Quantitative Real-time PCR Analysis of *Tobacco mosaic virus* in Individual Flue-cured Tobacco Seed

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Literature Review

Tobacco mosaic virus Infection and Symptoms

Tobacco has a rich history as a successful cash crop in the early settlement of America, and was intensively cultivated for tobacco production (Scholthof 2008). Tobacco crops often require high amounts of nutrients for development and growth in field conditions; therefore, they can deplete the soil of essential nutrients, which subsequently impacts the plants susceptibility to diseases (Scholthof 2008). The *Tobacco mosaic virus* (TMV) is one such virus that can have a negative impact on the growth and development of tobacco crops globally. TMV is characterized as a single stranded rod-shaped RNA virus that infects many different economically important crops. TMV is a member of the tobamovirus genus, and infects tobacco (*Nicotiana tabacum* L.), tomato (*Solanum lycopersicum* L.), pepper (*Capsicum annuum* L.), and many other Solanaceae crops. TMV has been identified as one of the simplest viruses known in relation to viral structure, hence the importance in the field of plant virology (Klug 1999). TMV structurally is made up of four basic protein units, two of which are designated for replication, along with the movement and coat protein (Susi and Wahlroos 2015). The single stranded positive sense RNA features of TMV's viral structure have made it one of the most stable viruses known (figure 1). TMV is an extremely hardy virus that withstands extreme heat, and can overwinter for extended periods of time. TMV can sustain heating above 90°C for 10 min and pH values ranging from 3 to 9 (Alonso et al. 2013). Additionally, TMV can remain infectious even after 50 yr of storage in a lab environment at 4°C, and 30 yr in dried tissue or plant debris in the field (Dorokhov et al. 2018). Since TMV is hardy and stable, it is very difficult for a farmer to control and nearly impossible to completely eradicate a TMV infestation once established in a field. Once a host

plant is infected with TMV via entry through an abrasion or wound, symptoms will be expressed within one or two weeks on newly developing plant tissue. The characteristic symptom associated with TMV infection is the appearance of light/dark green mottling or mosaic on developing leaf tissue. Along with these symptoms, infected plants can also show signs of leaf curling, stunted growth, and leaf yellowing. Chamberlain (1937) observed that when tobacco seedlings were infected with TMV early in development, the seedlings appeared to be smaller in size compared to healthy tobacco seedlings. All of these symptoms can have a detrimental effect on the growth and development of the host plant, which can lead to crop yield and economic losses. Once TMV infection is established in a field, mosaic infection can range from 2% to 95% (Chamberlain 1937). Strains of TMV infect tomato and pepper plants, causing poor yield, distorted fruits, delayed fruit ripening, and producing non-uniform fruit color developments (Scholthof 2000).

TMV Transmission and Replication

Tobamoviruses are different from a majority of other plant viruses because tobamoviruses are not transmitted horizontally by insects or other vectors (Dorokhov et al. 2018). Tobamoviruses have impacted tomato and pepper production globally and have been known to spread quickly through mechanical transmission (Kumar et al. 2011). Tobamoviruses are carried on seed, which could potentially lead to infection on germinating seedlings. Most literature classifies TMV as a seed borne virus that is unable to infect the embryo. Seed borne viruses are those that reside on the external surface of the seed and rarely lead to infection of the emerging seedling. Since early literature considers TMV unable to infect the embryo in seed, it is not defined as a seed transmitted virus. True seed transmitted viruses are defined as viruses that infect the embryo and lead to viral transmission to the emerging seedling. One exception to seed

transmitted viruses infecting embryos of a seed is the *Tobacco mosaic virus*. Mink (1993) states that TMV is carried as a contaminant on the seed surface and can infect seedlings during germination and early growth. Research on TMV transmission in tomato seeds (Taylor 1962), showed that TMV can be detected in and on the seed coats and in a small percentage of endosperms; however, it was not observed in the embryo. Taylor's results also indicated that true seed transmission of TMV probably does not occur in tomato, however, seedlings post germination could be accidently infected by the virus as a contaminate on the testa (Taylor 1962).

Factors that determine whether seed transmission occurs are host-virus interaction, timing of infection, and environmental factors such as temperature. TMV infection during transplanting can lead to higher percentages of infection than compared to TMV infection occurring post-transplant. Infection begins once TMV viral particles infect a host plant via entry through an abrasion or wound. Primary infection, the original infection which results from a dormant pathogen, accounts for approximately 15% of the disease incidence (Gooding and Todd 1976). Johnson et al. (1983) documented that primary infection was first observed three weeks after transplanting, and secondary spread began to occur between weeks four and seven after transplanting. Once a host plant is infected with TMV, secondary infection is the main cause of TMV spread and infection to other plants in close proximity. Secondary spread of TMV is mainly associated with mechanical transmission and poor sanitation practices, such as failure to wash hands, tools, and farming equipment with proper sanitation solutions. Secondary spread was documented to account for over 80% of all infection observed in field trials (Johnson et al. 1983). Tobamoviruses are easily spread through contact, including touching infected and healthy plants during important operations such as transplanting, pruning, tying, cultivation, spraying,

and harvesting (Dorokhov et al. 2018). Farmers and workers must be extremely cautious and mindful when handling tobacco plants to prevent TMV infection in early tobacco growing systems. Contaminated tools and the hands of workers after smoking cigarettes may also serve as an initial source of infection (Mila 2010). Experiments have shown that TMV can be detected in cigarettes and in the saliva of smokers, and can be classified as a secondary source to spread the virus.

TMV infection initially begins once TMV viral particles enter and infect a cell after mechanical damage of the cell wall and plasma membrane occurs (Dorokhov et al. 2018). A successful infection by a plant virus is defined by viral entry and accumulation in a cell, movement of the virus into neighboring cells, followed by systemic infection through the host plants vascular tissue (Liu and Nelson. 2013). Liu and Nelson (2013) also suggest that TMV replication occurs in association with the endoplasmic reticulum and both the movement and replication proteins of TMV associate naturally with the cellular membranes of the infected plant. The main steps of tobamovirus replication include the synthesis of viral replication proteins via translation of genomic RNA. Viral replication proteins then bind to the 5' region of genomic RNA in order to inhibit further translation of genomic RNA. The replication proteins bind to the 3' end of genomic RNA to initiate replication. The formation of viral replication protein complexes and the use of genomic RNA interact together within the host. Synthesis of complementary viral RNA is associated with the synthesis of progeny genomic RNA, allowing for TMV viral replication to occur throughout the cell in a host plant (Dorokhov et al. 2018). The movement protein of TMV allows for viral genetic material to move cell to cell in the host plant. The TMV movement protein is able to modify the plasmodesmata and open it for the transfer and movement of viral nucleic acid. Once the virus enters into a single cell of the plant, the entry

process of the virus is considered complete since the virus is able to move to other cells and tissue symplastically (Shaw 1999).

TMV Impact

TMV can lead to significant yield and financial losses in tobacco production. Viral diseases are known to be one of the most detrimental influences that cause significant yield losses and negatively affect production globally (Boualem et al. 2016). Chamberlain (1937) observed in the early 1900's, that mosaic infection at transplanting caused a loss in crop value from 50% to 60%. Estimated losses in flue-cured tobacco production due to TMV were more than one million dollars annually in North Carolina during 1960 to 1965 (Gooding 1969). In other countries, TMV is reported to cause heavy losses, partly through reduction in yield and partly through a lowering of the leaf quality (Chamberlain 1937). It is estimated that around 15% of global crop production was lost due to plant diseases, and around 47% of the plant diseases were plant viruses (Boualem et al. 2016). Johnson et al. (1983) found that tobacco plants inoculated with TMV 7, 35, or 49 days after transplanting in the field caused highly significant losses in yield, value, and quality. In many cases the quality of tobacco is lowered because of the poor color and small leaf size when infected by TMV. The significant economic loss farmers endure due to TMV in a tobacco cropping system makes viral detection methods extremely important to identify the presence of early pathogen infections.

Viral detection methods

Early plant pathogen detection is extremely important for effective disease diagnostics and disease management in a farming operation (Luchi et al. 2016). In the early 1900's most farmers had to base disease detection on visual symptoms. Today, many new serological and

nucleic acid based detection methods have been developed in the field of plant pathology and virology to facilitate the process of early plant disease diagnosis. In the early 2000's there were very few studies that were conducted for early infection detection with pathogens in asymptomatic plants (Luchi et al. 2016). The detection of plant pathogens in seed and asymptomatic tissue is difficult because only small amounts of the viral pathogen may be present. Sensitive detection techniques are therefore needed to effectively detect the presence of low viral concentrations residing in plant tissue and seed. Serological techniques were developed in the mid 1900's to produce rapid detection methods for selective pathogens in host plants. Serological tests were developed with specific antibodies to detect the presence or absence of a pathogen in plant tissue. One of the most well-known serological tests created for viral and pathogen detection is the enzyme linked immunosorbent assay (ELISA). ELISA tests are normally 96 well plate based assays that bind antibodies and proteins together to detect the presence of viruses or pathogens through a colorimetric indicator. If samples in an ELISA well show a colorimetric signal then it is considered positive for the presence of the targeted pathogen. Previous research on seed transmission of TMV in tobacco used ELISA tests to detect TMV in seeds, seed coats, and seedlings in green house and growth chamber tests (Wilkinson et al. 2006; unpublished data). This previous TMV research used pooled seed, seed coat, and seedlings samples to detect TMV. The researchers used pooled samples in their experiment because ELISA was not sensitive enough to detect presence of TMV infection in individual samples. Pooled samples did express positive yellow colorimetric indicating presence of viral infection in each sample type via ELISA. Since previous experiments used pooled samples to indicate infection, the researchers were unable to establish a definitive seed-borne transmission rate. ELISA is an extremely cost effective method for large sample virus detection; however, it is

less reliable when dealing with samples that have low virus titer (Dai et al. 2012). Serological assays also lacked sensitive detection methods in small sample sizes; therefore, the need for more sensitive viral detection methods was critical in establishing a true seed transmission rate of TMV infection.

The introduction of nucleic acid based detection methods has allowed for reliable and rapid detection of pathogens in small sample sizes and asymptomatic plant tissue. No other technique has advanced pathogen detection more than PCR (Schaad and Frederick 2002). Nucleic acid based techniques are now becoming the new standard for plant pathogenic detection (Schaad and Frederick 2002). Endpoint PCR assays have been applied in plant disease detection methods by using RNA samples of plant tissue that are further synthesized into cDNA for viral quantitation. Most traditional PCR assays establish viral quantitation of a pathogen in a qualitative yes/no format. In endpoint PCR, cDNA samples are amplified through a traditional PCR cycle on a thermocycler to create template DNA. That template DNA is then used in post PCR processes such as gel electrophoresis to detect visual bands for viral identification. The sensitivity of the bands produced on agarose gels quantitatively show presences of the virus.

The introduction of quantitative real-time PCR (RT-qPCR) is a more sensitive platform for pathogen and disease detection in micro scaled samples and asymptomatic tissue. Real-time PCR is an improved nucleic acid based technique compared to endpoint PCR in relationship to viral identification because of its low variability between samples and increased sensitivity. Reverse transcriptase PCR assays are used to identify RNA plant viruses and are known to be rapid and specific procedures (Kumar et al. 2011). The advantages of real-time PCR over traditional PCR methods consist of the elimination of post PCR product detection, less susceptible to cross contamination, less labor intensive, more user friendly operation, and faster

product detection between cycles. The development of RT-qPCR with true quantitation of target nucleic acids has been adopted from a medical research lab setting to a plant disease diagnostics lab setting (Mackay et al. 2002). RT-qPCR achieves relative quantitation of nucleic acids by using an internal control, such as 18s rRNA, and co-amplifying it with a target nucleic acid sample of unknown concentration. In general, the use of an internal rRNA control combined with replicates of each target sample is essential for quantitation of viral presence in a RT-qPCR format. Results in RT-qPCR are determined by the presence of a florescence signal generated above a background threshold, defined as a cycle threshold value (Ct value) (Caraguel et al. 2011). The Ct values define the cycle number during qPCR which detects the viral RNA in a given sample. RT-qPCR assays allow for quantitative real-time results to be achieved in association to viral concentration in a given sample.

TMV Control

Rapid disease detection methods are essential for farmers so that they can diagnosis diseases or pathogens present in their farming system and develop effective disease management programs to prevent pathogen infestations. Since TMV is a hardy virus and has the ability to survive in extreme conditions, management and disease control practices are important for TMV prevention. There are currently no known chemicals or viricides on the market that control and eradicate TMV. Normally, the best form of control dealing with TMV infection is prevention of possible viral inoculum. Farmers must take preventative control measures to limit the possible spread of TMV in a farming operation. Some preventative measures include proper sanitation of all tools and equipment, removal of crop debris from greenhouse and in field rows, purchasing of disease free seed, rouging of plants in field and greenhouse areas that exhibit TMV symptoms, and proper hygiene techniques when workers handle or come in contact with disease tissue

(PennState Extension 2015). The use of bleach solutions to disinfect tools and equipment help prevent viral spread in the greenhouse and field because it minimizes the initial source of viral inoculum. Farmers should also consider managing perennial weeds in field areas close to tobacco crops. Perennial weeds are great hosts for TMV inoculum and allow for TMV to overwinter in crop debris. By managing the perennial weeds known to be hosts for TMV, farmers can reduce the amount of initial viral inoculum present in the field. The discovery of a TMV resistant gene (N gene) in *Nicotiana glutinosa* L. has led to development of several TMV resistant tobacco cultivars. The N gene is a single locus dominate gene that is resistant against tobamoviruses (Padgett et al. 1993). These tobacco cultivars with N gene resistance exhibit a hypersensitive response on the infected leaf tissue to localize and contain the virus inside the plant to prevent viral transmission throughout the plant and eventually the field (Depta et al. 2018). The adoption of resistant cultivars can help prevent the introduction and spread of TMV in a given farming operation. It is best for farmers to incorporate a combination of control practices listed above to minimize the presence of TMV inoculum and spread of the virus.

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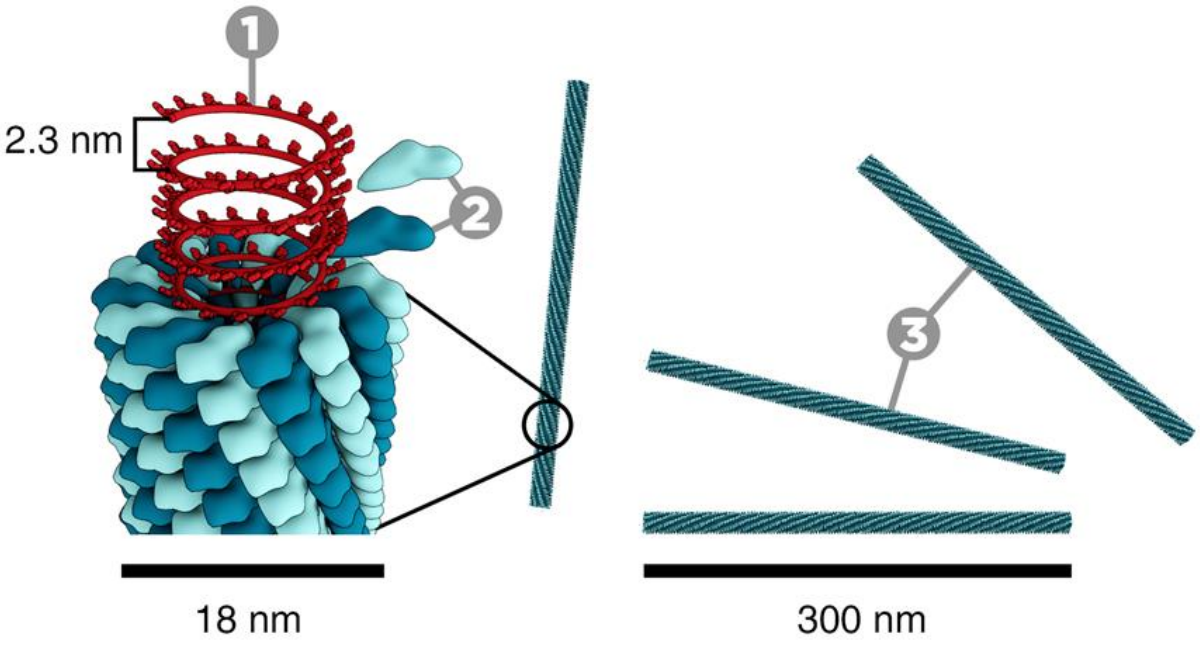
Figure 1. *Tobacco mosaic virus* (TMV) viral structure



The TMV viral structure: 1) TMV single stranded nucleic acid, 2) TMV coat protein subunits, and 3) rod shaped virus structure with coat proteins wrapped around the TMV nucleic acid.

Quantitative Real-time PCR Analysis of *Tobacco mosaic virus* in Individual Flue-cured Tobacco Seed

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Abstract

The *Tobacco mosaic virus* (TMV) has infected tobacco plants since the late 1800's, causing detrimental yield and economic losses in tobacco. TMV is classified as a seed borne virus because the virus infects tobacco seed as a contaminant on the seed coat surface. The purpose of this study was to investigate seed-borne transmission of TMV by examining the infestation route of tobacco seeds. Four crosses were performed using K 326 flue-cured cultivar: 1) self-pollinated, TMV infected, 2) self-pollinated, non-infected, 3) maternally TMV infected, and 4) paternally TMV infected. Tobacco seed were collected from three individual pods from each cross. Total RNA was extracted from 100 individual seeds per pod, and synthesized into cDNA for analysis. A reverse transcriptase quantitative PCR (RT-qPCR) assay was developed to analyze TMV concentrations within individual tobacco seeds. RT-qPCR was adopted over other traditional viral detection methods for its capability of generating fast quantitative results in real time. Results revealed distinct TMV concentration patterns and data suggest uneven distribution of TMV within individual seed pods. These results show evidence of maternal but not paternal seed-borne transmission of TMV. These findings may be useful in identifying the TMV infection route of entry in emerging tobacco seedlings to recognize TMV as a seed transmitted virus.

Introduction

The *Tobacco mosaic virus* (TMV) is a popular plant virus that historically has caused detrimental yield and economic losses in tobacco (*Nicotiana tabacum* L.) production globally. TMV is a member of the tobamovirus genus and is characterized as a rod shaped RNA virus. TMV also infects other economically important crops such as tomato (*Solanum lycopersicum* L.), pepper (*Capsicum annuum* L.), and many other members of the Solanaceae family (Scholthof 2000). Host plants infected with TMV usually exhibit visual symptoms such as mosaic patterns of light/dark green colored areas, leaf curling, and stunted growth. The symptoms produced by TMV infection reduce the quality and yield of the tobacco leaf. Extreme cases of TMV infection have resulted in losses of 50% to 60% (Chamberlain 1937). TMV is a hardy virus that can overwinter in plant debris and other perennial weeds (Gooding 1969). TMV's stability, along with the capability to overwinter, serves as concentrated viral inoculum for crops being established in future growing operations. TMV can remain dormant in dead tissue and becomes active once the virus enters into living cells where it can replicate and invade neighboring cells of a susceptible host.

TMV was one of the very first plant viruses purified and isolated (Scholthof 2008). Research has characterized TMV as a model virus system for the study of viral replication and transmission. Much debate has occurred on the topic of defining TMV as a true seed transmitted virus. Early literature depicts TMV as a seed borne virus that does not infect the seed embryo as the virus is present on the seed surface as a contaminate (De Assis Filho et al. 2000). More recent research (Wilkinson et al. 2006), demonstrated the virus to be present in bulked seed, seed coat, and seedling samples via ELISA detection. Researchers were unable to detect TMV in single seed and tissue samples due to the small sample weights. More sensitive forms of detection

methods were needed to effectively establish seed transmission rates of TMV. The adoption of nucleic acid detection techniques, reverse transcriptase quantitative polymerase chain reaction (RT-qPCR), allows for more sensitive viral detection methods to be achieved in small asymptomatic samples (Schaad et al. 2002). This technique can be used to detect virus concentrations to determine if parental tissue, maternal and paternal, influence the presence of TMV in tobacco seed.

Preliminary research conducted at Virginia Tech Southern Piedmont Agricultural Research and Extension Center (SPAREC) lab, indicated that TMV infection could be detected on tobacco seed, seed coats, leaf tissue, and pollen via RT-qPCR assays. The objective of this study was to determine the influence of parental tissue, maternal or paternal, on TMV in seed. RT-qPCR was used to identify the viral concentration in individual seeds from four different K 326 cultivar tobacco crosses. This research may provide insight into the viral movement of TMV in tobacco at the individual seed level.

Materials and Methods

Tobacco Seed Production

In 2016, approximately 100 K 326 cultivar flue-cured tobacco plants were grown in a geographically isolated field at the Virginia Tech Southern Piedmont Agriculture Research and Extension Center (SPAREC) located in Blackstone, VA. K 326 has been one of the most popular flue-cured tobacco cultivars for the past 30 yr. TMV infected plants have been grown in this field since the early 2000s. Standard production practices for seedling production, fertilization, transplanting, disease, weed, and insect control in flue-cured tobacco were followed (Reed et al. 2019). Seedlings were transplanted into the field in May and mechanically inoculated with TMV

inoculum after layby in late June. TMV inoculum was prepared by grinding 2 g of TMV infected leaf tissue with 20 mL 1% potassium phosphate and 1% celite (Fisher Scientific Inc., Suwanne, GA) buffer solution at pH 7.4 using a mortar and pestle. The infected leaf tissue and buffer mixture was filtered through cheesecloth and inoculum diluted to a 1:100 v/v. A sponge brush (Fisher Scientific Inc., Suwanne, GA) was dipped into the prepared TMV inoculum and rubbed across a palm sized immature leaf. The sponge brush created micro abrasions on the leaf surface, which allows for entry points for the virus to infect the plant. Typical symptoms developed 7 to 15 d after inoculation. Experimental controls were healthy, non-inoculated K 326 cultivar flue-cured tobacco plants grown in a geographically isolated field separate from the TMV infected field. Seed were collected from TMV infected K 326 self-pollinated, TMV infected K 326 x non-infected K 326 (maternally infected), non-infected K 326 x TMV infected K 326 (paternally infected), and K 326 non-infected self-pollinated plants. The TMV infected self-pollinated tobacco plants (positive control) were inoculated with TMV viral inoculum on the youngest actively growing leaf tissue. The non-infected self-fertilized tobacco plants (negative control) were inoculated with virus free buffer inoculum similar to that of the positive control plants. Seed for maternally infected crosses were produced by using healthy virus free pollen to pollinate the stigma of TMV infected K 326 flowers using a cotton swab. Seed for paternally infected crosses were produced by using pollen from TMV infected K 326 to cross pollinate the stigma of non-infected K 326. The self-pollinated TMV infected and maternally TMV infected tobacco plants were grown in the TMV field, while the self-pollinated non-infected and paternally TMV infected tobacco plants were grown in the TMV-free field to minimize potential exposure to viral inoculum. Mature seed pods were collected in early September. Three individual pods were collected for each of the four crosses. Each seed pod was crushed by hand

and sieved to separate seed based on size. The seed were separated using 600 µm and 500 µm sieves (Gilson Company Inc., Lewis Center, OH). The 600 µm sieve caught the seed pod debris/trash, the 500 µm sieve caught the large seed, and the pan caught the remaining small seed. All seed collected by the 500 µm sieve, which is similar in size to commercial seed, was used in this study. A separate set of virus free sieves were used for seed separation of the non-infected healthy seed. One hundred seed from three different pods for each of the four treatments were analyzed, with the exception of 200 seed for pods 1 and 2 of the self-pollinated TMV infected.

RNA extraction

Whole individual seed were homogenized in a 1.5 mL microcentrifuge tube (USA Scientific, Ocala, FL) using a DeWalt 18v power drill (Home Depot, Atlanta, GA) and polypropylene pestles (USA Scientific, Ocala, FL). RNA was extracted from each seed using a Direct-zol MicroPrep Kit™ (Zymo Research, Irvine, CA). After the seed was ground, the manufactures protocol was followed for complete RNA extraction. The final RNA solution from each sample was stored in a -20°C freezer until used for cDNA synthesis.

cDNA synthesis

The RNA extracted was used for cDNA synthesis following a protocol developed by Dr. Tim Sit of North Carolina State University. Reagents include: deoxynucleotide (dNTP) (New England Biolabs, Ipswich, MA), random primers (ThermoFisher Scientific, Carlsbad, CA), RNase inhibitor murine (New England Biolabs, Ipswich, MA), protoscript II reverse transcriptase (New England Biolabs, Ipswich, MA), 10X DTT (New England Biolabs, Ipswich, MA), and reverse transcriptase buffer (New England Biolabs, Ipswich, MA). Six microliters

dH_2O, 1 µL 10 mM dNTP, 2 µL 60 µM random primers, and 3 µL of total RNA sample were combined in a 0.5 mL microcentrifuge tube (USA Scientific, Ocala, FL). Samples were vortexed and incubated at 70°C on a dry heat block (Fisher Scientific, Sawanne, GA) for 5 min, followed by chilling on ice for 4 min. Four microliters 5X ProtoScript II buffer, 2 µL 0.1 M DTT, 1 µL RNase inhibitor murine (40 U/µL), and 1 µL ProtoScript II reverse transcriptase (200 U/µL) were added to the sample tubes. Samples were pipetted up and down five times to mix the solution. Samples were vortexed and incubated at room temperature for 5 min followed by incubation on a dry heat block at 42°C for 45 min. The samples were denatured by incubating at 80°C on a dry heat block for 5 min. The final cDNA samples were then stored in a -20°C freezer until used for RT-qPCR.

Reverse-transcriptase quantitative PCR (RT-qPCR)

Data were collected quantitatively using a ThermoFisher QuantStudio 3 (ThermoFisher Scientific, Carlsbad, CA) real-time PCR system and by following a RT-qPCR protocol developed by Dr. Tim Sit of North Carolina State University. Forward and reverse primer sets for 18*S* ribosomal RNA internal control and TMV specific sequences were purchased from Eurofin Genomics. The TMV primer sets for RT-qPCR were TMV_114 forward: 5'-GGATATGTCTAAGTCTGTTGC-3', TMV_249 reverse: 5'-CAGACAACTCGGGTGCG-3' (Eurofin Genomics, Louisville, KY), and the internal control rRNA primer sets were NT_1165 forward: 5'-CGGCGATGCGCTCCTG-3', NT_1238 reverse: 5'-TACAGAGCGTAGGCTTGCTTTG-3' (Eurofin Genomics, Louisville, KY). Samples were analyzed in triplicate through RT-qPCR for both the TMV primer sets and rRNA internal control primer sets on a 96 well optical qPCR plate (ThermoFisher Scientific, Carlsbad, CA). RT-qPCR samples were carried out in 20 µL reactions for each well, which contained 1 µL of cDNA

sample and 19 µL of reaction mix. The reaction mix was created by combining 8.4 µL of dH2O, 0.6 µL of forward/reverse primer mix (10 pmoles/µL of each), and 10 µL of 2X SYBR Green Supermix (ThermoFisher Scientific, Carlsbad, CA). The dH_2O, primers, and 2X SYBR Supermix volumes were combined in a 1.5 mL microcentrifuge tube (USA Scientific, Ocala, FL) and placed on ice. The reaction mixes were vortexed and spun down using a benchtop mini microcentrifuge (USA Scientific, Ocala, FL) before use. The 19 µL of reaction mix was pipetted into each well, 3 wells per sample in a qPCR plate. One microliter of first strand cDNA from each sample was added to their specific well. Positive and negative control samples were analyzed on each qPCR plate. TMV infected leaf tissue and dH_2O were designated as the positive and negative controls respectfully. Once the 96well qPCR plate was loaded with samples and positive/negative controls, an optical adhesive film (ThermoFisher Scientific, Carlsbad, CA) was used to seal the plate prior to the start of a qPCR run. ThermoFisher Scientific software was used to calculate the average Ct value for each triplicate sample. The average Ct values of the TMV primer mix was compared to the average Ct values of the 18*S* internal control mix to determine the viral concentration for each sample. Average Ct values for each sample were plotted on a graph, using GraphPad Prism 8 software, to illustrate the distribution of viral concentration among the four crosses. All 100 seeds from three pods of each tobacco cross were plotted on a graph to show the TMV viral distribution among all samples, seed pods, and tobacco plant crosses.

Results

TMV detection in seed

The concentration of TMV in individual seed was determined through RT-qPCR analysis (Fig. 1). The threshold for positive samples was arbitrarily defined at a Ct value of 32 based on the range of values for self-pollinated non-infected seed. Of the 500 seed analyzed for the self-pollinated TMV infected plant, 493 (98.6%) seed were found to be infected with TMV. There were 279 seed out of 300 (93%) seed infected with TMV for the maternally TMV infected cross. The paternally TMV infected cross and the self-pollinated non infected plant had 2 seed out of 300 (0.006%) and 10 seed out of 300 (0.03%) infected with TMV respectively. These results demonstrate a higher percentage of TMV in seed for self-pollinated TMV infected plants and maternally TMV infected crosses compared to seed from paternally TMV infected and self-pollinated non-infected plants (Figure 1). Results revealed distinct TMV concentration trends and data suggest uneven distribution of TMV within individual seed pods. The TMV Ct value and 18*S* Ct value were compared for each sample to determine a viral titer among the tobacco crosses. The $\log_{10}$ viral titer was calculated for each pod and averaged for each tobacco cross. The average $\log_{10}$ viral titer was found to be -2.5 for self-pollinated TMV infected, -3.31 for maternally TMV infected, -6.79 for paternally TMV infected, and -6.88 for self-pollinated non-infected plant. The average viral titer showed a similar trend as revealed from the infected seed percentage data (Figure 2). These data show that seed harvested from maternally TMV infected crosses produce more infected seed and seed have a higher viral titer than seed derived from paternally TMV infected crosses. The data suggests that TMV is primarily maternally seed transmitted and TMV infected pollen has little to no effect on TMV infection in seed.

Discussion

Early viral detection techniques are important for the rapid detection of pathogens that infect economically important crops. The diagnosis of plant pathogens is important for crop improvement and disease control strategies (Kumar et al. 2011). Nucleic acid based techniques are sensitive enough to detect viral presences in small plant tissue or seed samples. Previous research had to bulk seed and plant material for viral detection because of the lack of sensitive detection methods available. Wilkinson et al. (2006) found bulk seed coat samples infected with TMV from a range of 71% to 100% via ELISA. Pooled seedling samples grown from seed collected from TMV infected plants ranged from 2% to 77% TMV infection (Wilkinson et al. 2006). Although this research demonstrated seed transmission of TMV in flue-cured tobacco, the rate of transmission could not be determined because bulked samples were used. Other previous seed transmission work reported TMV infection from seed to seedlings to be 1% to 10% in tomatoes and pepper plants (Chitra et al. 1999). Most of the current literature on seed transmission of plant viruses examines the seed transmission percentages from seed to seedling. Previous ELISA research demonstrated TMV infection in bulked K 326 seedlings from maternally TMV infected crosses to be 80% and 100% (Brown et al. 2014). K 326 seedlings from paternally TMV infected crosses demonstrated TMV infection to be 0% to 18% (Brown et al. 2014). Brown et al. (2014) presented ELISA data that revealed significantly higher percent TMV positive seedling samples in maternally TMV infected crosses compared to paternally TMV infected crosses. There is no current literature that studies maternal and paternal transmission of plant viruses for the individual seed level. This study is the first report of TMV being expressed as a maternally seed transmitted virus.

Results indicate that the majority of TMV infected seed were harvested from maternally TMV infected K 326 tobacco plants. The self-pollinated TMV infected plants and the maternally TMV infected crosses result in similar TMV seed infection rates. Data suggests that the maternal tissue is responsible for a majority of the TMV seed infection rate. In contrast, paternally TMV infected crosses and self-pollinated non-infected plants shared common results, where lesser amount of TMV is observed. The lack of TMV infection of seed in paternally TMV infected crosses suggests that pollen has little to no effect on TMV transmission in seed. In regards to TMV Ct values of paternally TMV infected crosses and self-pollinated non-infected plants, the Ct values that were found to be below the designated Ct value threshold had extremely low 18*S* values, resulting in very low viral titer concentrations. This data provides insight into how TMV is transmitted through seed. These findings may be useful in identifying the TMV infection route of entry in emerging tobacco seedlings.

Literature Cited

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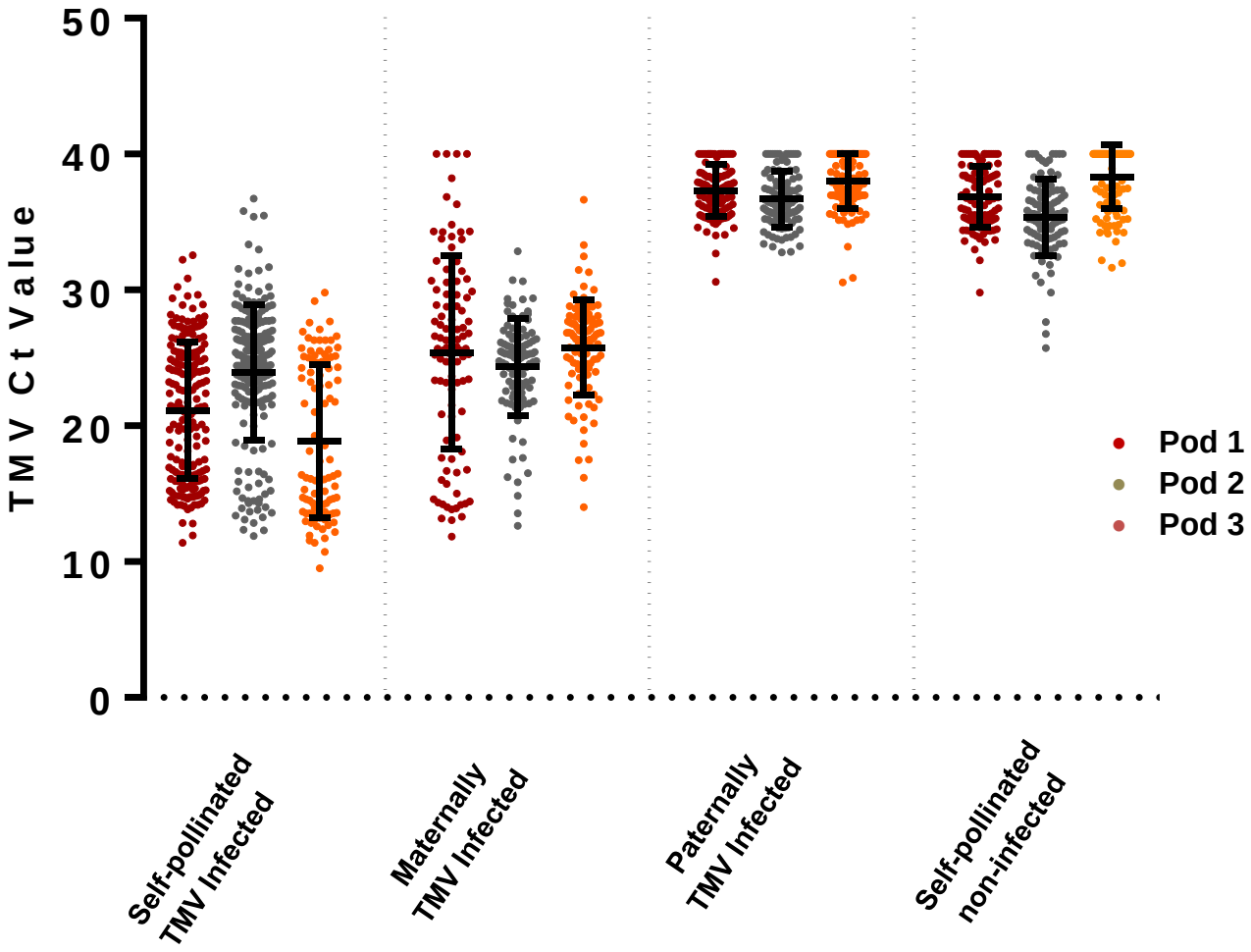
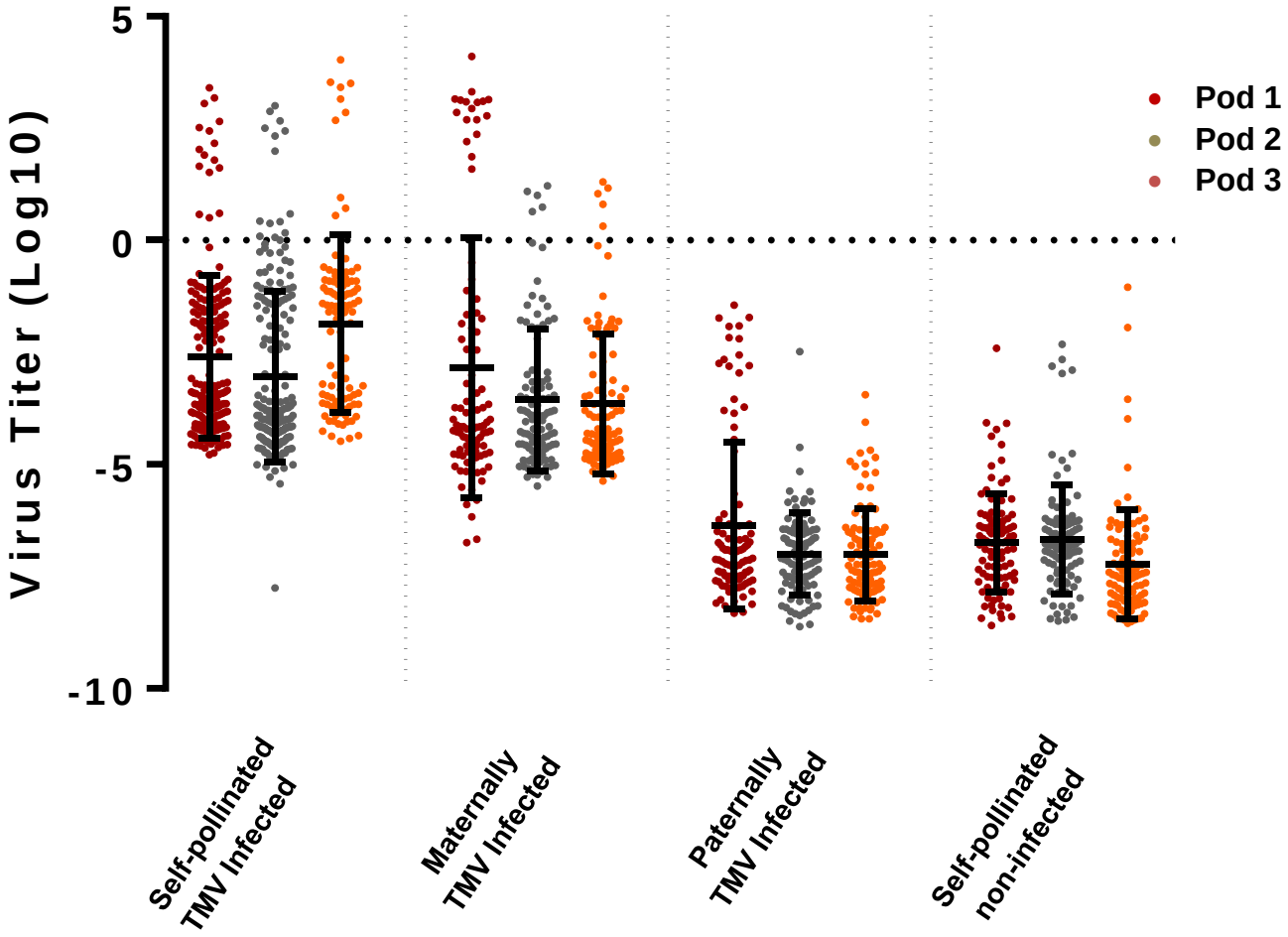
Figure 1. *Tobacco mosaic virus* (TMV) cycle threshold (Ct) value of infected seed samples from four K 326 cultivar flue-cured tobacco crosses generated by RT-qPCR.



Sample size was 100 seed for all pods with the exception of self-pollinated TMV infected pods 1 and 2, where 200 seed were examined. The average Ct value for each pod of all four K 326 cultivar tobacco crosses is indicated by the middle black bar; error bars indicate the variability of the data.

Figure 2. *Tobacco mosaic virus* (TMV) viral titer of infected seed samples from four K 326 cultivar flue-cured tobacco crosses generated by RT-qPCR.



Sample size was 100 seed for all pods with the exception of self-pollinated TMV infected pods 1 and 2, where 200 seed were examined. The average viral titer concentration for each pod of all four K 326 cultivar tobacco crosses is indicated by the middle black bar; error bars indicate the variability of the data.