Quantitative Real-Time PCR Analysis of Individual Flue-Cured Tobacco Seeds and Seedlings Reveals Seed Transmission of Tobacco Mosaic Virus

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ABSTRACT

Tobacco mosaic virus (TMV) is an extensively studied RNA virus known to infect tobacco (*Nicotiana tabacum*) and other solanaceous crops. TMV has been classified as a seedborne virus in tobacco, with infection of developing seedlings thought to occur from contact with the TMV-infected seed coat. The mechanism of TMV transmission through seed was studied in seed of the K 326 cultivar of flue-cured tobacco. Cross pollinations were performed to determine the effect of parental tissue on TMV infection in seed. Dissection of individual tobacco seeds into seed coat, endosperm, and embryo was performed to determine TMV location within a seed, while germination tests and separation of the developing seedling into seed coat, roots, and cotyledons were conducted to estimate the percent transmission of TMV. A reverse-transcriptase quantitative PCR (RT-qPCR) assay was developed and used to determine TMV concentrations in individual seed harvested from pods that formed on plants from TMV-infected and noninfected crosses. The results showed maternal transmission of TMV to

tobacco seed and seedlings that developed from infected seed, not paternal transmission. RT-qPCR and endpoint PCR assays were also conducted on the separated seed coat, endosperm, and embryo of individual seed and separated cotyledons, roots, and seed coats of individual seedlings that developed from infected tobacco seed to identify the location of the virus in the seed and the subsequent path the virus takes to infect the developing seedling. RT-qPCR and endpoint PCR assay results showed evidence of TMV infection in the endosperm and embryo, as well as in the developing seedling roots and cotyledons within 10 days of initiating seed germination. To our knowledge, this is the first report of TMV being detected in embryos of tobacco seed, demonstrating that TMV is seedborne and seed-transmitted in flue-cured tobacco.

Keywords: RT-qPCR, seedborne transmission, seed transmission, *Tobacco mosaic virus*, virology

Tobacco mosaic virus (TMV) is a single stranded, positive-sense RNA virus that causes detrimental crop losses worldwide (Lewis and Rose 2010). It is one of the most recognizable and economically important viruses infecting tobacco (*Nicotiana tabacum* L.), tomato (*Solanum lycopersicum* L.), pepper (*Capsicum annuum* L.), and other members of the Solanaceae family (Scholthof 2004). TMV is mechanically transmitted, which allows for the virus to be readily transmissible to an entire crop. Host plants infected with TMV usually exhibit visual symptoms such as a mosaic pattern on infected leaves, leaf curling, and stunted growth, thus reducing the yield and quality of the crop. Crop losses due to TMV have been upward of 2% in multiple states, ultimately resulting in more than \$10 million in losses per year for U.S. tobacco growers (Melton et al. 2000).

TMV is one of the simplest viruses known but its densely packed structural features also make it one of the most stable viruses (Dorokhov et al. 2018). The virus is able to overwinter in the seed coat and plant debris, where it can remain dormant for decades (Gooding 1986). TMV is very readily transmitted between plants, making it very difficult to control. Any sort of breach on the leaf's surface facilitates an easy entry point for the virus and, once a plant

becomes infected, it will always harbor the virus (Scholthof 2004). There are multiple ways to reduce transmission of TMV such as sanitation of equipment and tools and rogueing of infected plants but the most effective means of TMV management is through the use of resistant cultivars (Lewis and Rose 2010).

TMV is a member of the *Tobamovirus* genus and is known to be a seedborne virus (de Assis Filho and Sherwood 2000; Kumar et al. 2011; Maule and Wang 1996; Mink 1993). Much debate has occurred about defining TMV as a seed-transmitted virus in tobacco. Earlier reports described TMV as a seedborne virus that does not infect the embryo but, instead, is present as a contaminant on the surface of the seed coat (de Assis Filho and Sherwood 2000). More recent research (Wilkinson et al. 2006) demonstrated seed transmission of TMV by detecting the virus in bulked seed coats and samples of seedlings that developed from the seed, via enzyme-linked immunosorbent assay (ELISA), but the researchers were unable to establish a definitive TMV transmission percentage as a result of the pooled sampling method. Detection methods that are more sensitive than serological assays need to be adopted in order to detect a limited amount of TMV RNA in a given sample.

Seed transmission of TMV in tomato and pepper is common and well established but seed transmission of TMV in tobacco is unknown (Beczner et al. 1997). Cicek and Yorganci (1991) found 98, 18, and 7% seed transmission of TMV in tomato, pepper, and eggplant, respectively, and Taylor et al. (1961) found TMV in both the internal and external tissues of tomato seed harvested from infected fruit. Gilmer and Wilks (1967) found a high percentage of seed transmission of TMV in apple seed. However, Taylor et al. (1961) found that the seedlings became infected with TMV as they were being transplanted, a procedure that readily transmits the virus, whereas Gilmer and Wilks (1967) reported that the seedlings were infected prior to transplanting.

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Seed transmission of many plant viruses has been determined (Mink 1993; Sastry 2013) but the precise mechanisms of seed transmission have yet to be demonstrated for TMV in tobacco seed. In this study, we examined seed transmission of TMV in tobacco using three objectives: (i) determining the effect of maternal or paternal tissue on infection of seed by TMV (cross pollination test), (ii) localizing TMV within individual tobacco seed (seed dissection test), and (iii) establishing percent transmission of TMV in tobacco seedlings (seed germination test). A reverse-transcriptase quantitative PCR (RT-qPCR) assay was developed as part of this study to enable quantification of TMV in individual seed, seed components, and seedling tissues. Endpoint PCR assays, using primers targeting a different region of the TMV genome, were used to verify the RT-qPCR results. To our knowledge, this is the first report of the association between tobacco embryo infection and subsequent seed transmission of TMV.

MATERIALS AND METHODS

Seed production. The flue-cured tobacco cultivar K 326 was grown in fields at the Virginia Tech Southern Piedmont Agricultural Research and Extension Center (SPAREC) in Blackstone, VA, U.S.A. Seed for the cross pollination tests of TMV-infected and noninfected plants (infected maternal plant with noninfected paternal plant, and vice versa) were produced in 2016, and seed for the seed dissection and germination tests were produced in 2017. Tobacco plants used for TMV experiments were grown in a separate field 150 m from other tobacco fields. Plants were grown using standard agronomic practices for fertilization, transplanting, disease, and insect control in Virginia (Reed 2018).

Inoculation of plants. Each field-grown K 326 plant was inoculated using a sponge brush (Fisher Scientific, Pittsburgh, PA, U.S.A.) dipped in TMV inoculum or 1% potassium phosphate buffer (control treatment) and rubbed against a leaf approximately 13 cm in size. TMV inoculum was prepared by grinding 2 g of TMV-infected tobacco leaf tissue in 20 ml of 1% potassium phosphate buffer (Fisher Scientific) with 1% Celite (Fisher Scientific). The pH of the resultant sap inoculum was adjusted to 7.4 and the inoculum was filtered through cheesecloth to create a 1:10 dilution. A 0.5-ml aliquot of the filtered suspension was added to 4.5 ml of 1% potassium phosphate buffer to create a 1:100 dilution of TMV inoculum. The TMV strain used for inoculum was obtained from Dr. Sue Tolin of Virginia Tech. All plants inoculated with TMV in 2016 and 2017 were grown 150 m from other tobacco fields at SPAREC to prevent spread of TMV. Noninfected, self-pollinated plants (negative control treatment) of the flue-cured tobacco cultivar K 326 were grown in a geographically isolated field 150 m from the TMV-infected field in 2016.

Self-pollinated plants of K 326 infected with TMV served as the positive control treatment. The plants were inoculated with TMV after layby in 2016. Negative control plants were inoculated with 1% potassium phosphate buffer after layby in 2016. For the seed dissection and germination tests, three TMV-infected plants of K 326 were each inoculated with TMV after layby in 2017.

Pollination of plants for a cross pollination test. Seed pods of maternally infected crosses of K 326 were produced using virus-free pollen to pollinate the stigma of TMV-infected K 326 plants with a cotton swab. Seed pods of paternally infected plants of K 326 were produced using pollen from TMV-infected K 326 plants to pollinate the stigmas of noninfected K 326 plants with a cotton swab. Positive control and maternally infected plants were grown in the isolated field designated for TMV-infected plants. Negative control and paternally infected plants were grown in the isolated TMV-free field to minimize potential exposure to TMV inoculum. Sixteen total plants were pollinated to generate maternal and paternal crosses. One plant was selected randomly for the maternal cross and one plant was selected for the paternal cross. Cross pollination was prevented by enclosing the terminal inflorescence

in a Delnet pollinating bag (Delstar Technology Inc., Middletown, DE, U.S.A.). Three pods were collected from each plant for individual seed analysis.

Collection of seed. Mature individual seed pods for each test were collected on a weekly basis. The seed from each pod were separated by size using 600-, 500-, and 425- μ m sieves (Gilson, Middleton, WI, U.S.A.). Trash from the seed was retained on the 600- μ m sieve, large seed on the 500- μ m sieve, medium seed on the 425- μ m sieve, and a catch-pan at the very bottom collected the small seed. All seed from each size were kept in separate envelopes but only large seed were used for each test.

For the cross-pollination tests, three mature seed pods (pods 1, 2, and 3) were selected randomly from each of the positive control, negative control, TMV-infected K 326 $\times$ noninfected K 326 (maternally infected), and noninfected K 326 $\times$ TMV-infected K 326 (paternally infected) plants. For the seed dissection and germination tests, three seed pods (pods A, B, and C) were sampled randomly from each of three infected plants (plants 1, 2, and 3).

Seed dissection. Seed were soaked in a 1.5-ml microcentrifuge tube (USA Scientific, Ocala, FL, U.S.A.) containing sterilized deionized water at room temperature for 24 h prior to dissection to allow for easy removal of the seed coat. The separation process was done under a dissecting microscope (Meiji Techno, San Jose, CA, U.S.A.) and consisted of four steps: (i) removal of the seed coat, (ii) washing of the endosperm with the embryo still inside to remove remnant pieces of seed coat adhering to the endosperm, (iii) removal of the embryo from the endosperm, and (iv) washing of the embryo (Supplementary Fig. S1). A 24-well microtiter plate (USA Scientific) was filled with sterilized deionized water and was used for all of the wash steps. All utensils were dipped in 95% ethanol and flame sterilized in between each step to prevent cross contamination.

A P49.0-cm-diameter, medium-fine porosity piece of filter paper (Fisher Scientific) was soaked with sterilized deionized water and placed under the microscope. A single seed was removed from a microcentrifuge tube and placed on the wet filter paper on the microscope. The seed coat was removed using 114-mm straight, superfine pointed forceps (Fisher Scientific) to hold the seed and a microscalpel (PFM Medical Standard Incision Blade, Köln, Germany) to scrape off the seed coat gently. The seed coat was then placed inside one of the 1.5-ml microcentrifuge tubes. The endosperm with the embryo still inside was washed in one of the sterilized deionized water-filled wells for 30 s to ensure that there were no remaining pieces of seed coat on the outside of the endosperm. The seed was removed from the water and placed back under the microscope. The embryo was removed from the endosperm by gentle application of pressure with the microscalpel, and was washed for 30 s in a different sterilized deionized water-filled well than the well previously used. The separated endosperm and embryo were placed in different 1.5-ml microcentrifuge tubes. All three seed components were placed on ice immediately after separation and stored at -20°C until they were used for RNA extraction. Data were collected from 100 seeds for pod A of all three plants, and from 50 seed for pods B and C of all three plants.

Seed germination. Germination of TMV-infected seed was evaluated for 100 seeds from pod A and 50 seeds from pods B and C in a growth chamber (Sanyo Scientific, Wood Dale, IL, U.S.A.) with cycles of 16 h of light and 8 h of darkness at 22°C and 70% relative humidity for 10 days (Reed 2018). To serve as a control treatment, 50 seeds from healthy plants of the K 326 flue-cured tobacco cultivar were germinated using the same conditions as the TMV-infected seed. Seed were placed on 8.255-cm-diameter blotter paper (Hoffman Manufacturing, Inc., Corvallis, OR, U.S.A.) dampened with sterilized deionized water in a Petri dish 100 mm in diameter by 15 mm tall (Fisher Scientific) and the forceps were flame sterilized between each seed to minimize contamination. Seed were considered to have germinated if cotyledons and primary roots were observed. Germinated seed were counted and dissected into three

parts: the seed coat, cotyledons, and root (Supplementary Fig. S2). Forceps and microscalpels were flame sterilized between each cut to prevent cross contamination. Plant samples were placed in separate 1.5-ml microcentrifuge tubes and stored at -80°C prior to being assayed for virus by RT-qPCR assay and endpoint PCR assays.

RNA extraction. Seed, separated seed tissues, and separated seedling tissues were each ground using a DeWalt 18V power drill and polypropylene pellet pestles (USA Scientific). Total RNA of seed and seed tissues were extracted using TRIzol (Life Technologies, Carlsbad, CA, U.S.A.) in conjunction with the Direct-zol RNA MicroPrep Kit (Zymo Research, Irvine, CA, U.S.A.) following the manufacturer's instructions. Total RNA of seedling tissues were extracted using TRIzol with the Direct-zol RNA MiniPrep Plus Kit (Zymo Research) following the manufacturer's instructions. The extracted RNA was eluted into RNase-free microcentrifuge tubes and stored at -20°C until use for cDNA synthesis. Total RNA yield was typically $<1\text{ ng}/\mu\text{l}$ for each sample.

cDNA synthesis. The RNA-primer annealing reaction mixture consisted of a total volume of $12\text{ }\mu\text{l}$ and contained $6\text{ }\mu\text{l}$ of sterilized deionized water, $1\text{ }\mu\text{l}$ of 10 mM dNTPs (New England Biolabs, Ipswich, MA, U.S.A.), $2\text{ }\mu\text{l}$ of $60\text{ }\mu\text{M}$ random hexamer primers (Thermo Fisher, Waltham, MA, U.S.A.), and $3\text{ }\mu\text{l}$ of RNA sample in a 0.5-ml microcentrifuge tube. The reaction mixture was vortexed and incubated for 5 min at 70°C to denature RNA, followed by incubation on ice for $3\text{ to }4\text{ min}$ to anneal the primers. To complete the first-strand cDNA reaction mixture, $4\text{ }\mu\text{l}$ of $5\times$ ProtoScript buffer, $2\text{ }\mu\text{l}$ of 0.1 M dithiothreitol, $1\text{ }\mu\text{l}$ of Murine Rnase inhibitor ($40\text{ U}/\mu\text{l}$), and $1\text{ }\mu\text{l}$ of Protoscript II Reverse transcription ($200\text{ U}/\mu\text{l}$) (all from New England Biolabs) were added to the tube and vortexed. Samples were incubated for 5 min at room temperature, followed by 45 min at 42°C and 5 min at 80°C to denature the reverse transcription. After incubation, cDNA samples were stored at -20°C .

RT-qPCR. Quantitative data were collected using a Thermo Fisher QuantStudio 3 real-time PCR instrument (Thermo Fisher). Forward and reverse primers (Eurofin Genomics, Louisville, KY, U.S.A.) were used to amplify target TMV genome-specific sequences (TMV primer working solution) and *N. tabacum* 18S ribosomal RNA internal control sequences (18S primer working solution). TMV primers were designed to conserve TMV sequences within the polymerase open reading frame (ORF) (Liu and Nelson 2013). This target was chosen to represent true viral genome abundance versus other ORFs that also measure gene expression because they are expressed via subgenomic RNAs. The TMV primers were forward, $5'\text{-GGATATGTCTAAGTCTGTTGC-}3'$, and reverse, $5'\text{-CAGACAACCTCGGGTGCG-}3'$. The internal 18S rRNA control primer set was forward, $5'\text{-CGGCGATGCGCTCCTG-}3'$, and reverse, $5'\text{-TACAGAGCGTAGGCTTGCTTTG-}3'$. Both primer sets had amplification efficiencies of 89% . Each TMV primer reaction mixture consisted of $8.4\text{ }\mu\text{l}$ of sterilized deionized water, $0.3\text{ }\mu\text{l}$ of TMV forward primer ($10\text{ pmol}/\mu\text{l}$), $0.3\text{ }\mu\text{l}$ of TMV reverse primer ($10\text{ pmol}/\mu\text{l}$), and $10\text{ }\mu\text{l}$ of Fast SYBR Green Master Mix (Thermo Fisher) for a total volume of $19\text{ }\mu\text{l}$. Each 18S primer reaction mixture consisted of $8.4\text{ }\mu\text{l}$ of sterilized deionized water, $0.3\text{ }\mu\text{l}$ of 18S forward primer ($10\text{ pmol}/\mu\text{l}$), $0.3\text{ }\mu\text{l}$ of 18S reverse primer ($10\text{ pmol}/\mu\text{l}$), and $10\text{ }\mu\text{l}$ of Fast SYBR Green Master Mix for a total volume of $19\text{ }\mu\text{l}$. Primer mixtures were aliquoted into microtiter plates in triplicate for each sample ($19\text{ }\mu\text{l}$ per well by three wells per sample).

Assays were performed in 96-well optical PCR plates sealed with optical film (Applied Biosystems, Foster City, CA, U.S.A.). Fourteen samples were run in triplicate for both TMV and 18S primers. Appropriate positive (cDNA from TMV-infected leaf tissue) and negative (sterilized deionized water) control samples were used in all PCR experiments. Plates were processed using a QuantStudio 3 Real-Time PCR system. RT-qPCR conditions were as follows: 1 cycle at 95°C for 20 s , 40 cycles at 95°C for 1 s , 40

cycles at 60°C for 20 s , 1 cycle at 95°C for 15 s , 1 cycle at 60°C for 1 min , and 1 cycle at 95°C for 15 s . The average cycle threshold (Ct) values were recorded for each sample using the standards calculated by QuantStudio Design and Analysis Software v1.4 (Thermo Fisher).

Endpoint PCR and gel electrophoresis. To validate the results from the RT-qPCR assays, and to determine a conservative infection threshold value for seed tissues and seedling tissues, endpoint PCR assays were performed. Tob-Uni forward and reverse primers (Letschert et al. 2002) (Eurofin Genomics) were used to amplify a different region of the virus from that targeted in the RT-qPCR experiments. The PCR mixture in a total volume of $25\text{ }\mu\text{l}$ consisted of the following: $9.5\text{ }\mu\text{l}$ of sterilized deionized water, $1\text{ }\mu\text{l}$ of $5'$ forward primer ($10\text{ pmol}/\mu\text{l}$; TobUni2), $1\text{ }\mu\text{l}$ of $3'$ reverse primer ($10\text{ pmol}/\mu\text{l}$; TobUni1), $12.5\text{ }\mu\text{l}$ of One Taq $2\times$ Master Mix (New England Biolabs), and $1\text{ }\mu\text{l}$ of cDNA (the same cDNA used for RT-qPCR experiments). The PCR mixture was incubated in a Bio-Rad C1000 Touch Thermal Cycler (Bio-Rad, Hercules, CA, U.S.A.) using the following conditions: 94°C for 5 min , 94°C for 1 min , 60°C for 45 s , 68°C for 1 min for 40 cycles, 68°C for 5 min , and holding at 12°C .

PCR fragments were visualized by horizontal gel electrophoresis (Bio-Rad) in $1\times$ Tris-acetate EDTA buffer (Promega Corporation, Madison, WI, U.S.A.) on a 1% agarose gel (Promega Corporation) using a 100-bp DNA Ladder (New England Biolabs) to estimate the size of the fragments. The DNA ladder was loaded into the first and last well, and consisted of the following: $4\text{ }\mu\text{l}$ of sterilized deionized water, $1\text{ }\mu\text{l}$ of 100 bp DNA ladder and $1\text{ }\mu\text{l}$ of $6\times$ loading dye (New England Biolabs). Twenty samples for each seed tissue component and each seedling tissue component were prepared using a mixture of $2\text{ }\mu\text{l}$ of sterilized deionized water, $3\text{ }\mu\text{l}$ of PCR mixture, and $1\text{ }\mu\text{l}$ of $6\times$ loading dye per sample. Appropriate negative (sterilized deionized water) and positive (cDNA from a TMV-infected seed) control samples were used for electrophoresis. Samples were electrophoresed at 90 V for 45 min , and stained with ethidium bromide (Sigma Aldrich, St. Louis, MO, U.S.A.) at $2\text{ }\mu\text{g}/\text{ml}$ followed by destaining in sterilized deionized water for $20\text{ to }30\text{ min}$. Gels were examined in a Gel Doc-It system (UVP, Upland, CA, U.S.A.) and samples were considered positive for TMV if a well-defined band was observed.

Data analysis. Quantitative data from the RT-qPCR assays were transferred into QuantStudio Design and Analysis Software v1.4 (Thermo Fisher). Mean TMV Ct values and mean 18S Ct values were transferred into Microsoft Office 365 ProPlus Excel for Windows (Excel; Microsoft, Redmond, WA, U.S.A.). Data from all plants and pods were combined for analysis. Mean Ct values for seed, seed tissues, and seedling tissues were copied into GraphPad Prism 7.04 for Windows (GraphPad Prism, San Diego, CA, U.S.A.) to construct tree graphs.

The relative abundance of TMV RNA was calculated according to Pfaffl (2001), assuming 89% amplification efficiencies for each primer pair. Normalized 18S, normalized virus ratio, and $\log_{10}$ transformation virus values were calculated in Excel as Normalized 18S ($N18S$) = $(1.89/\text{mean Ct } 18S)$, Normalized virus (NV) = $(N18S/1.89^{\text{mean Ct virus}})$, and Virus titer = $\log_{10}(NV)$.

$\log_{10}$ values for seed, seed tissues, and seedling tissues were copied into GraphPad Prism to construct tree graphs. The incidence (%) of germination of tobacco seedlings was calculated by dividing the number of seed that germinated by the total number of seed sown. The incidence (%) of TMV infection detected in each seed tissue component and each seedling tissue component was calculated using conservative Ct threshold values generated from the endpoint RT-PCR assays. Any samples below the designated Ct value were considered positive and the incidence (%) of infection was calculated based on the number of positive samples for each tissue component divided by the total number of samples for each tissue component.

GraphPad Prism was used for statistical analysis of virus titer means among plant components within each pod using an analysis of variance (ANOVA) and Tukey's multiple comparisons of the $\log_{10}$ normalized data. SAS 9.4 for Windows (SAS Institute Inc., Cary, NC, U.S.A.) was used for statistical comparison of plant components among plants and pods by ANOVA and Tukey's multiple comparisons of the $\log_{10}$ normalized data. *P* values < 0.05 were considered significant.

RESULTS

TMV detection in cross-pollination tests. The threshold for positive samples was arbitrarily defined at a Ct value of 32 based on the range of values for negative control plants (Fig. 1). Of 500 seeds analyzed from the positive control plants, 98.6% were infected with

TMV (Supplementary Table S1). Of 300 seeds analyzed from the maternally infected cross, 93% were infected with TMV. Seed samples from the paternally infected cross had a 0.006% incidence of infection, and seed samples from the negative control plants had 0.03% incidence of TMV infection. These results demonstrated a greater percentage of TMV in seed from the positive control plants and maternally infected crosses compared with seed from the paternally infected crosses and negative control plants (Fig. 1). The TMV and 18S Ct values were compared for each sample to determine a normalized viral titer ratio among the tobacco crosses. The average $\log_{10}$ viral titer was -2.5 for positive control plants, -3.31 for maternally infected plants, -6.79 for paternally infected plants, and -6.88 for the negative control plants (data not shown). The relative viral titer distribution was similar across all samples (Fig. 2). Seed pods harvested from maternally infected crosses had a

Fig. 1. Tobacco mosaic virus (TMV) cycle threshold (Ct) value of infected seed samples from four K 326 cultivar flue-cured tobacco crosses generated by reverse-transcriptase quantitative PCR assays. Error bars represent the mean and standard deviation of the data.

Fig. 2. Tobacco mosaic virus (TMV) viral titer value of infected seed samples from four K 326 cultivar flue-cured tobacco crosses generated by reverse-transcriptase quantitative PCR assays. Error bars represent the mean and standard deviation of the data.

greater percentage of infected seed than seed pods harvested from paternally infected crosses. The data suggest that TMV is primarily seed transmitted maternally, and paternal (pollen) sources of TMV have little to no effect on TMV infection of tobacco seed.

Statistical analysis for seed dissection and germination tests. Highly significant ($P < 0.0001$) differences among plants and pods were observed for relative TMV titer for the seed coats, endosperms, and embryos in the seed dissection test (Table 1). There were no significant differences among plants for relative virus titer in cotyledons, roots, or seed coats in the seed germination test (Table 1). In contrast, highly significant differences ($P < 0.0001$) were observed among pods for relative virus titer in all three seedling components. There were highly significant ($P < 0.0001$) plant-pod interactions for relative virus titer in seed and seedling components. Therefore, data were presented for individual pods (Table 1).

ANOVAs also showed highly significant differences in TMV titer among the seed coats, endosperms, and embryos within a pod (Table 2). Highly significant ($P < 0.001$) differences in relative TMV titer among roots, cotyledons, and seed coats within each pod were also observed (Table 3). Seed components within each individual pod were found to be highly significantly different in terms of TMV titer ($P < 0.0001$). Mean relative virus titers for the seed coats were significantly greater than for the endosperms, which were significantly greater than that of the embryos in each pod (Table 4). Seed coats, endosperms, and embryos had mean relative $\log_{10}$ virus titer ranges of -2.564 to 1.074 , -5.883 to 1.162 ,

and -7.038 to -3.589 , respectively. Within each pod, there was a statistically significant difference among seedling components for virus titer as determined by one-way ANOVA (Table 4). Seed coats had a significantly greater relative viral titer than cotyledons and roots in all pods. Roots had a significantly greater relative virus titer than cotyledons in four pods (1A, 1C, 2C, and 3A). There was no significant difference in relative viral titer between cotyledons and roots for the other five pods (Table 4).

Location of TMV in tobacco seed. RT-qPCR results indicated that TMV was detected in each of the seed and seedling components. A similar concentration gradient of TMV accumulation was detected across all tobacco pods and plants tested, with the greatest viral concentrations detected in the seed coats, followed by the endosperms and the embryos (Fig. 3). Mean Ct values for all seed components showed a slightly greater TMV concentration from pods B and C of plant 1 compared with pod A of the same plant (Fig. 3A). Overall, seed from pod B of plant 2 had lower Ct values, indicating greater TMV concentrations compared with seed from pods A and C (Fig. 3B). Seed from all three pods sampled from plant 3 had similar Ct values (Fig. 3C).

Seed had a Ct value range from 15 to 35. Gel results showed fluorescent bands on all nine gels for the endpoint PCR assays, indicating TMV accumulation in all three seed components (Fig. 4; 2 of 9 gels depicted). Generally, samples with Ct values from 15 to 31 also expressed a band of amplified DNA by endpoint PCR assay, demonstrating that TMV was present, whereas seed samples with Ct values > 31 did not show DNA bands, indicating that TMV was not detected.

Relative viral titers for each seed component showed the same trend as Ct values: the seed coats had the greatest relative viral

TABLE 1. Analysis of variance of tobacco mosaic virus (TMV) virus titer for tobacco seed and seedling components of K 326 flue-cured tobacco plants infected with TMV^z

Component, sources of variation	df	P value
Embryo		
Plant	2	<0.0001
Pod	2	<0.0001
Plant $\times$ pod	4	<0.0001
Error	591	...
Total	599	...
Endosperm		
Plant	2	<0.0001
Pod	2	<0.0001
Plant $\times$ pod	4	<0.0001
Error	591	...
Total	599	...
Seed coat		
Plant	2	<0.0001
Pod	2	<0.0001
Plant $\times$ pod	4	<0.0001
Error	591	...
Total	599	...
Cotyledon		
Plant	2	0.5996
Pod	2	<0.0001
Plant $\times$ pod	4	<0.0001
Error	591	...
Total	599	...
Root		
Plant	2	0.6157
Pod	2	0.0012
Plant $\times$ pod	4	0.0019
Error	591	...
Total	599	...
Seed coat		
Plant	2	0.5928
Pod	2	<0.0001
Plant $\times$ pod	4	<0.0001
Error	591	...
Total	599	...

^z Tobacco seed were collected in 2017 from field-grown K 326 flue-cured tobacco cultivar infected with TMV. Tobacco seedling components were separated 10 days after seeding and evaluated for TMV by reverse-transcriptase quantitative PCR.

TABLE 2. One-way analysis of variance comparing $\log_{10}$ values of the seed coats, endosperms, and embryos from each tobacco seed collected from each individual pod that was harvested from K 326 flue-cured tobacco plants infected with tobacco mosaic virus (TMV)^z

Plant, pod	Sources of variation	df	P value
1			
A	Seed component	2	<0.001
	Error	296	...
	Total	298	...
B	Seed component	2	<0.001
	Error	146	...
	Total	148	...
C	Seed component	2	<0.001
	Error	147	...
	Total	149	...
2			
A	Seed component	2	<0.001
	Error	296	...
	Total	299	...
B	Seed component	2	<0.001
	Error	144	...
	Total	146	...
C	Seed component	2	<0.001
	Error	147	...
	Total	149	...
3			
A	Seed component	2	<0.001
	Error	296	...
	Total	298	...
B	Seed component	2	<0.001
	Error	147	...
	Total	149	...
C	Seed component	2	<0.001
	Error	147	...
	Total	149	...

^z Data represent results of one-way analysis of variance using $\log_{10}$ -transformed data from each pod. The three seed components were the seed coat, endosperm, and embryo from each seed sampled from the individual pods. Seed were collected in 2017 from field-grown K 326 flue-cured tobacco cultivar infected with TMV. Pod A, $n = 300$; pods B and C, $n = 150$.

concentration, followed by the endosperms and embryos (Fig. 5). For two of the nine pods, pod B of each of plants 1 and 2 demonstrated less variability in $\log_{10}$ relative viral titers for seed within a pod. However, the other seven pods had greater variability among seed within a pod (Fig. 5). Plants and pods were selected randomly; therefore, there was no significance to less variability in TMV detection in seed sampled from pod B of the two plants. All samples with relative $\log_{10}$ viral titers > 0 were considered TMV positive because this indicated relative TMV levels greater than 18S rRNA levels. In general, more of the seed coats tested positive for TMV than both the endosperms and embryos (Fig. 5).

TABLE 3. One-way analysis of variance from comparing $\log_{10}$ values of cotyledons, roots, and seed coats from each seed collected from each individual pod that was harvested from K 326 flue-cured tobacco plants infected with tobacco mosaic virus (TMV)^z

Plant, pod	Sources of variation	df	P value
1			
A	Seedling component	2	<0.001
	Error	297	
	Total	299	
B	Seedling component	2	<0.001
	Error	147	
	Total	149	
C	Seedling component	2	<0.001
	Error	147	
	Total	149	
2			
A	Seedling component	2	<0.001
	Error	297	
	Total	299	
B	Seedling component	2	<0.001
	Error	147	
	Total	149	
C	Seedling component	2	<0.001
	Error	147	
	Total	149	
3			
A	Seedling component	2	<0.001
	Error	297	
	Total	299	
B	Seedling component	2	<0.001
	Residual	147	
	Total	149	
C	Seedling component	2	<0.001
	Residual	147	
	Total	149	

^z Seedling components were separated 10 days after seeding and evaluated for TMV by reverse-transcriptase quantitative PCR. Seed were collected in 2017 from field-grown K 326 flue-cured tobacco cultivar infected with TMV. Seedling components were the roots, cotyledons, and seed coats from each germinated seed. Pod A, $n = 300$; pods B and C, $n = 150$.

Detection of TMV in seed germination test. Healthy flue-cured seed of the tobacco cultivar K 326 had a germination incidence of 92% (data not shown). Germination percentages varied across plants and pods; seed from pod B of plant 1 had the greatest germination at 94%, and seed from pod C of plant 3 had the lowest germination at 50% (data not shown). Seed from all plants and pods, except for pod B of plant 1, had a germination percentage less than that of the control plants ($<92\%$). TMV Ct values had a consistent distribution across all plants and pods (Fig. 6). The seed coats had lower TMV Ct values compared with cotyledons and roots for all plants and pods. Standard deviation showed that seed coats had the most variation in mean TMV Ct values of the seedling components for all plants and pods. Ct values for cotyledons and roots were similar for seed harvested from all pods.

A range of TMV Ct values from 15 to 35 was used to establish a conservative threshold for TMV-infected samples. All samples that displayed a DNA band were considered positive for TMV. When testing the range of TMV Ct values by endpoint RT-PCR assay, amplified product was visible in the endpoint assay for all three tobacco seedling components. These results validated the presence of virus in the cotyledons, roots, and seed coats. According to endpoint RT-PCR results, the maximum TMV Ct value detected for positive cotyledon samples was 29.596 (Fig. 4B). A sample with a Ct value of 30.576 was negative; therefore, samples with Ct values > 30 were considered negative. Roots had a maximum mean TMV Ct value of 30.729 to detect a positive sample. A sample with a Ct value of 31.494 was negative; therefore, any Ct value > 31 was considered negative. Seed coats had a maximum mean TMV Ct value of 31.851 for positive samples. A sample with a Ct value of 33.251 was negative; therefore, any samples with a Ct value > 33 were considered negative.

Relative virus titer was $\log_{10}$ transformed in order to make trends more easily discernible. RT-qPCR data showed a similar relative virus titer distribution across all samples (Fig. 7). The seed coats had greater relative virus titers compared with cotyledons and roots for all plants and pods. Virus titer for cotyledons and roots were similar in all pods. Standard deviation showed that seed coats had the most variation in TMV titers of the plant components assayed (Fig. 7).

Percent TMV infection of seed and seedling components. Endpoint PCR results determined a conservative Ct value threshold for the seed component samples: 30 for seed coats, 32 for endosperms, and 31 for embryos, meaning that samples with less than or equal to the threshold value were positive for TMV. The incidence for each component infected with TMV was calculated using the threshold Ct values determined from endpoint PCR (Table 5). Of 1,800 seed component samples, 1,201 were considered to be infected with TMV: 81% of the seed coats, 77% of the endosperms, and 48% of the embryos (Supplementary Table S2). This indicated that, although more seed coats were infected with TMV, infection was still prevalent in the embryos. The number

TABLE 4. Tobacco mosaic virus (TMV) viral titer ($\log_{10}$ of normalized virus) means for each tobacco seed and seedling component of K 326 flue-cured tobacco plants infected with TMV^z

Plant	Pod	Seed dissection test			Seed germination test		
		Embryo	Endosperm	Seed coat	Cotyledon	Root	Seed coat
1	A	-5.965 c	-3.606 b	-0.738 a	-4.218 c	-3.749 b	-0.051 a
1	B	-4.415 c	-1.684 b	0.518 a	-3.957 b	-3.759 b	-0.297 a
1	C	-3.589 c	-1.384 b	0.659 a	-4.768 c	-4.015 b	-0.843 a
2	A	-6.050 c	-4.709 b	-2.127 a	-3.939 b	-3.521 b	-0.217 a
2	B	-4.061 c	-1.162 b	1.074 a	-3.419 b	-3.924 b	1.771 a
2	C	-7.038 c	-4.914 b	-2.564 a	-5.624 c	-4.330 b	-1.896 a
3	A	-5.910 c	-3.878 b	-1.224 a	-4.387 c	-3.506 b	-0.135 a
3	B	-5.226 c	-3.383 b	-0.226 a	-4.597 b	-4.333 b	0.631 a
3	C	-5.883 c	-5.883 b	-1.350 a	-4.059 b	-3.502 b	-1.047 a

^z Seed were collected in 2017 from field-grown K 326 flue-cured tobacco cultivar infected with TMV. Seedling components were separated 10 days after seeding. Pod A, $n = 300$; pods B and C, $n = 150$. Means within a row within a test followed by the same letter are not significantly different at the 0.05 probability level using the Tukey test.

of seed coats infected with TMV ranged from 52% for pod C of plant 2 to 100% for pod C of plant 1 and pod B of plant 2. The incidence of infected endosperm samples ranged from 40% for pod C of plant 3 to 100% for pod C of plant 1 and pod B of plant 2. The incidence of infected embryos ranged from 14% from pod C of plant 2 to 94% from pod C of plant 1.

The incidence of seedling components infected with TMV was calculated using TMV Ct cutoff values for each component (the

cotyledon Ct < 30, root Ct < 31, and seed coat Ct < 33) (Supplementary Table S3). All samples that were below the cutoff were considered infected with TMV. The incidence of cotyledons infected ranged from 2% of the seedlings grown from seed harvested from pod B of plant 3 to 24% of the seedlings that grew from the seed harvested from pod B of plant 1 (Table 5). The incidence of TMV infection of roots ranged from 8% of the seedlings grown from seed harvested from pod A of plant 2 and pod

Fig. 3. Determination of tobacco mosaic virus (TMV) cycle threshold (Ct) value of infected K 326 flue-cured tobacco seed component samples generated by reverse-transcriptase quantitative PCR assays for **A**, plant 1; **B**, plant 2; and **C**, plant 3. Tobacco plants were grown in the field in 2017 and infected with TMV. Error bars represent the mean and standard deviation of the data.

C of plant 2 to 40% of seedlings that grew from seed harvested from pod B of plant 1. Seed coats had a percent infection range from 30% in seed harvested from pod C of plant 2 to 100% in seed harvested from pod B of plant 2 (Table 5).

DISCUSSION

Viral detection techniques are important during the early developmental stages of a plant for the rapid detection of pathogens that infect economically important crops (Kumar et al. 2011). The diagnosis of plant pathogens is important for crop improvement and disease control strategies (Kumar et al. 2011). Biological assays can be time consuming and sometimes can lead to ambiguous results (Gumus and Paylan 2013). Serological assays such as ELISA have previously been used to detect TMV in bulk tobacco samples but were not sensitive enough to be utilized on individual seed or seedling components containing limited amounts of virus (Wilkinson et al. 2006). Nucleic-acid-based analysis such as endpoint RT-PCR assays and RT-qPCR assays are among the most sensitive techniques for detecting small amounts of nucleic acids, and can be used to quantify relative gene expression (Fraga et al. 2014). Similar to TMV, there was limited evidence that Cucumber mosaic virus (CMV) was seedborne and seed transmitted on pepper seed. Ali and Kobayashi (2010) germinated 200 seeds for 7 to 8 days and found a 29.5% incidence of seed transmission based on CMV-infected seedlings and 59.5% incidence of infected seed coats using a RT-PCR assay. In another test, 108 seeds germinated from CMV-infected plants and sampled 4 weeks postgermination revealed that none of the plants showed symptoms of CMV but 12% tested positive for CMV by RT-PCR assay, which confirmed that CMV was seedborne and seed transmitted (Ali and Kobayashi 2010). In this TMV tobacco study, we report for the first time the use of an RT-qPCR assay as a diagnostic tool to detect TMV in individual flue-cured tobacco seed and seedlings to confirm seed transmission.

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 had TMV infection incidences ranging from 0 to 18% (Brown et al. 2014). Brown et al. (2014) presented ELISA data that revealed a significantly greater percentage of TMV-positive seedling samples from maternally TMV-infected crosses compared with paternally TMV-infected crosses. To our knowledge, there have been few studies that examined maternal versus paternal influences on seed transmission of plant viruses (Harth et al. 2017; Liu et al. 2014; Yang et al. 1997) at the individual seed level. This study is the first report using nucleic acid assays to confirm TMV as a maternally seed-transmitted virus.

Results indicated that the majority of TMV-infected seed were harvested from maternally infected K 326 tobacco plants. The positive control plants and the maternally infected crosses resulted in similar incidences of TMV seed infection. These data suggest that the maternal tissue is responsible for a majority of TMV seed infection. In contrast, paternally infected crosses and negative control plants in this study had similar results, with a smaller percentage of seed, if any, displaying a positive result for TMV in the RT-qPCR assay. The few positive samples may have been due to mispriming during the PCR and potential cross contamination. Overall, the distribution of the data are most illustrative for the crosses. The lack of TMV infection of seed harvested from paternally infected crosses suggested that infected pollen had little to no effect on TMV infection of seed and seed transmission despite the greater incidence of infected seed harvested from the positive control plants that were self pollinated with TMV-infected pollen. The TMV Ct values of paternally infected crosses and negative control plants were below the designated Ct value threshold. This was often due to extremely low 18S values, resulting in very low relative viral titer concentrations. In 147 cases, the viral Ct values could not be measured; in those incidences, the Ct value for TMV

was arbitrarily assigned a value of 40. If the 18S Ct values were also high, this would lead to a false-positive result. These data provide insight into how TMV is transmitted in tobacco seed.

Seed transmission of TMV in tobacco has been a controversial topic for years. Seed transmission of a virus is commonly associated with embryo infection (de Assis Filho and Sherwood 2000; Mink 1993). It has generally been accepted that TMV is seedborne but not seed transmitted in tobacco because most previous research was only able to find the virus on the seed coat, which did not always lead to an infected seedling when the seed was planted. Benoit and Maury (1976) observed TMV accumulation in the seed coat but not in the endosperm or the embryo, whereas Taylor (1962) observed the virus in the seed coat as well as in the endosperm. Together, no definitive results could be concluded because they were unable to fully separate the seed tissues, thus leading to the conclusion that TMV was in the seed coat but not in the embryo. The results presented in this study show that endosperm and embryo infection by TMV did occur in 77 and 48% of the samples, respectively, that were tested. There is always the remote possibility that, despite best attempts to mitigate cross contamination of the various components, there was some carryover of tissue between rinsing steps. However, if this were the case, we wouldn't expect to observe the high relative TMV concentrations in the embryo from trace amounts of endosperm tissue. This is the first report of TMV infection in the embryos of tobacco seed.

Statistical analyses illustrated highly significant differences for relative virus titer among the plants, pods, and seed components, with all sources of variation having *P* values of < 0.0001. Variation in embryonic infection among seed pods can be due to differences in the timing of infection or permeability of the embryo tissue (Maule and Wang 1996; Sastry 2013). The virus can move across tissues because the inoculated leaf cells are connected symplastically by the plasmodesmata. TMV can move from infected areas of a plant to new areas that may develop into the maternally derived seed coat. The seed coats develop from maternal tissue (Sastry 2013), which

Fig. 4. Representative images of endpoint PCR used to demonstrate a tobacco mosaic virus (TMV) cycle threshold (Ct) value range based on reverse-transcriptase quantitative PCR assay results for **A**, plant 1, pod A embryos and **B**, plant 3, pod A cotyledons. **A**, Lanes 2 to 21 represent the following Ct values: 2 = 22.530, 3 = 22.620, 4 = 23.396, 5 = 23.727, 6 = 24.043, 7 = 24.456, 8 = 24.669, 9 = 24.774, 10 = 25.197, 11 = 25.506, 12 = 25.652, 13 = 25.874, 14 = 27.191, 15 = 27.523, 16 = 28.198, 17 = 28.827, 18 = 29.295, 19 = 29.902, 20 = 33.287, and 21 = 33.428. Lane 22 represents a TMV-positive control (+) while lane 23 represents a TMV-negative control (-). **B**, Lanes 2 to 21 represent the following Ct values: 2 = 21.042, 3 = 25.607, 4 = 27.611, 5 = 27.889, 6 = 29.596, 7 = 29.450, 8 = 30.576, 9 = 30.930, 10 = 31.226, 11 = 31.450, 12 = 32.390, 13 = 32.600, 14 = 33.640, 15 = 33.958, 16 = 34.047, 17 = 34.379, 18 = 34.405, 19 = 34.595, 20 = 35.127, and 21 = 35.157. Lane 22 represents a TMV-negative control (-) while lane 23 represents a TMV-positive control (+). K 326 flue-cured tobacco plants were grown in the field in 2017 and infected with TMV.

explains why TMV was most commonly found in the seed coats in previous research and this study. During the later stages of development, the endosperm becomes impermeable, which may block the movement of viruses (Yim and Bradford 1998). Although this study did not examine the permeability of the endosperm, Argerich and Bradford (1989) showed that mature tomato seed lacking a permeable endosperm failed to measure leachate from aged seed via electroconductivity tests. A similar loss of endosperm

permeability in the later stages of tobacco seed development would prevent apoplastic movement of TMV into the embryo and passively protect the embryo from infection. Transcellular movement of TMV could not happen in senescing tissue after development is complete and the seed has dried. Therefore, there would be a limited window of time during which tobacco embryos are susceptible to TMV infection. RT-qPCR assay data in this study demonstrated that root and cotyledon tissue from germinated tobacco seed were infected with

Fig. 5. Determination of relative tobacco mosaic virus (TMV) virus titer ($\log_{10}$ of normalized virus) of infected K 326 flue-cured tobacco seed component samples generated by reverse-transcriptase quantitative PCR assays for **A**, plant 1; **B**, plant 2; and **C**, plant 3. Tobacco plants were grown in the field in 2017 and infected with TMV. Error bars represent the mean and standard deviation of the data.

TMV, thus demonstrating seed transmission of TMV in flue-cured tobacco. Cotyledons from infected seedlings had incidences of infection ranging from 2 to 24% depending on the plant and pod, and roots had infection ranging from 8 to 40%. Chamberlain (1937) documented that 0.12% of tobacco seedlings grown from infected plants tested positive for TMV. Although this number was relatively low, it is still significant because 0.12% of infected seedlings could serve as a primary infection source. Wilkinson et al. (2006)

(unpublished data) reported that 2 to 71% of pooled seedlings were TMV positive when TMV-infected K 326 flue-cured tobacco seed were planted in growth chamber tests. When compared with other plants that TMV can affect, the infection rates differ. de Assis Filho and Sherwood (2000) used ELISA for detection of TMV in *Arabidopsis thaliana* L., and showed that 0% of seedlings from TMV-infected seed tested positive for TMV. In contrast, Demski (1981) reported that 28% of roots and 3% of shoots from TMV-infected *N. glutinosa* were considered

Fig. 6. Tobacco mosaic virus (TMV) cycle threshold (Ct) value distribution of infected germinated K 326 flue-cured tobacco seed components generated by reverse-transcriptase quantitative PCR assays for **A**, plant 1; **B**, plant 2; and **C**, plant 3. Tobacco plants were grown in the field in 2017 and infected with TMV. Error bars represent the mean and standard deviation of the data.

positively infected based on a bioassay. Sevik and Kose-Tohumcu (2011) observed that 23.5% of tomato seedlings grown from TMV-infected seed tested positive by ELISA. There is evidence that TMV can infect seedlings from a variety of plants such as tobacco, tomato, and pepper. It is crucial to understand how this virus infected the seedlings and where TMV might reside in the seed. This tobacco study reported that the number of TMV-infected seedling components varied among seed pods. There were significant differences among pods for

virus titer in cotyledons, roots, and seed coats of seed harvested from the pods. Seed coats had a significantly greater mean viral titer compared with the cotyledons and roots for all plants. In earlier studies, it was assumed that seed pooled from several pods might increase the variability in results. To minimize this variation, we sampled seed from individual pods and used the seed samples for dissection of seed components and seed germination tests. This variation in incidence of TMV infection observed could reflect differences in the timing of

Fig. 7. Tobacco mosaic virus (TMV) viral titer ($\log_{10}$ of normalized virus) distribution of infected germinated K 326 flue-cured tobacco seed components generated by reverse-transcriptase quantitative PCR assays for **A**, plant 1; **B**, plant 2; and **C**, plant 3. Tobacco plants were grown in the field in 2017 and infected with TMV. Error bars represent the mean and standard deviation of the data.

TABLE 5. Incidence (%) of tobacco mosaic virus (TMV) in seed and seedling components of K 326 flue-cured tobacco plants infected with TMV

Plant	Pod	Seed dissection test ^y			Seed germination test ^z		
		Embryo	Endosperm	Seed coat	Cotyledon	Root	Seed coat
1	A	35	78	79	16	14	99
1	B	82	96	98	24	40	70
1	C	94	100	100	10	20	86
2	A	23	71	68	11	8	86
2	B	88	100	100	20	26	100
2	C	14	54	52	4	8	30
3	A	24	87	83	8	12	79
3	B	54	66	94	2	32	94
3	C	20	40	56	4	16	84

^y Based on a TMV cycle threshold (Ct) value of seed coats Ct < 30, endosperms Ct < 32, and embryos Ct < 31. Threshold values were determined by endpoint PCR. All samples that had a TMV Ct value lower than the threshold associated with each component was considered to be a TMV-infected sample. Pod A, n = 100 samples; pods B and C, n = 50 samples.

^z Based on a TMV Ct threshold value of cotyledons Ct < 30, roots Ct < 31, and seed coats Ct < 33. Threshold values were determined by endpoint PCR. All samples that had a TMV Ct value lower than the threshold associated with each component was considered to be a TMV-infected sample. Pod A, n = 100 samples; pods B and C, n = 50 samples.

infection and developmental stage or position of the pods and seed on the plant. Seedlings were only incubated for 10 days due to fungal contamination beyond that time point. If seed had been surfaced sterilized, an extended germination period potentially could have been evaluated but surface sterilization potentially could affect TMV infection. An extended germination time may have led to a greater percentage of the cotyledons and roots showing the presence of TMV because the infection would have had more time to increase in viral titer.

The novel findings in this report provide further insight into the transmission of TMV from tobacco seed to seedling but further experiments are required to verify these results. The results of this study demonstrate that tobacco embryo infection by the virus does occur and, together with successful transmission of TMV to the seedlings that develop from the seed, seed transmission of TMV in flue-cured tobacco has now been established.

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