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Biological and molecular interactions between bell pepper endornavirus and two tobamoviruses

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Peppers (*Capsicum* spp.) are native plants to the Americas. They are cultivated worldwide for direct human consumption and industrial purposes. Peppers can be infected by acute plant viruses, which cause a variety of diseases and crop losses. However, many *Capsicum* species can also be infected by persistent viruses. These are emerging viruses and they do not cause apparent disease and are transmitted only vertically. Using two near-isogenic lines of bell pepper cv. Marengo, biological and molecular interactions between the persistent virus bell pepper endornavirus (BPEV) and two acute viruses, pepper mild mottle virus (PMMoV) and tobacco mild green mosaic virus (TMGMV), were evaluated by symptom expression, enzyme-linked immunosorbent assay, and RT-qPCR. The relative titer of BPEV decreased at least two-fold at 14 days after infection when BPEV-infected plants were single infected with TMGMV or in mixed infection of PMMoV and TMGMV. The presence of BPEV was associated with symptom reduction in pepper plants infected with single and mixed infections of PMMoV and TMGMV. This suggests that the ubiquitous infection of BPEV may trigger the plant immune response, and therefore, BPEV is active when the plant is infected with PMMoV and/or TMGMV.

KEYWORDS

acute viruses, emerging viruses, *Endornaviridae*, persistent viruses, *Virgaviridae*

1 Introduction

Peppers (*Capsicum* spp.) are native plants to the Americas. Among the domesticated *Capsicum* species, *C. annuum* is the most commonly cultivated (1–4). According to the symptoms caused in their hosts, viruses can be grouped as persistent or acute (5). Persistent plant viruses do not cause symptoms, lack cell–to–cell movement, and are transmitted only vertically. In contrast, acute viruses are associated with symptoms and can be transmitted vertically and horizontally. Endornaviruses are persistent viruses, and in plants, they do not cause symptoms, although male sterility and cellular organelles malformations have been

associated with the presence of an endornavirus in *Vicia faba* and *C. annuum*, respectively (6, 7). These viruses are emerging viruses, and they have been recently reported in many economically important crops (6, 8–12). In pepper, bell pepper endornavirus (BPEV) has been reported worldwide (8, 13–18).

A mixed viral infection occurs when more than one virus species or strain inhabits the same host in a simultaneous or subsequence infection (19, 20). In horticultural crops, mixed acute viral infections are very common, although in many cases, they are unrecognizable due to the difficulty of distinguishing the specific symptoms each virus can cause. Research on acute viruses co–infecting the same plant has shown that mixed infection can result in synergistic interactions (21–24). A classic example of synergism is the product of a mixed infection between sweet potato feathery mottle virus (SPFMV) and sweet potato chlorotic stunt virus (SPCSV). Infections of sweet potato with SPFMV alone result in relatively mild foliar symptoms that include vein mottle and ringspots. When infected with SPCSV, sweet potato shows only mild chlorosis. However, when sweet potato is mixed infected with SPCSV and SPFMV, the symptoms consist of severe mosaic, leaf distortion, and plant stunting (22, 25, 26). Furthermore, it has been shown that in sweet potato plants infected with SPFMV, infection of SPCSV increases the titers of SPFMV (26). Mukasa et al. (27) showed that SPCSV also increased the titer of sweet potato mild mottle virus. Similarly, soybean plants co–infected with soybean mosaic virus and bean pod mottle virus developed more severe symptoms compared to a single infection of these two viruses (28, 29). In cucumber plants, double infection by cucumber mosaic virus (CMV) and zucchini yellow mosaic virus increased the virus titer of CMV (30).

Many acute viruses infect bell pepper, causing severe fruit yield and quality loss. The major viruses that infect pepper include members of the families *Potyviridae*, *Bromoviridae*, *Bunyaviridae*, *Geminiviridae*, and *Virgaviridae*. The genus *Tobamovirus* includes ssRNA viruses in the family *Virgaviridae*. Pepper mild mottle virus (PMMoV) and tobacco mild green mosaic virus (TMGMV) are species of this genus with worldwide distribution causing economically important diseases of pepper (31). These viruses are abundant and ubiquitous in nature. Despite the numerous mixed infections of acute viruses in pepper, not much research has been conducted on their combined effect on the crop. Therefore, the objective of this investigation was to use tissue containing natural single and mixed infections of the acute viruses PMMoV and TMGMV from a previous investigation (32) to evaluate biological and molecular interactions between BPEV, PMMoV, TMGMV infections in two near–isogenic lines (NIL) of bell pepper cv. Marengo (33). The interactions were evaluated using a symptom scale to determine the percentage of symptom appearance, enzyme–linked immunosorbent assay (ELISA), and quantitative reverse transcriptase polymerase chain reaction (RT–qPCR).

2 Materials and methods

2.1 Plant material

Two bell pepper NILs developed in a previous investigation (33) were used in this study. Seeds of the two NILs were planted in 10–cm square pots containing a soil mix that consisted of 1.5 parts of soil, 1.5 parts of sand, and 3 parts of potting mix (Miracle–Gro® Lawn Products, Inc., Marysville, OH). Before planting, seeds were treated with 10% sodium phosphate tribasic dodecahydrate (Sigma–Aldrich Co., St. Louis, MO). Seedlings were kept in a growth room with an average temperature of 23°C and under artificial light (54W/120V 60Hz/4.0A Lamps) with 15 h dark/9 h light photoperiod.

2.2 Source and increase of PMMoV and TMGMV

Tissue containing single and mixed infections of PMMoV and TMGMV was used. Isolates of these viruses were those used in previous investigations (32). PMMoV was increased in Tabasco pepper (*C. frutescens*) and TMGMV in *Nicotina tabacum*. Mechanical inoculations of single or mixed infection of the viruses were conducted on 30–day–old bell pepper cv. Marengo plants grown in a greenhouse in 5.6–L clay pots using the soil mix described previously. Symptomatic tissue was harvested two weeks after inoculation and used for virus purification.

2.3 Virus purification

Viruses were purified using a method described for the purification of tobamoviruses (34). Briefly, leaves were ground in sodium phosphate buffer (0.5 M NaH_2PO_4, pH 7.2) using a blender. Homogenized tissue extract was clarified with 8% butanol. Four percent polyethylene glycol (PEG 6000) combined with low–speed centrifugation (8,000 g) was used to concentrate the virus. The concentration of purified virus was measured at an absorbance of 280 nm in a nanodrop ND–1000 spectrophotometer (NanoDrop® Technologies, Inc., Wilmington, DE). The purified virus was diluted with phosphate buffer (0.05 M, pH 7.2) to a final concentration of 0.05 mg/ml. Purified virus samples were negatively stained with 2% phosphotungstic acid pH 7.0 and observed with a JEOL JSM–1400 (Jeol Inc., Peabody, MA) transmission electron microscope.

2.4 Virus inoculations

Thirty–day–old plants were used to perform single and mixed mechanical inoculations. The inoculum consisted of purified PMMoV, TMGMV, and a mixture of purified PMMoV with TMGMV diluted in phosphate buffer (0.05 M, pH 7.2) to a final concentration of 0.05 mg/ml. Three leaves of each test plant of the two NILs previously dusted with silicon carbide (carborundum) were inoculated using sterile cotton swabs. The inoculated leaves were rinsed immediately with distilled water. Four plants per treatment were inoculated. Four plants of each NIL were also mock–inoculated using phosphate buffer. Virus–inoculated and mock–inoculated plants were kept in the dark overnight before placing them under the lights to avoid leaf damage. The mechanical inoculation experiments consisted of eight treatments: BPEV–free/Mock–inoculated, BPEV–infected/Mock–inoculated, BPEV–free/

PMMoV, BPEV–infected/PMMoV, BPEV–free/TMGMV, BPEV–infected/TMGMV, BPEV–free/Tobamovirus Mixed, BPEV–infected/Tobamovirus Mixed.

2.5 Symptom evaluation

Symptoms were recorded daily from the upper half canopy for at least 14 days after inoculation (DAI). Values were assigned to each type of symptom described in Supplementary Figure 1 and recorded as follows: no symptoms [1], mild mottle, vein clearing, and leaf wrinkling/rugose [2], necrosis [3], severe necrosis [4], leaf dropping [5], necrosis or severe necrosis with leaf dropping [6]. If more than one symptom was recorded per plant, only the most severe symptom (higher value) was used to convert to the percentage of symptom appearance. Values were converted to percentage of symptom appearance using the following disease scale: 1 = 0%, 2= >0–20%, 3 = 21–40%, 4 = 41–60%, 5 = 61–80%, and 6 = 81–100%. Four independent experiments were performed per treatment, and four plants were recorded for symptom evaluation.

2.6 Virus quantification using ELISA

The enzyme–linked immunosorbent assay was used to determine the success of mechanical inoculations with single and mixed infection of PMMoV and TMGMV and to perform relative viral quantification. Leaf tissue from each treatment was collected, and 0.05 g was used to perform ELISA using PMMoV and TMGMV detection kits following the instructions and reagents provided by the manufacturer (Agdia® Elkhart, IN). Alkaline phosphatase was used as a substrate for the enzymatic reaction. The absorbance representing relative virus titer was measured using a microplate at 405 nm in an ELISA plate reader (Model EL311 SX, Bio–Tek™ Instrument Inc., Winooski, VT), and the relative titer of PMMoV and TMGMV was determined at 7 and 14 DAI. Two independent experiments were performed. Three biological repetitions (two technical repetitions each) were used to perform the readings.

2.7 Viral RNA extraction

Approximately 0.5 g of tissue from the two NILs showing systemic symptoms by the tobamoviruses was collected and ground in liquid nitrogen using a sterilized mortar and a pestle. Ground samples were kept at –70°C and used in total RNA extractions. The same amount of tissue was collected from mock–inoculated plants. Total RNA was extracted from 100 mg of leaf tissue using the PureLink® Plant RNA Reagent Kit (Invitrogen, Carlsbad, CA). DNA was digested with PerfeCta® DNase I (Qiagen, Beverly, MA). Total RNA was eluted in 50 µl of RNase–free water (Ambion®, Life Technologies™, Carlsbad, CA), and the RNA concentration and quality were determined in a spectrophotometer (NanoDrop®). All samples were diluted to a final concentration of 50 ng/µl. Diluted RNA was used to perform RT–qPCR.

2.8 Primer and probe design

Forward and reverse primers were designed using the nucleotide sequence of the viral RNA–dependent RNA polymerase (RdRp) of BPEV, PMMoV, and TMGMV available in the GenBank (35) (Table 1). An evaluation of hairpin and self–complementation of the primers was conducted using the BLAST sequence alignment search tool from the National Center for Biotechnology Information (NCBI). The Universal ProbeLibrary Assay Design Center tool of Roche (Roche Molecular Systems, Inc., Indianapolis, IN) was used to design probes and primers (TaqMan® FAM). Primers and probes were also designed for ubiquitin–conjugating enzyme 3 (UBI–3) (36) to be used as a reference gene for the normalization of relative viral RNA quantification (Table 1).

2.9 RT–qPCR reactions

Each RT–qPCR reaction had a total of 11 µl distributed as follows: 2 µl of RNA template, 5 µl of iTaq Universal probe reaction mix (2x), 0.25 µl of iScrip reverse transcriptase, 0.5 µl of both forward and reverse primers, 0.2 µl of fluorogenic probe, and 2.55 µl of nuclease–free water. All components were added in the order provided by the manufacturer (iTaq™ Universal Probes One–Step Kit, Hercules, CA). The reaction mix was placed in Hard–Shell Low–Profile 96–Well Semi–Skirted PCR plates and sealed with an optically transparent film (Microseal 'B' Adhesive Seals, Bio–Rad Laboratories, Inc., Hercules, CA). The reaction was performed on a CFX96 Touch™ Real–Time PCR Sequence Detection System (Bio–Rad Inc., Hercules, CA, USA). Forty cycles of the following PCR thermal cycler were conducted for each sample: reverse transcription reaction (10 min at 50°C), polymerase activation and DNA denaturation (1 min at 95°C), amplification reactions consisted of denaturation (2 min at 95°C), annealing/extension + plate read (30 sec at 54°C). Two independent experiments were performed. Three biological repetitions (two technical repetitions each) were used to perform the RT–qPCR reactions.

2.10 Virus detection

The presence of BPEV, PMMoV and TMGMV was tested 7 DAI by electrophoretic analysis of viral dsRNA. DsRNA was extracted from fresh or desiccated plant tissues using the method of Khankhum et al. (37).

2.11 Experimental design and data analysis

The Cq values for each NIL were compared side by side. To determine the relative RNA viral titer for each virus, data was transformed by using the algorithm $2^{-\Delta\Delta Cq}$ described in the Real–Time Guide of Bio–Rad (38). The average fold change titer of BPEV, PMMoV, and TMGMV was determined by normalization of the data to the reference gene (UBI–3). A completely randomized

TABLE 1 Primers and probes used in RT–qPCR.

Virus	Gene	Probe	Primer
BPEV	RdRp	5′–GAGGAGAG–3′	F = 5′–GCACAACAGTCATTTTAACTGGA–3′ R = 5′–CCAGTCAATCTCATGGCATC–3′
PMMoV	RdRp	5′–ATTCCAGC–3′	F = 5′–ATACGCTGTCGCTTTGCAC–3′ R = 5′–AGTGCTGCCCCAAATTCAT–3′
TMGMV	RdRp	5′–CTGGTTGC–3′	F= 5′–GCTGCAGGACTACTCGAAA–3′ R= 5′–CAAGTCTGGTGCGTTGAA–3′
Reference	UBI–3	5′–GCAGTGGA–3′	F = 5′–TGGAAGTATTTGCCTTGATATTCTC–3′ R = 5′–GCAGGACCTTCGATATGGTT–3′

BPEV, bell pepper endornavirus; PMMoV, pepper mild mottle virus; TMGMV, tobacco mild green mosaic virus; RdRp, RNA–dependent RNA polymerase; and UBI–3, ubiquitin–conjugating enzyme 3.

design was used. Data from repeated experiments were analyzed to determine if they could be combined (39). The averaged data obtained from each pepper NIL was analyzed by One–Way ANOVA using SPSS (IBM© SPSS© Statistics Version 24) and R software. The comparisons were considered statistically significant at $p < 0.05$. Only the average percentage of symptom appearance is presented because the data was converted from non–categorical values.

3 Results

3.1 Virus purification and detection

The three viruses, BPEV, PMMoV, and TMGMV, were consistently detected by electrophoretic analysis of viral dsRNA. A representative result of the electrophoretic analyses of dsRNAs extracted from Marengo bell pepper with single and mixed infection of viruses (BPEV, PMMoV, and TMGMV) is shown in Figure 1D. Moreover, PMMoV and TMGMV were also detected by ELISA (Figures 2A, B). Electron microscopy analysis of the purified virus preparation of PMMoV and TMGMV showed the presence of rigid rod–shaped virus particles of approximately 300–450 nm long, which is the typical size for members of the genus *Tobamovirus* (Figures 1A–C).

3.2 Symptom evaluation

Plants of both NILs mixed infected with PMMoV and TMGMV began to show symptoms 5 DAI. At that time, symptoms on the inoculated leaves consisted of mild mottle and vein clearing. At 7 DAI, the BPEV–free NIL inoculated only with TMGMV showed mild mottle, leaf wrinkling, and systemic necrosis. In contrast, the BPEV–infected NIL showed only mild mottle and leaf wrinkling (Supplementary Figure 2). A similar symptom pattern compared to TMGMV was observed in the two NILs mixed infected with PMMoV and TMGMV (Supplementary Figure 2). The BPEV–free NIL single infected with TMGMV and mixed infected with PMMoV and TMGMV consistently showed more severe symptoms until 14 DAI (Figure 3). Nevertheless, necrosis was observed at 13 DAI on inoculated leaves (in single infection of TMGMV and mixed infection with PMMoV and TMGMV) of some plants of the BPEV–infected NIL. Plants of both NILs single infected with PMMoV began to show symptoms 6–7 DAI. In both NILs infected with PMMoV, similar symptoms were observed during the 14 DAI period. Symptoms consisted of mild mottle, vein clearing, and leaf wrinkling (Supplementary Figure 2).

3.3 Virus quantification using ELISA

At 7 DAI, the relative amount of PMMoV determined by ELISA was higher in the BPEV–free NIL than in plants of the BPEV–infected NIL, however, the differences were not statistically significant (Figure 2A). The same trend was observed at 7 DAI when PMMoV was mixed infected with TMGMV. However, the titer of PMMoV in the mixed infection was significantly lower ($p < 0.05$) in the BPEV–infected plants compared with single infection of PMMoV in the BPEV–free plants. An opposite trend was observed in TMGMV at 7 DAI. In single infections of TMGMV and mixed infection with PMMoV, there was a significantly higher titer of TMGMV in the BPEV–infected plants than in the BPEV–free plants (Figure 2B). At 14 DAI, both NILs showed a similar viral titer of PMMoV and TMGMV in single and mixed infections.

3.4 Viral RNA quantification using RT–qPCR

In RT–qPCR, high Cq values indicate a lower nucleic acid concentration of the target template (38). In this investigation, each target (BPEV, PMMoV, and TMGMV) that was relatively quantified was compared to the BPEV–free NIL that was inoculated with the respective tobamovirus. In general, the Cq values of BPEV were higher than the Cq values of PMMoV and TMGMV at 7 and 14 DAI (Figure 2F). Therefore, the relative RNA titer of BPEV was lower compared to the titer of PMMoV and TMGMV in single and mixed infections at 7 and 14 DAI. However, BPEV showed higher titer (with no significant statistical differences) when BPEV–infected plants were infected with TMGMV at 7 DAI. The titer of BPEV decreased at least two–fold at 14 DAI when BPEV–infected plants were single infected with TMGMV or in mixed infection of PMMoV and TMGMV (Figure 2C).

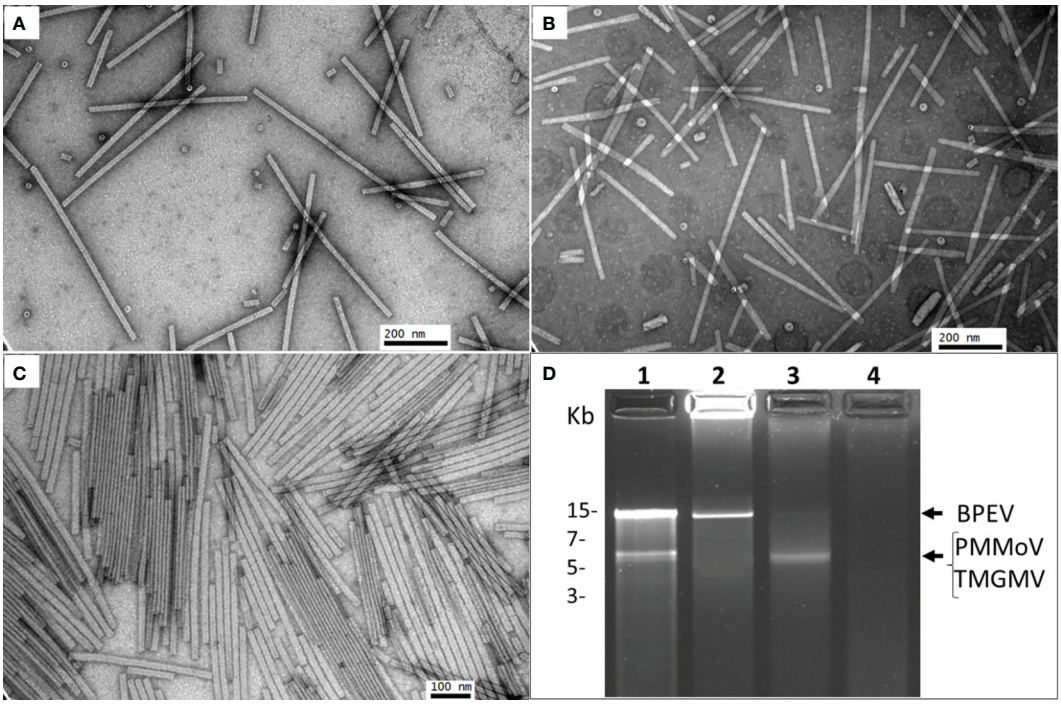

FIGURE 1
Electron micrographs showing purified preparations of PMMoV and TMGMV and electrophoretic analysis of dsRNA. **(A)** Purified PMMoV. **(B)** Purified TMGMV. **(C)** Purified PMMoV and TMGMV. **(D)** Agarose (1.2%) gel electrophoresis of dsRNA extracted from two bell pepper cv. Marengo near–isogenic lines infected with PMMoV and TMGMV. Lane 1= triple infection of BPEV, PMMoV and TMGMV, lane 2= single infection of BPEV, lane 3= single infection of PMMoV, and lane 4= negative control. The gel was run for 2 h at 70 V.

The RNA accumulation of PMMoV did not show significant changes at 7 and 14 DAI, however, the titer of PMMoV was two–fold higher in the BPEV–free NIL compared with the BPEV–infected NIL at 7 DAI (Figure 2D). An opposite trend in titer accumulation of PMMoV–infected plants was observed at 14 DAI (Figure 2D). Overall, the RNA titer accumulation of PMMoV was similar at 7 and 14 DAI when it was mixed infected with TMGMV in both NILs. The RNA titer of TMGMV at 7 DAI was higher in the BPEV–infected NIL (Figure 2E). However, when plants were mixed infected with PMMoV and TMGMV at 7 DAI, the relative RNA titer of TMGMV was higher in the BPEV–free NIL. The same trend was observed in the RNA titer of TMGMV in mixed infection of PMMoV and TMGMV 14 DAI, however, the differences were not statistically significant. A Pearson correlation analysis was performed comparing the virus titer obtained by ELISA and RT–qPCR (Supplementary Table 1). A positive correlation was observed in the single infection of PMMoV and TMGMV in the BPEV–free NIL at 7 DAI. The titer of TMGMV was negatively correlated in mixed infection of PMMoV and TMGMV in both NILs at 7 and 14 DAI.

4 Discussion

There has been extensive research conducted in the area of mixed acute viral infections that cause severe damage to economically important crops (22, 28, 29, 40–42). However, limited research has been conducted on the interaction between emerging persistent plant viruses, the host, and acute viruses. In plant viruses, cross–protection is conferred to a plant by previous inoculation of the plant with a mild strain of the same virus (43, 44). This may be associated with the activation of gene silencing through the production of small RNAs, which play an important role in plant defense against viral diseases (43, 45, 46). This activation of the plant immune system has also been reported in closely related viral species. Although distantly related, it is possible that this can also occur with acute viruses infecting plants that host persistent viruses since their early stages of development.

Under the experimental conditions of this investigation, symptoms of single infection of TMGMV and mixed infection of PMMoV and TMGMV in mechanically inoculated plants were more severe in the BPEV–free NIL than in the BPEV–infected NIL. Moreover, the relative virus titer of TMGMV measured by RT–qPCR was higher in plants of the BPEV–free NIL than in plants of the BPEV–infected NIL. This suggests that interference or competition occurred between BPEV, TMGMV, and PMMoV for host resources used for early replication. There have been reports of the activation of small RNAs in the interaction of BPEV and bell pepper (14). Furthermore, a significant number of plant–defense–related genes have been shown to be up–regulated in endornavirus–infected plants (47, 48). This indicates the activation of host gene silencing and supports the hypothesis that endornaviruses have an active role in the infected host.

At 14 DAI, BPEV–infected plants single infected with TMGMV and mixed infected with PMMoV and TMGMV showed symptoms similar to those observed in the BPEV–free plants at 7 DAI. This

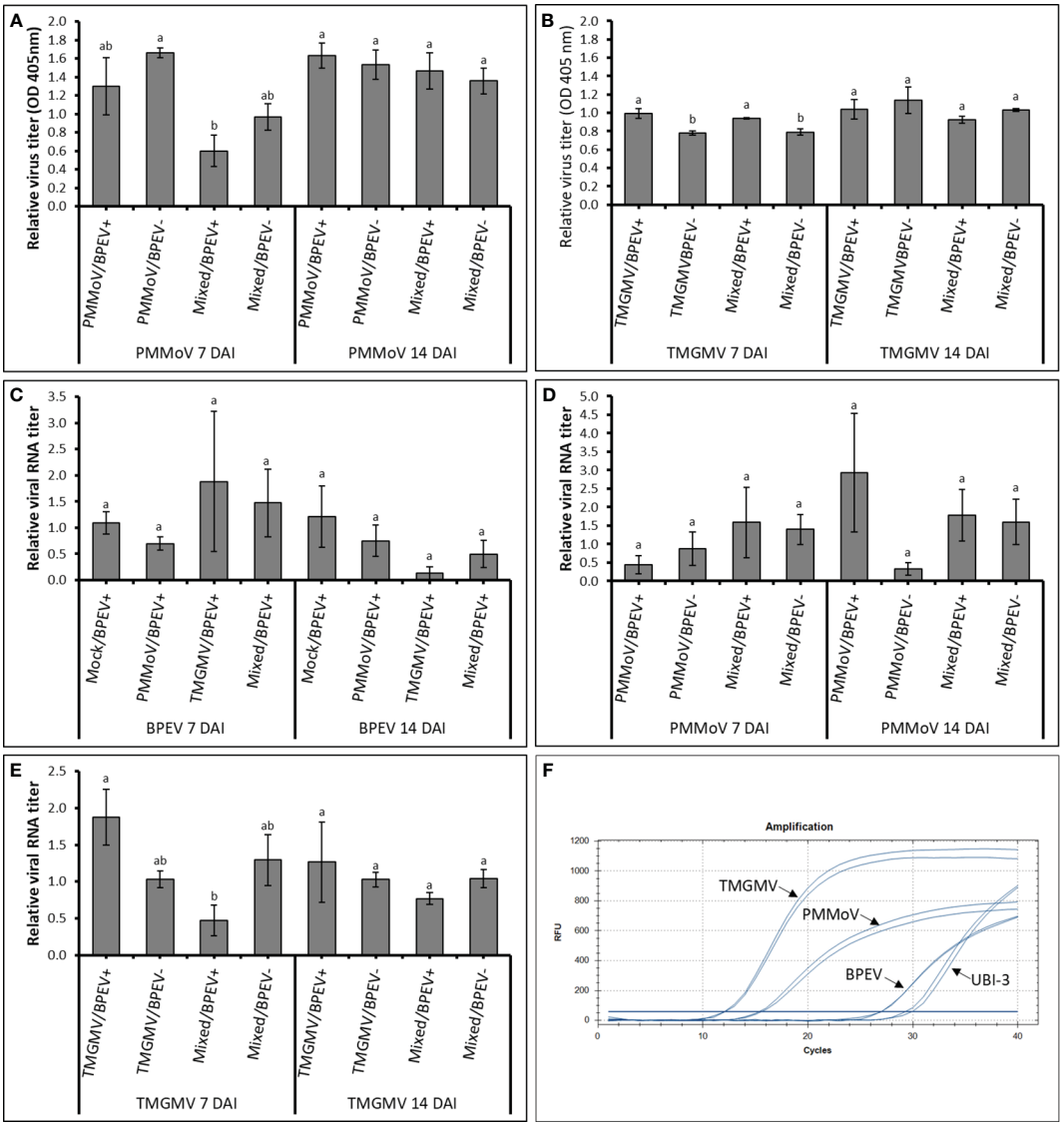

FIGURE 2
Relative quantification titers of PMMoV, TMGMV, and BPEV by ELISA and RT–qPCR at 7 and 14 DAI in two bell pepper cv. Marengo near–isogenic lines. **(A)** Relative virus titer of PMMoV quantified by ELISA. **(B)** Relative virus titer of TMGMV quantified by ELISA. **(C)** Relative viral RNA titer of BPEV quantified by RT–qPCR. **(D)** Relative viral RNA titer of PMMoV quantified by RT–qPCR. **(E)** Relative viral RNA titer of TMGMV quantified by RT–qPCR. **(F)** Amplification of different targets using RT–qPCR. Two independent experiments were performed. Three biological repetitions with two technical repetitions were included in the one–way ANOVA analysis. Treatments were analyzed within each day. Values with the same letters indicate no statistical difference between treatments at $p < 0.05$. Bars indicate the standard error. For all treatments, n= 6. BPEV= bell pepper endornavirus, PMMoV= pepper mild mottle virus, TMGMV= tobacco mild green mosaic virus, DAI= days after inoculation, UBI–3= ubiquitin–conjugating enzyme 3, and OD= optical density at 405 nm.

reaction may be related to the amount of acute virus accumulation, which may have reached a point in which the plant defense mechanisms cannot interfere with virus replication. In this investigation, the amount of inoculum of acute viruses might be relatively large compared to the amount of inoculum that naturally infects a pepper plant. Pepper plants become naturally infected by tobamoviruses mainly by mechanical plant contact with contaminated materials that include tools, equipment, seed coat, or other plants. Therefore, conducting experiments simulating natural mechanical inoculation could help to confirm the symptom patterns observed under the conditions of this investigation.

Khankhum (49) observed a synergistic interaction in single, double, and triple infection of acute viruses with two endornaviruses in common bean (*Phaseolus vulgaris*). It has been demonstrated that co–infection of two acute viruses resulted in synergistic interactions (22, 26, 27, 50–52). In these reports, one of the co–infecting viruses served as an enhancer, allowing higher accumulation of the other virus in the host. The synergistic interactions of these viruses have been associated with the suppression of the host defense mechanisms by viral proteins associated with RNA–silencing suppression (53). In our investigation, a triple infection of BPEV, PMMoV, and TMGMV did not result in a synergetic interaction. We hypothesize that BPEV

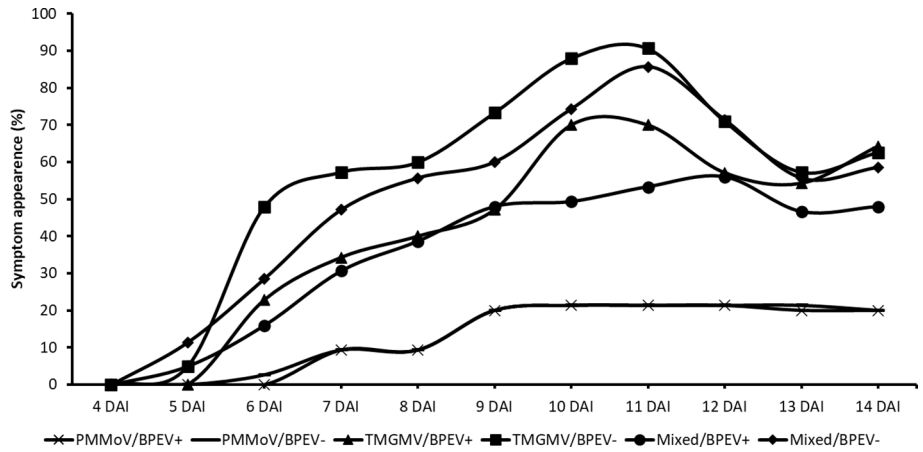

FIGURE 3
Percentage of symptom appearance in two near–isogenic lines of bell pepper cv. Marengo, one infected with BPEV (BPEV+) and the other free of BPEV (BPEV–) after mechanical inoculation with single and mixed infections of PMMoV and TMGMV. Figure represents the average value of four independent experiments. For all treatments, n= 16. BPEV, bell pepper endornavirus; PMMoV, pepper mild mottle virus; TMGMV, tobacco mild green mosaic virus; and DAI, days after inoculation.

could suppress protein expression in acute viruses. For example, the lack of coat protein has been associated with the expression of less severe symptoms caused by TMV (54). It is not known if the BPEV–free NIL is more susceptible to single infection of TMGMV or mixed infection of PMMoV and TMGMV due to the lack of BPEV. It is possible that the absence of BPEV might allow single and mixed infection of tobamoviruses to suppress the mechanisms of plant defense by suppressing RNA silencing of the host, as suggested for other viruses (27, 53, 55).

In this investigation, co–infection of BPEV and TMGMV and triple infection of BPEV, PMMoV, and TMGMV appear to result in an antagonistic interaction. We hypothesize that one or two mechanisms can be triggering these reactions: *1)* the interaction is a response to a pre–activation of the plant defense by BPEV, or *2)* there is a competition for the host resources among viruses during replication. It is possible that BPEV helps the host to express proteins such as catalases, which are known to be involved in the decomposition of reactive oxygen species (ROS) triggered by viral infections in the host (42). If this is the case, BPEV might not necessarily suppress the replication of tobamoviruses in single and mixed infections. Instead, BPEV could help the plant have less ROS accumulation, rendering less severe symptoms. It is also well known that phytohormones like jasmonic acid (JA), salicylic acid, and ethylene play an important role in plant immunity (56). However, there are viruses, including rice ragged stunt virus, that can suppress JA–mediated defense in order to facilitate virus infection (57).

In future studies, it is important to explore the interactions of BPEV with the host and other viruses under abiotic stress. BPEV might not only play a role in activating the defense mechanisms to biotic agents, but also might have an adaptive effect on the host by reducing stress caused by abiotic factors. It has been demonstrated that under some abiotic stresses, viruses may have beneficial effects on their hosts. *Arabidopsis halleri* inhabits soils contaminated by heavy metals and it has been suggested that the ability of this plant to survive these conditions might be conferred by the persistent virus Arabidopsis halleri partitivirus 1 (58) (59). Xu et al. (60) showed that infections of cucumber mosaic virus (CMV) improved drought tolerance in several plant species and enhanced the freezing tolerance of beets. In the same study, *Nicotiana benthamiana* plants inoculated with CMV, TMV, or brome mosaic virus were significantly more resistant to drought stress than non–inoculated plants.

Transmitting BPEV and other endornaviruses to their hosts still represents a major challenge, and the development of an inoculation method for persistent viruses is necessary to confirm the interactions and effects of these viruses on the host. Nevertheless, the use of the NILs of bell pepper cv. Marengo has been helpful in studying the role of BPEV in the host reaction to biotic agents (7, 33, 48). There are some cases in which viruses have been engineered to be transmitted horizontally and used as biological control. One classical example is the mycovirus Cryphonectia parasitica virus 1 (CHV1), which has been integrated into the nucleus of the host to be transmitted sexually (61). The success of transmission of CHV1 was somewhat erratic. This could be improved by using engineered viruses to be transmitted horizontally. However, developing engineered viruses represents a major challenge, especially because it requires a deep understanding of the molecular and biological interaction with the host and the environment.

In summary, the results obtained in this study suggest that the presence of BPEV is associated with symptom reduction in pepper plants infected with single and mixed infections of two tobamoviruses, PMMoV and TMGMV. We hypothesize that the ubiquitous infection of BPEV may trigger the plant immune response, and therefore, BPEV is active when the plant is infected with PMMoV alone or in combination with TMGMV. Further experiments that involve other endornavirus–infected plants and other acute viruses should be conducted to further understand the roles and effects of endornaviruses co–infecting the host with acute viruses. The possible role of endornaviruses in the reduction of

symptoms caused by acute viruses may be investigated by studying virus–plant protein interactions and the expression of genes known to trigger the plant immune system.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

CE: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Writing – original draft. SK: Formal Analysis, Methodology, Writing – review & editing. RV: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fviro.2023.1267692/full#supplementary-material

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