $F_{2:3}$ families from individual F_2 plants segregating 3:1 for TI 1473 x NC 567 after inoculation with tobacco mosaic virus.

Cross	No. of plants		Chi-Square		df
	LL	Mosaic	3:1	P-value	
TI 1473 x NC 567 1 5	54	18	0.0	0.99	1
TI 1473 x NC 567 1 21	60	14	1.46	0.20-0.30	1
TI 1473 x NC 567 2 73	56	19	0.0	0.99	1
TI 1473 x NC 567 4 2	56	19	0.0	0.99	1
TI 1473 x NC 567 4 9	55	19	0.02	0.80-0.95	1
TI 1473 x NC 567 4 68	55	20	0.11	0.70-0.80	1
TI 1473 x NC 567 5 13	56	16	0.30	0.50-0.70	1
TI 1473 x NC 567 5 54	58	17	0.22	0.50-0.70	1
TI 1473 x NC 567 6 4	60	16	0.63	0.30-0.50	1
TI 1473 x NC 567 6 29	59	17	0.28	0.50-0.70	1
Total	569	175	3.02		10
Heterogeneity			2.15	0.95-0.99	9

Table 8. Segregation analysis and heterogeneity test of $F_{2:3}$ families from individual F_2 plants segregating 15:1 for TI 1473 x NC 567 after inoculation with tobacco mosaic virus.

Cross	No. of plants		Chi-Square		df
	LL	Mosaic	15:1	P-value	
TI 1473 x NC 567 1 52	71	4	0.11	0.70-0.80	1
TI 1473 x NC 567 2 21	67	8	2.50	0.05-0.20	1
TI 1473 x NC 567 2 33	68	7	1.22	0.20-0.30	1
TI 1473 x NC 567 4 21	69	5	0.03	0.80-0.95	1
TI 1473 x NC 567 4 54	68	6	0.44	0.50-0.70	1
TI 1473 x NC 567 5 3	74	1	3.09	0.05-0.20	1
TI 1473 x NC 567 5 57	73	2	1.64	0.20-0.30	1
TI 1473 x NC 567 6 20	70	5	0.02	0.80-0.95	1
TI 1473 x NC 567 6 30	71	4	0.11	0.70-0.80	1
TI 1473 x NC 567 6 58	69	3	0.53	0.30-0.50	1
TI 1473 x NC 567 7 3	72	3	0.65	0.30-0.50	1
TI 1473 x NC 567 7 31	67	8	2.50	0.05-0.20	1
TI 1473 x NC 567 7 50	71	4	0.11	0.70-0.80	1
TI 1473 x NC 567 7 75	69	6	0.40	0.50-0.70	1
Total	979	66	13.35		14
Heterogeneity			13.34	0.25-0.50	13

Chapter 4

Tobacco Mosaic Virus Movement in Susceptible and Resistant Flue-Cured Tobacco Cultivars after Different Inoculation Times and Methods

Abstract

The N gene, which is responsible for tobacco mosaic virus (TMV) resistance in tobacco (*Nicotiana tabacum* L.), acts by inducing cell death, thus preventing TMV movement in the plant. However, the gene does not always completely restrict viral movement. Systemic spread of virus is occasionally observed in TMV resistant flue-cured cultivars, which may result in plant death. Virus movement was evaluated in resistant and susceptible field grown flue-cured tobacco cultivars inoculated using different methods at different times throughout the growing season. One susceptible cultivar (K 326), one homozygous resistant cultivar (NC 567), and three heterozygous resistant hybrids (NC 297, RGH4, Speight H20) were inoculated with TMV in the field at transplanting, at layby, and at topping. Corollas, stamens, pistils, roots, pods, seed, pollen, and leaves at the 3rd, 7th, and 13th stalk position from the bottom of the plant were sampled and tested for presence of TMV using immunostrip test kits, as well as tested for biological activity using infectivity assays. All plant parts of K 326 contained virus regardless of the time of inoculation. Virus was not present in any NC 567 plant part. The resistant hybrids consistently contained virus in the suckers and roots, with less frequent detection of TMV in the corollas and pistils. Virus was only detected in resistant plants exhibiting systemic necrosis. The N gene results in good protection most of the time, however systemic necrosis of TMV in hybrids is correlated with viral movement into some of the plant parts.

Introduction

Tobacco mosaic virus (TMV) infects commercially grown tobacco (*Nicotiana tabacum* L.) and causes yield losses in the crop each year. The virus is mechanically spread and prevention of the virus is only achieved through careful crop management and farm sanitation. However, resistance can also control the disease and keep the virus from

spreading throughout the tobacco crop. The N gene, derived from *N. glutinosa*, has been transferred into flue-cured and burley tobacco. The gene product produced by the N gene interacts with any TMV replicase elicitor that enters the plant. At the point of infection, the N gene induces a hypersensitive response in which the cells in and surrounding the infection point die. This programmed cell death keeps the virus from moving throughout the plant and, more importantly, the resistance keeps the virus from infecting the rest of the crop.

The N gene, a single dominant gene, provides complete TMV resistance in most commercially grown burley tobacco (Legg et al., 1979). The N gene is also used in several flue-cured tobacco cultivars. However, flue-cured tobacco cultivars deriving resistance from *N. glutinosa* show reduced yield and lower quality compared to the same cultivars absent the N gene (Chaplin et al., 1961). Cultivars with the N gene in the heterozygous state also have reduced agronomic characteristics compared to cultivars without the N gene, but the negative attributes are not as substantial as plants containing the N gene in the homozygous condition (Chaplin et al., 1966).

Since the heterozygous condition provides resistance and better yield and quality, many plant breeders are developing flue-cured tobacco hybrids with the N gene in the heterozygous condition as opposed to cultivars with the N gene in the homozygous state. However, both homozygous and heterozygous resistant plants sometimes have leakage of the infecting TMV particles and viral movement within resistant plants occurs. The temperature sensitive N gene allows viral movement in homozygous resistant plants at temperatures higher than 32°C and localizes TMV with the formation of lesions at temperatures lower than 32°C (Richael and Gilchrist, 1999). Therefore, a resistant plant with a substantial amount of viral leakage, before the N gene localizes the virus, can be severely damaged or die. The results of viral movement followed by localization of the virus could be more detrimental to a farmer than susceptible plants infected with TMV.

The objective of this study was to evaluate virus movement in field-grown, TMV-resistant and susceptible flue-cured tobacco cultivars inoculated using different methods

at different periods during the growing season. Plant parts were sampled at the end of the growing season to determine if and where the virus had moved. Also of importance is whether viral leakage in resistant plants results in a potential source of inoculum for either the current crop or future crops through over-wintering. Infectivity assays were used to determine whether virus that had moved throughout a plant was still biologically active.

Materials and Methods

A field experiment was conducted in 2001 at the Southern Piedmont Agricultural Research and Extension Center in Blackstone, VA. Standard production practices for seedling production, fertilization, transplanting, disease, weed, and insect control in flue-cured tobacco were followed (Reed et al., 2000) with a few exceptions. No equipment as permitted in the field after layby to minimize plant-to-plant spread of TMV. Therefore, no late season insecticides were applied. Also, no plants were topped except for the topping inoculation treatment and no sucker control chemicals were applied. A TMV-susceptible cultivar (K 326), a TMV-resistant inbred cultivar (NC 567), and three TMV-resistant hybrid cultivars (Speight H20, RGH4, and NC 297) were transplanted in the field on May 13th. Seedlings were transplanted 51 cm apart within the row and 122 cm between rows.

The experimental design was a split block with four replications. There were three plants per plot for a total of 12 plants of each cultivar for each treatment. The eight treatments included four inoculation treatments at transplanting (13 May), two inoculation treatments 49 days (3 July) after transplanting (DAT), which will be referred to as layby, one inoculation treatment 70 DAT (24 July), referred to as topping, and one treatment was a non-inoculated control.

TMV inoculum was prepared by grinding 0.5 g of infected tissue in 1% K_2PO_4 buffer with 1% Celite at pH 7.0 and diluting the final inoculum to 1:100 v/v. The four inoculation treatments at transplanting involved inoculating three different sites, the root, stalk, or a leaf of the transplant. The first inoculation treatment at transplanting involved

swirling seedling roots for 5 to 10 s in the TMV inoculum immediately prior to transplanting (root). The second inoculation treatment involved dipping a wooden skewer in the TMV inoculum and jabbing the skewer into the stalk of the seedling at ground level (jab). The third transplant inoculation treatment involved gently rubbing a newly expanded leaf of the transplant with a foam-tipped swab (sponge). The last inoculation treatment at transplanting involved rubbing a TMV infected leaf on the surface of a newly expanded leaf of the transplant (tleaf). The newly expanded leaf to be inoculated was located between the 2nd or 3rd leaf position from the top of the plant. Inoculated leaves were identified with a string tag. Two inoculation treatments were carried out at layby. Layby inoculations were performed on leaves between the 2nd and 4th leaf position from the top of the plant. Inoculated leaves were approximately 16cm long and 8 cm wide. The first layby treatment, the lleaf treatment, also involved rubbing a TMV-infected leaf on the surface of a newly expanded leaf. The second inoculation treatment at 49 DAT involved cutting the petiole near the base of the leaf and rubbing inoculum on the cut surface of the petiole with a foam-tipped swab (midrib). For the last inoculation treatment, plants were topped 70 DAT. The plant was topped at 18 leaves with TMV-contaminated clippers (stalk). The clippers were contaminated by rubbing a TMV-infected leaf on the cutting surface of the clippers before each clipping. These methods were selected to simulate possible natural means of spread of the virus.

Various parts of the plant were sampled at the end of the growing season and tested for presence of TMV. Immunostrip test kits (Agdia, Elkhart, ID) were used to determine if TMV was present in plant parts. A 0.15 g sample of plant tissue was collected and ground in the test bag containing buffer. A test strip was inserted into the suspension of ground tissue in buffer. A positive reaction resulted in two lines on the test strip, while a negative reaction gave only one control line.

Leaves were sampled using a number 6 cork-borer to remove a disk of tissue from five randomly selected locations. The five disks weighed approximately 0.15 g. The bottom, middle, and top leaves were sampled at approximately the 3rd, 7th, and 13th leaf position from the bottom of the plant. No plants were topped except those inoculated at

topping to minimize inadvertent spread of TMV. Plants inoculated at topping did not receive sucker control treatments and flower samples were collected from the resultant suckers. One corolla was sampled from each plant as well as three pistils from each plant. NC 567 and K 326 produced seed, so one pod was sampled from each plant and the seed from that pod was also tested for viral presence. Five stamens from both NC 567 and K 326 were collected from each plant for testing. Different numbers of plant parts were taken in order to sample approximately 0.15 g of tissue. Pollen was collected from 5 to 10 anthers from each plant, placed in microcentrifuge tubes, and 300 μL of immunostrip buffer was added to each tube. Each sample was shaken before testing. Finally, each plant of all five cultivars was dug up and approximately 0.15 g of fibrous root tissue was collected for testing.

The tissue buffer suspension remaining from the serological tests was used to conduct the infectivity assays. One leaf of a Xanthi plant was inoculated with a cotton swab dipped in Celite for each plot to determine whether TMV was biologically active when positive immunoassays were observed. Leaves were checked 2 to 3 d after inoculation for lesion formation. Lesions appearing on leaves were counted. All infectivity assays were carried out on Xanthi plants grown in the greenhouse.

Results and Discussion

Visual observations

The tleaf treatment was the most effective inoculation method at transplanting. Leaves of each inoculated K 326 plant developed mosaic symptoms (Table 2). A hypersensitive response was observed on every inoculated leaf of the inbred (NC 567) and the three TMV resistant hybrids. No other symptoms were observed anywhere else on the resistant cultivars.

In contrast, only 50% (6/12), 25% (3/12), and 17% (2/12) of the K 326 plants inoculated with the sponge, jab, and root dip treatments, respectively, exhibited mosaic symptoms 14 d after inoculation, indicating that these transplant inoculation treatments were not as effective in virus transmission (Table 1). No visible symptoms were

observed on the four resistant cultivars after the sponge, jab, or root dip treatments. Leaf burning was observed on the inoculated leaf of all cultivars inoculated with the sponge method. This occurrence was possibly due to a virus overdose of the leaf. Inoculated leaf tissue may have desiccated and become physically damaged before the virus had time to consistently spread through the leaf laminar cells to reach the vascular system or cause a hypersensitive response. All K 326 plants that became infected at transplanting were severely stunted throughout the growing season.

Results of the two layby treatments, lleaf and midrib, were similar to the observations seen after the tleaf treatment in K 326 (Table 1 and 2). Mosaic symptoms were observed on the leaves of all twelve K 326 plants receiving either of the two layby treatments. The lleaf treatment, which involved rubbing a newly expanded leaf with an infected leaf, caused a hypersensitive response on the inoculated leaf of the resistant inbred and hybrids. In contrast, no visible symptoms were observed on any of the resistant plants receiving the midrib treatment in which inoculum was rubbed onto the cut petiole. Approximately two weeks after the initial hypersensitive response was observed on the inoculated leaf for the lleaf treatment, a systemic necrosis developed along the stalk in 50% (6/12), 33% (4/12), and 17% (2/12) of RGH4, NC 297, and Speight H20 plants, respectively. The blackening of the stalk due to systemic necrosis averaged 25 cm in length and always originated from the point of inoculation. The necrosis followed the leaf midrib into the stalk of the plant and moved along the vascular system. The systemic necrosis was responsible for the premature death of one RGH4 and one Speight H20 plant as well as the prevention of flower formation in three RGH4 plants and one Speight H20 plant. NC 567, on the other hand, did not develop any systemic necrosis from the lleaf treatment.

Topping K 326 plants with TMV-contaminated clippers resulted in 100% of the plants showing mosaic symptoms on the top, most actively growing leaves (Table 1 and 2). No immediate symptoms were observed in the resistant inbred or the resistant hybrid cultivars. However, 58% (7/12) of RGH4 plants and 50% (6/12) of NC 297 and Speight H20 plants developed systemic necrosis visible on the stalk within 2 to 3 wk after

inoculation with contaminated clippers. In contrast, only 17% (2/12) of the NC 567 plants showed this sign of systemic virus movement. The systemic necrosis caused a black streak down the stalk averaging 17 cm in length. The systemic necrosis seen on resistant plants inoculated at topping always originated from the point of inoculation (where the plant was topped).

Non-inoculated resistant plants did not develop any necrotic symptoms throughout the growing season. The non-inoculated susceptible cultivar K326 finished the growing season with 2 out of the 12 plants infected through inadvertent inoculation. One plant became infected before any samples were collected, while the second K 326 plant did not show symptoms until corolla samples were collected. Therefore, the second K 326 plant may have become infected during the sampling process.

Serological Tests and Infectivity Assays

Tobacco mosaic virus was detected in all plant parts sampled from symptomatic K 326 plants (Table 2). Root samples from inoculated K 326 plants were always associated with positive immunostrip results, as were leaf samples collected from plants inoculated at transplanting. However, positive immunoassay results were not always obtained for leaf samples taken near or below the approximate inoculation site. Positive immunoassays were almost always observed for leaf samples collected at the 7^{th} and 13^{th} leaf positions from plants inoculated at layby, but positive immunoassays for samples from the 3^{rd} leaf position were only observed for 6 or 7 of the 12 samples collected for the layby midrib and leaf inoculation treatments, respectively. Positive immunoassay reactions were largely confined to the 13^{th} leaf position for samples from plants inoculated at topping. Immunoassay results were always negative for samples from non-symptomatic K 326 plants. Infectivity assays from root and leaf samples that had positive immunostrips always resulted in the development of lesions on Xanthi plants (data not shown).

Flower parts were also collected from K 326 for serological testing. The flower parts tested included the corolla, pistil, stamen, and pollen. In addition, mature pod and

seed samples were also taken. However, many flower parts could not be sampled from some treatments. Inoculation of K 326 plants at transplanting caused stunting that resulted in either no flowers or very meager flower formation. The large number of samples required and the subsequent amounts of time needed for each immunoassay test also resulted in many of the flowers over-maturing (drying up) before some samples could be taken. The last reason for inadequate pod and seed samples was insect damage. No insecticides were sprayed on the field to limit field traffic and therefore, TMV spread. Insects destroyed many of the pods and seed. Therefore, flower, pod, and seed samples are reported as the number of positive immunostrips out of the number of symptomatic plants sampled.

With only a few exceptions, immunoassays of flower parts from symptomatic K 326 plants resulted in positive reactions (Table 2). All stamen, pod, and seed samples taken from symptomatic K 326 plants resulted in positive immunostrip tests. However, corollas, pistils, and pollen from symptomatic plants did not always display positive immunostrip tests. The inconsistent immunostrip results may be the result of experimental error associated with the large numbers of samples handled each day. The inconsistent immunoassays observed from pollen samples may have resulted from the small amount of pollen available for sampling. Sample sizes for flower parts were generally small, preventing conclusions other than a general agreement with previous reports that TMV particles move through infected tobacco plants into all flower parts (Allard, 1914; Allard, 1915). Infectivity assays (data not shown) from corolla and stamen samples that gave positive immunostrip tests also resulted in lesion formation on Xanthi plants. In contrast, when positive immunostrip tests were observed from pistil, pod, and seed samples, the resultant infectivity assays resulted in either low lesion numbers compared to corolla and stamen samples or the failure of any lesions to appear on Xanthi leaves.

Serological testing failed to detect TMV in any plant part sampled from NC 567 regardless of the inoculation time or method. No positive immunostrips were observed for any TMV-resistant hybrid cultivar inoculated at transplanting or by the midrib method

at layby. However, positive immunostrips were observed from root samples from all three resistant hybrid cultivars (NC 297, RGH4, and Speight H20) when leaves were inoculated at layby (Table 3). In cases where positive immunostrips were observed from resistant hybrids, all samples came from a plant showing systemic necrosis. In contrast, the topping inoculation resulted in 1 of 6, 0 of 7, and 0 of 6 systemic necrotic NC 297, RGH4, and Speight H20 plants, respectively, giving a positive immunostrip test for root samples. It is possible that the one positive immunoassay sample collected from NC 297 roots at topping may actually belong with the NC 297 lleaf treatment results. This switch would be more consistent with the results seen in RGH4 and Speight H20. Nevertheless, there was virus movement into the roots of the resistant hybrids when plants were inoculated at layby and systemic necrosis was observed. However, when systemic necrosis was observed after topping inoculation, virtually no viral movement into the roots was detected. Three weeks between the topping inoculation and the date when plant samples were collected may not have been long enough to allow the virus to move from the top of the plant into the roots. Another possible explanation is related to developmental changes in plants at the topping stage, when older inoculated plants may not send as many of their resources into the already mature parts of the plants (Gibbs and Harrison, 1976). As the plant limits the resources it sends to older tissue, virus may not travel as extensively as it would into younger actively growing tissue.

Positive immunostrip reactions were generally not observed for leaf samples from the resistant hybrids NC 297, Speight H20, and RGH4. In some cases for NC 297 and RGH4, the leaf inoculated at layby occupied the 7th leaf position, in which case positive immunoassay reactions were observed (Table 3). The virus detected was from placement of the virus onto the leaf from inoculation and not from viral movement. There were also several cases in which suckers were sampled from NC 297 and RGH4 plants in place of the leaf sample for both the lleaf and stalk treatments. The suckers were sampled due to the necrosis seen on the leaves of the suckers. One ground sucker was sampled in place of the 3rd leaf for one plant each of NC 297 and RGH4 receiving the lleaf treatment. One axilary sucker originating from the 7th leaf position was sampled in place of the leaf sample from a NC 297 plant receiving the stalk treatment. A RGH4 and a NC 297 plant

receiving the lleaf treatment each had one sucker originating from the 13th leaf position sampled instead of the 13th leaf. Suckers originating from the 13th leaf position were also sampled from three NC 297 plants inoculated at topping. All suckers tested positive for TMV presence as observed by positive immunotest strips (Table 4).

Like K 326, pistils and corollas were not always sampled from the resistant hybrids. Delayed sampling due to the amount of time needed to collect all the flower samples contributed to sample over-maturity and thus small sample sizes. In some instances, no flower formation was evident due to systemic necrosis of the plant. Limited detection of TMV was observed in the corolla for NC 297 and RGH4, while detection of TMV in the pistil was confined to only one RGH4 plant. This occasional detection of TMV in flower parts from NC 297 and RGH4 suggests movement of TMV into flower parts of resistant hybrids, similar to K 326, may occur in some cases, but the exact nature of these circumstances is unknown.

Conclusions

Tobacco yield and quality is inferior when TMV is not controlled. The susceptible cultivar K 326 gave clear evidence of the effect of TMV inoculation on young plants. Inoculated plants became stunted for the rest of the season with small and deformed leaves. The later inoculation dates did not stunt the plants but discolored the newly growing leaves with the mottling effect. TMV moved quite readily throughout all plant parts of K 326. Virus was found in the leaves, corolla, pistil, stamen, pollen, pod, seed, and roots of K 326 plants regardless of the time the plant was inoculated. However, virus did not readily move into the mature leaves after layby and topping treatments. After conducting infectivity assays (data not shown), infectious virus was recovered from K 326 when inoculum was taken from infected leaves, roots, corollas, and stamens. While biological activity was also seen for some pistil, pod, and seed inoculum, the lesion numbers observed on Xanthi leaves were lower.

The resistant inbred NC 567 did not show many detrimental effects of TMV inoculation. Only 2 of 12 inoculated plants developed systemic necrosis on the stalk, and

no NC 567 plants tested positive for TMV in any plant part sampled. However, transmission in the suckers of NC 567 cannot be ruled out since no suckers were tested.

The resistant hybrids did not fare as well as NC 567 and virus was consistently detected in late season suckers, and most importantly roots. Occasionally, small amounts of virus were detected in the flower parts. However, sample sizes were too small to draw any conclusions. Interestingly, no virus was found in leaves of resistant hybrids unless the leaf had been previously inoculated. This data may suggest that virus moves down to the roots of a plant, and into the actively growing tissues like flowers, but not into mature leaves. Similar results were seen in TMV infected tomato plants (Samuel, 1934) and in the systemic K 326. Active virus was recovered from roots, corollas, and suckers of resistant hybrid plants, but the lesion numbers in our infectivity assays were generally lower than seen with K 326, implying a lower titer of TMV in resistant hybrids. Roots of resistant hybrids may therefore, potentially serve as an over wintering source of TMV though the likelihood seems lower than for K 326 based on lesion numbers. The only time virus was detected in any part of a resistant hybrid was when the plant displayed systemic necrosis originating from the point of inoculation.

The resistant hybrids had more plant parts testing positive for TMV after layby and topping inoculation treatments than for the earlier transplanting treatments. This may be explained by the very nature of the N gene. Since the N gene is temperature sensitive, the N gene allows more viral movement on hot days when the temperature is above 32°C and thus more systemic necrosis occurs during hotter periods. This systemic necrosis seems to be a clue to finding plant parts infected with the virus. The earlier transplanting treatments occurred on 13 May with a high temperature of 23°C. Daily air temperatures never exceeded 32°C until 12 June. These temperatures were initially cool enough to result in good control of virus. However, later inoculation treatments were performed in the middle of summer when hot periods were observed. The layby treatments occurred on 3 July. The high temperature for that day was 26°C, but it was followed by two consecutive days with high temperatures of 33°C and 32°C. The topping treatment occurred on 24 July with a high temperature of 32°C, followed by a 33°C day. The first

day of sampling occurred on 23 August in which 19 more days with high temperatures above 32°C occurred after the layby treatments. Eleven more days above 32°C occurred after the topping treatments and before the first sampling date. Both the layby and topping treatments each resulted periods of above 32°C weather and may be responsible for the increased systemic necrosis seen. Both inoculation times also occur on or before a day of threshold temperatures. The resistant hybrids give very good protection against TMV, but that control may be compromised if inoculation takes place during hotter periods.

The fact that the homozygous resistant cultivar NC 567 gave better control than the heterozygous resistant inbred may suggest that a gene dose effect is present. The N gene in the homozygous state may give better protection than the N gene in the heterozygous state.

Resistant hybrids still appear to be a good way of managing TMV as long as the resistant lines are also managed well. Resistant hybrids did show viral movement into the roots of plants and thus may serve as a source of over-wintering virus. However, the overwintering sources should not be as abundant in a field with TMV-infected resistant cultivars as opposed to a field with TMV-infected susceptible cultivars. There was TMV movement into the roots of every infected susceptible plant. However, only a small proportion of the resistant hybrids displayed systemic necrosis, and only necrotic plants actually had virus move into the roots. Therefore, overwintering sources in resistant hybrids should be less important reservoirs of TMV compared to a susceptible cultivar.

Although 4 of 7 inoculation treatments resulted in a 100% infection rate for K 326, only two inoculation treatments resulted in systemic necrosis in resistant hybrids. This suggests that when and how the virus enters a resistant hybrid may influence if and how TMV will move systemically through a plant. It appears that conditions must be just right for TMV to induce systemic necrosis in resistant hybrids, reducing the likelihood of TMV causing any major problem in those cultivars. However, if a large source of TMV inoculum is near resistant hybrids, say from a nearby field with infected susceptible

cultivars, the likelihood of exposing a resistant hybrid cultivar at the right time and place may increase, with detrimental effects.

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Table 1. Infection efficiency of field grown susceptible cultivar K 326 inoculated at different times during the growing season using different inoculation methods.

Inoculation Time and Method	Infection Efficiency
Transplanting	
Leaf	100 a[a]
Sponge	50 b
Jab	25 c
Root Dip	17 c
Layby	
Leaf	100 a
Midrib	100 a
Topping	
Stalk	100 a

[a] Means followed by the same letter are not significantly different at P=0.05 based a least significant difference.

Table 2. Number of positive immunostrip assays relative to the number of symptomatic plants sampled from field grown susceptible cultivar K 326 inoculated at different times during the growing season using different inoculation methods.

Inoculation Time and Method	Sample Size (No. plants)	No. Symptomatic Plants	Number of positive immunoassay samples									
			Roots	3rd Leaf	7th Leaf	13th Leaf	Corolla	Pistil	Stamen	Pollen	Pod	Seed
Transplanting												
Leaf	12	12	12	12	12	12	2/2[ac]	4/4	3/3	1/1	-[b]	-
Sponge	12	6	6	6	6	6	1/2	1/1	-	-	-	-
Jab	12	3	3	3	3	3	1/2	1/1	1/1	0/3	1/1	-
Root Dip	12	2	2	2	2	2	0/1	-	-	-	-	-
Layby												
Leaf	12	12	12	7	12	12	10/10	9/10	6/6	6/7	10/10	4/4
Midrib	12	12	12	6	11	11	12	9/10	7/7	2/2	12/12	5/5
Topping												
Stalk	12	12	12	0	1	12	5/5	7/7	4/4	5/5	4/4	2/2
Non-inoculated Control												
	12	2	2	1	1	1	1	2	2	0	1	1

[a]Flower, pod, and seed from symptomatic K 326 plants were not sampled from all plants due to insufficient formation from stunted K 326 plants or from samples maturing before samples could be collected. These results are presented as the number of positive immunostrip assays from the number of symptomatic plants sampled.
[b]Dashes indicate that no sample from a symptomatic K 326 plant was collected and tested.
[c] Fractions represent the number of positive immunostrips out of the number of samples taken from systmic necrotic plants.

Table 3. Number of positive immunostrip assays relative to the number of systemic necrotic plants sampled from field grown resistant cultivars inoculated at different times during the growing season using different inoculation methods.

Inoculation Time and Method	Sample Size (No. plants)	No. Plants (Sytemic Necrosis)	Number of positive immunoassay samples									
			Roots	3rd Leaf	7th Leaf	13th Leaf	Corolla	Pistil	Stamen	Pollen	Pod	Seed
NC 567												
Topping												
Stalk	12	2	0	0	0	0	0	0	0	0/1[a]	0	0/1
NC 297												
Layby												
Leaf	12	4	3	0/3	2[b]	0/3	3	-[c]				
Topping												
Stalk	12	6	1	0	0/5	0/3	1/5	0/5				
RGH4												
Layby												
Leaf	12	6	6	0/5	5[b]	0/5	1/3	-				
Topping												
Stalk	12	7	0	0	0	0	0/6	1				
Speight H20												
Layby												
Leaf	12	2	2	0	0	0	0/1	-				
Topping												
Stalk	12	6	0	0	0	0	0	0				

[a]Flower, pod and seed from resistant plants showing systemic necrosis were not sampled from all plants due to either samples maturing before samples were able to be collected or failure of flower formation due to severe systemic necrosis. These results are presented as the number of positive immunostrip assays from the number of systemic necrotic plants sampled.
[b] Samples were collected from inoculated leaf.
[c]Dashes indicate that no sample from a resistant plant showing systemic necrosis was collected and tested.

Table 4. Number of positive immunostrip assays relative to the number of suckers sampled from field grown resistant cultivars inoculated at different times during the growing season using different inoculation methods.

Inoculation samples Time and Method	Sample Size (No. plants)	No. Plants (Sytemic Necrosis)	Number of positive immunoassay 3rd Leaf Suckers	7th Leaf Suckers	13th Leaf Suckers
NC 297					
Layby					
Leaf	12	4	1/1[a]	-[b]	1/1
Topping					
Stalk	12	6	-	1/1	3/3
RGH4					
Layby					
Leaf	12	6	1/1	-	1/1

[a] Fractions represent the number of positive immunostrips out of the number of suckers sampled. Sampled suckers originated from the leaf position shown except for the 3rd leaf suckers which originated from the soil.
[b] Dashes indicate that no sample from a sucker was collected and tested.

Vita

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Christopher Alexander Bagley is the son of Grayson and Nancy Bagley of Lunenburg, VA. He was born on August 11, 1978 in Petersburg, VA, and raised on a small farm in Victoria, VA. Christopher attended Central Senior High School, graduating third in his class in June 1996. He then attended Virginia Tech, where he graduated Summa Cum Laude in May 2000. Christopher received he Bachelor Degree in biology with a concentration in microbiology/immunology and a minor in chemistry. While attending college, he worked at the Southern Piedmont Agricultural Research and Extension Center during the summer months. It was here, that Christopher worked with Dr. Carol Wilkinson in the tobacco plant-breeding program. After taking great interest in the agricultural research seen at the research station, Christopher started working on his master's degree under Dr. Carol Wilkinson. Christopher graduated with his Master of Science degree from Virginia Polytechnic Institute and State University in December 2001.