Interactions Between Geminiviruses in a Naturally Occurring Mixture: *Pepper huasteco virus* and *Pepper golden mosaic virus*

J. Méndez-Lozano, I. Torres-Pacheco, C. M. Fauquet, and R. F. Rivera-Bustamante

First, second, and fourth authors: Departamento de Ingeniería Genética, Centro de Investigación y Estudios Avanzados del IPN, U. Irapuato, Apartado Postal 629, Irapuato, Gto, México; and third author: ILTAB, Danforth Plant Science Center, St. Louis, MO.

Current address of J. Méndez-Lozano: CIIDIR-IPN, Unidad Sinaloa, Apdo Postal 280, Guasave, Sin. México.

Current address of I. Torres-Pacheco: INIFAP-Campo Experimental Bajío. Apdo Postal 112, Celaya, Gto., México.

Accepted for publication 12 September 2002.

ABSTRACT

Méndez-Lozano, J., Torres-Pacheco, I., Fauquet, C. M., and Rivera-Bustamante, R. F. 2003. Interactions between geminiviruses in a naturally occurring mixture: *Pepper huasteco virus* and *Pepper golden mosaic virus*. *Phytopathology* 93:270-277.

Pepper huasteco virus (PHV) and *Pepper golden mosaic virus* (PepGMV) are found in mixtures in many horticultural crops in Mexico. This combination constitutes an interesting, naturally occurring model system to study several aspects of virus-virus interactions. Possible interactions between PHV and PepGMV were studied at four levels: symptom expression, gene expression, replication, and movement. In terms of symptom expression, the interaction was shown to be host-dependent because antagonism was observed in pepper, whereas synergism was de-

tected in tobacco and *Nicotiana benthamiana*. PHV and PepGMV did not generate viable pseudorecombinant viruses; however, their replication is increased during mixed infections. An asymmetric complementation in movement was observed because PHV was able to support the systemic movement of PepGMV A whereas PepGMV did not support the systemic distribution of PHV A. Heterologous transactivation of both coat protein promoters also was detected. Several conclusions can be drawn from these experiments. First, viruses coinfecting the same plant can interact at several levels (replication, movement) and in different manners (synergism, antagonism); some interactions might be host dependent; and natural mixed infections could be a potential source of geminivirus variability by generating viable tripartite combinations that could facilitate recombination events.

Geminiviruses belong to the family *Geminiviridae* and take their name from the unique structure of their twin, or geminate, particle. Their genomes consist of either one (monopartite) or two (bipartite) covalently closed circular single-stranded DNA (ssDNA) molecules. The family *Geminiviridae* has been divided into four genera according to their host range, insect vector, and genome organization (*Mastrevirus*, *Curtovirus*, *Begomovirus*, and *Topocuvirus*) (24). *Pepper huasteco virus* (PHV) and *Pepper golden mosaic virus* (PepGMV, previously known as *Texas pepper virus*, TPV) are members of the genus *Begomovirus*; both viruses have a bipartite genome (DNAs A and B), are transmitted by whiteflies, and naturally infect a series of dicotyledonous crops such as pepper, tomato, tomatillo, and tobacco.

Mixed infections with two or more geminiviruses infecting the same plant are common in tropical and subtropical areas (15, 30,36). Virus interactions in those mixed infections could play an important role leading to the development of complex diseases (8,26,38). The type of interactions between co-infecting viruses can range from synergism, where symptoms are stronger than those of individual viruses (27), to interference, where one virus

affects the infective cycle of the second virus (19). Possible interactions also include events such as recombination (4,17,26,30,31) and complementation (3,7,20). These interactions also can occur at different levels of the virus cycle (i.e., gene expression, replication, movement, or encapsidation) (5,14).

Several studies on geminivirus interactions have been reported. However, in most cases, the virus combination used as a model involves viruses that do not interact in nature (e.g., *African cassava mosaic virus* [ACMV] and *Tomato golden mosaic virus* [TGMV] or TGMV and *Bean golden mosaic virus*-Guatemala [BGMV-GT]) (9,25,34) or they have been carried out using experimental hosts (e.g. *Nicotiana benthamiana*) which are seldom infected in nature by the selected viruses. Therefore, extrapolation of those results to a natural condition must be done with caution. Nevertheless, interesting observations have been raised. For example, studies with *Bean dwarf mosaic virus* (BDMV) and *Tomato mottle virus* (ToMoV) demonstrated recombination in relatively short-term laboratory experiments (17). More recently, studies on cassava mosaic disease in Africa and cotton diseases in Pakistan provided direct evidence for the ability of begomoviruses to recombine as a result of a natural mixed infection (4,8,26,30).

In Mexico, PHV and PepGMV have been reported as the most important viral pathogens in horticultural crops. They were initially detected in the same geographic area (northeastern Mexico and southern Texas) and both have been characterized at the molecular level (12,21,33,37). More recent surveys have shown that both viruses are now well distributed throughout Mexico in crops such as pepper, tomato, tomatillo, and tobacco (2,36). Although single infections are common, a high percentage of the infected plants contain both viruses, showing that PHV-PepGMV is the predominant infecting mixture in such crops (21). Therefore, this combi-

Corresponding author: R. F. Rivera-Bustamante;
E-mail address: rrivera@ira.cinvestav.mx

The sequence of PepGMV component B has been deposited as GenBank Accession No. AF499442.

*The e-Xtra logo stands for "electronic extra" and indicates that the online article contains supplemental material not included in the print edition. Figure 1 is in color online.

nation constitutes an interesting system to study some of the mechanisms involved in virus-virus interactions of a naturally occurring mixture. In this report, we describe various aspects of the interaction between PHV and PepGMV in mixed infections of several solanaceous hosts.

MATERIALS AND METHODS

Viruses. Infectious clones of PHV (pIGV22 and pIGV21) and PepGMV (pTPVA and pTPVB) have been described earlier (12,36). Plasmids pJMPHVAGFP and pJMPepGMVAGFP correspond to the respective viral component A, in which the coat protein (CP) gene was replaced with that encoding the green fluorescent protein (GFP). Both chimeric components are cloned in pBluescript. The chimeric viruses were shown to be functional (expressing GFP) and to replicate at levels similar to the wild-type (WT) virus. The GFP version used presents a change at position 65, where the WT serine residue was replaced by a threonine, resulting in a higher quantum yield of fluorescence and a reduced photo bleaching compared to the WT version (16). The GFP construct (pCat-GFP) was provided by C. Reichel.

Protoplast electroporation. For transient expression, protoplasts were prepared from a dark-grown tobacco BY-2 cell culture 3 days after subculturing, basically as described by Watanabe et al. (40). One million protoplasts were inoculated by electroporation with 2 µg each of the respective clone of DNA-A (PHV A or pJMPHVAGFP) and DNA-B (12) plus 40 µg of sheared herring sperm DNA. In the case of tripartite mixtures, protoplasts were transfected with 2 µg of each cloned component plus 40 µg of sheared herring sperm DNA. Electroporation of protoplasts was performed in 0.4-cm cuvettes at 250 V and 500 µF using a Gene Pulser (Bio-Rad). After electroporation, protoplasts were diluted in 10 ml of protoplast culture medium (PCM) (Murashige and Skoog salts mixture at 2.2 g liter⁻¹, 1% [wt/vol] sucrose, myo-inositol at 0.1 g liter⁻¹, 2,4-dichlorophenoxyacetic acid [2-4-D] at 0.2 mg liter⁻¹, thiamine-HCl at 1 mg liter⁻¹, 0.4 mannitol, pH 5.8), transferred to petri dishes, and incubated at 29°C in darkness. Protoplasts were harvested 48 h postinoculation (hpi) for DNA isolation and analysis by fluorescence microscopy.

Plant inoculations. Two-week-old seedlings of *N. benthamiana* or *N. tabacum* cv. Xanthi nc, grown in Magenta boxes, or pepper plants at the four-leaf stage, were inoculated by particle bombardment as reported previously (12). In each experiment 10 plants were inoculated for each virus (or mixture) using 0.5 µg of each viral DNA (A + B) per plant. Plants were observed every day for symptom development and the inoculated leaves as well as the newly emerging leaves were harvested for DNA extraction and

polymerase chain reaction (PCR), dot, or Southern blot hybridization analysis up to 26 days postinoculation (dpi). For symptoms evaluation, an arbitrary scale from 1 (mildest) to 10 (strongest symptom in that species) was used. In the case of pepper, the scale was: 1 = small yellow spots visible only against a strong light, 2 = groups of yellow spots visible, 3 = yellow spots start to form a net, 4 = net completely visible, 5 = mild rugosity detected in the first leaves showing symptoms, 6 = some curling detectable, 7 = all symptomatic leaves show curling and rugosity, 8 = symptomatic leaves are smaller, 9 = symptomatic plants show general distortion, and 10 = severe stunting of the plant with curling and rugosity. Similar scales were used for tobacco and *N. benthamiana*. However, the strongest symptom observed on each species was different; therefore, the scales are not directly comparable. For the comparison of the symptoms induced by each virus and the mixture, each inoculated plant (10 per experiment) was graded and an average was calculated as well as the standard deviation. An analysis of variance test was performed to verify if the differences between the symptoms were statistically different.

Virus replication assays. All inoculated plants and transfected protoplasts were assayed for viral DNA. For Southern blot hybridization analysis, DNA extraction from systemically infected leaf samples was carried out as described previously (12). DNA extraction from protoplast preparations was essentially performed as described (22). Samples containing the same amount of total nucleic acids (5 to 10 µg) were analyzed by agarose gel electrophoresis. Transfer of nucleic acids to Hybond N+ membranes (Amersham Pharmacia) was performed under alkaline conditions according to standard procedures (29). Blots then were hybridized using virus-specific nonradioactive probes (Gene Images random prime labeling module, Amersham Pharmacia) from the intergenic regions of the respective virus: PHV, PCR fragment of 350 bp obtained with primers 240 to 241 (36); primer coordinates: 240, 2,490 to 2,514; 241, 184 to 207; PepGMV, PCR fragment of 288 bp obtained with specific primers JM23 (5'-TGGTGTAGGACTCCAGCAGAATC-3') and JM24 (5'-TAGGCCACACCTTG-GTCACCAAG-3'); primer coordinates: JM23, 2,494 to 2,516; JM24, 145 to 168. As recently suggested, numbering starts, in both cases, at the adenosine in the nicking site located in the stem-loop structure: T*ACC. In all hybridization analyses, high stringency conditions were used to eliminate possible cross-hybridization signals. Hybridization conditions were 65°C, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate (SDS), and 5% Dextran sulfate; final washing conditions were 65°C, 0.5× SSC and 0.1% SDS.

Fluorescence microscopy for GFP detection. For light and fluorescence microscopy, 20 µl of a protoplast suspension contain-

TABLE 1. Symptom expression in plants inoculated by particle bombardment with *Pepper huasteco virus* (PHV), *Pepper golden mosaic virus* (PepGMV), or a PHV/PepGMV mixture^z

Host	PHV	SD	PepGMV	SD	Mix	SD
Pepper genotypes						
Ancho San Luis	7.0	0.9	9.0	0.7	8.0	0.9
Sonora Anaheim	6.5	0.7	10.0	0.5	8.5	0.4
Jalapeño Tres Lomos	5.0	1.4	9.0	1.7	7.0	1.5
Mirasol La Blanca 74	7.0	1.8	8.0	0.9	7.0	1.1
Serrano 25 Pánuco	6.0	1.5	8.0	0.7	7.0	0.8
28 L-Serrano 2-(M)-3-1	5.0	1.9	10.0	1.6	6.0	1.7
Ancho Rojo Carmin	6.0	0.9	9.0	1.8	7.0	1.5
Piquín BG54	5.0	1.7	9.0	1.3	7.0	1.3
Chile de árbol 3555	6.0	1.8	9.0	1.5	7.0	1.4
Mean	5.9 bc	...	9.0 a	...	7.1 b	...
<i>Nicotiana benthamiana</i>	7.0	...	8.0	...	10.0	...
<i>N. tabacum</i>	5.5	...	8.0	...	10.0	...

^z Arbitrary scale (1 to 10) for symptom severity. In the case of pepper, the mean value for all genotypes is shown. SD = standard deviation. Statistics analysis (analysis of variance) showed that the values obtained for PepGMV and for the mixture are significantly different ($P < 0.05$, $n = 20$ /genotype). In the case of tobacco and *Nicotiana benthamiana*, the responses were much more homogeneous (SD = 0.5 and 0.6, respectively). Again, differences in values for PepGMV and Mix were statistically significant. Lowercase letters represent the groups that resulted after the statistical analysis (Tukey Test).

ing approximately 2×10^3 protoplasts was transferred to a glass slide and analyzed with a Nikon (Melville, NY) Optiphot 2 UD microscope. Visualization of GFP fluorescence in the whole plant was performed with an FITC filter cube (470- to 490-nm excitation filter, 505-nm dichroic mirror, and 520-nm barrier filter). For analysis of GFP expression, inoculated and apical leaves were removed and analyzed under the microscope. Documentation was done by photography with Kodak EPH p1600 film. Original 35-mm slides were scanned and processed with Adobe PhotoShop software (Adobe Systems, San Jose, CA). Fluorescence also was detected and recorded with a confocal laser-scanning microscope (Olympus BX50) with wavelengths of excitation and emission of 488 and 510 nm, respectively. Digital images obtained with the confocal microscope also were processed with Adobe PhotoShop software.

Fig. 1. Symptoms induced in plants inoculated with *Pepper huasteco virus* (PHV), *Pepper golden mosaic virus* (PepGMV), and the mixture. **A, B, and C,** Pepper, **D, E, and F,** *Nicotiana benthamiana*, and **G, H, and I,** tobacco plants were inoculated with **A, D, and G,** PHV, **B, E, and H,** the mixture PHV/PepGMV, or **C, F, and I,** PepGMV using a biolistic procedure. Inoculated plants were transferred to a growth chamber for symptom expression and evaluated at 20 days postinoculation. **E and H,** A synergism can be observed in tobacco and *N. benthamiana* plants inoculated with the mixture; whereas, in pepper, the mixture induced less severe symptoms (**B**) when compared with the PepGMV-infected plants (**C**).

RESULTS

Symptom expression in single and mixed infections. The mixture of PHV and PepGMV initially was detected in north-eastern Mexico in the late 1980s. However, more recent surveys have shown that both viruses currently are distributed widely in all horticultural areas in Mexico, with pepper being the most common host for both viruses. For plant–geminivirus interactions, the commercial cv. Sonora Anaheim was chosen as the experimental host due to its high homogeneity in several phenological (i.e., germination and flowering) and phytopathological (time for symptom appearance and severity) parameters. In this host, PHV induces symptoms with an average intensity of 5 (in an arbitrary scale of 1 to 10), whereas PepGMV produces a more severe response with an intensity of 10 (Table 1; Fig. 1). Interestingly, when both viruses were inoculated as a mixture, the severity of the symptoms induced was reduced for PepGMV and increased for PHV (Table 1; Fig. 1). To verify whether or not the decrease in symptom severity was dependent on the pepper genotype, similar experiments also were performed using eight additional pepper genotypes (including other commercial cultivars as well as wild pepper species), and three different inoculation procedures (biolistics, grafting, and whiteflies). The results obtained with all pepper genotypes inoculated with the biolistic procedure are shown in Table 1. In all genotypes, the mixture of PepGMV and PHV induced attenuated symptoms in systemically infected pepper when compared with those induced by PepGMV alone. Similar results were obtained when the plants were inoculated using grafting or whiteflies (data not shown). These results suggested an antagonistic interaction between PHV and PepGMV in pepper plants at the level of symptom expression.

To determine if the antagonistic interaction in symptom expression also occurred in other hosts, similar experiments were performed with *N. benthamiana* and *N. tabacum* Xanthi-nc. Again, the intensity of the symptoms induced by PepGMV was more severe in both *Nicotiana* spp. than was that induced by PHV. However, contrary to the results observed in pepper, the plants inoculated with the mixture (PHV/PepGMV) showed an increased symptom severity in both *Nicotiana* spp. (Table 1; Fig. 1). In addition, the symptoms appeared as early as 3 dpi when both viruses were co-inoculated; whereas, in single infections, the symptoms appeared 6 to 8 dpi. This suggested that, in the case of tobacco and *N. benthamiana*, the presence of both viruses resulted into a synergetic interaction.

PHV–PepGMV interactions at the level of replication. The ability to create viable pseudorecombinant viruses by exchanging

TABLE 2. Detection of viral DNA in pseudorecombination, single and mixed infections in protoplasts and plants^w

Inoculated DNA ^y	Protoplasts				Plants				Infectivity ^z
	Detection of viral DNA ^x								
	PHV		PepGMV		PHV		PepGMV		
	A	B	A	B	A	B	A	B	
PHV A + B	+	+	na	na	+	+	na	na	19/20
PepGMV A + B	na	na	+	+	na	na	+	+	20/20
PHV A + PepGMV B	+	na	na	–	–	na	na	–	0/20
PepGMV A + PHV B	na	–	+	na	na	–	–	na	0/20
PHV A + B + PepGMV A + B	+	+	+	+	+	+	+	+	20/20
PHV A + B + PepGMV A	+	+	+	na	+	+	+	na	20/20
PepGMV A + B + PHV A	+	na	+	+	–	na	+	+	20/20
Mock	–	–	–	–	–	–	–	–	0/15

^w Protoplasts (tobacco) or plants were inoculated with the proper DNA combinations and analyzed for virus replication by Southern or dot blot hybridization using specific probes. In the case of inoculated plants, virus replication was analyzed in noninoculated, apical tissue. Experiments were repeated in pepper, tobacco, and *Nicotiana benthamiana* plants, except in the case of tripartite inoculation, where only pepper and *N. benthamiana* plants were used. The experiments in protoplasts were repeated three times with replicas for each DNA combination.

^x PHV = *Pepper huasteco virus*, PepGMV = *Pepper golden mosaic virus*, + = viral DNA detected, – = no viral DNA detected, na = not applicable.

^y Mock (protoplasts) = carrier DNA alone; Mock (plants) = pBluescript DNA alone.

^z Symptomatic plants/inoculated plants.

genomic components is probably the most studied virus–virus interaction for bipartite geminiviruses. For PHV and PepGMV, an analysis of their intergenic region showed that the iterative elements involved in replication specificity were different; therefore, it was predicted that these viruses could not form viable pseudorecombinant entities (1). To corroborate whether the DNA A of either virus (PHV or PepGMV) was able to mediate replication of the DNA B of the second virus and cause a systemic infection on the inoculated plants, the proper combinations were inoculated into pepper or tobacco plants. Neither one of the pseudorecombinants induced a systemic infection in pepper after 45 dpi, implying a genomic incompatibility. To verify that this incompatibility was at the level of replication, the same pseudorecombinant mixtures were transfected into tobacco protoplasts. Again, neither PHV nor PepGMV DNA A was able to *trans*-replicate the heterogenomic DNA B to detectable levels. The levels of viral replication were monitored 24, 48, and 70 hpi. These results suggest that the incapacity to generate viable pseudorecombinant viruses is due at least to inability to *trans*-replicate the heterogenomic components (Table 2).

In addition to the pseudorecombination assays, it was also of interest to see if the replication of one virus could affect the replication of the second virus when both pathogens were co-inoculated. *N. tabacum* plants were inoculated with both viruses and virus accumulation was analyzed by Southern blots using virus-specific probes. There was no evidence, in either case, that the replication of one virus could interfere with the replication of the second virus because the levels of replicative forms (RFs) for both viruses were never reduced in the mixed-infected plants. On the contrary, in both cases, it was common to observe that the RF levels of both viruses, as detected by Southern blot hybridization analysis, were elevated in the doubly infected plants compared with those observed in single infections (Fig. 2). Similar results were obtained with pepper plants, indicating that the increased viral accumulation is not host dependent (data not shown).

Elevated RF levels in mixed infection could be achieved by increased replication rate in the infected cells or by increasing the numbers of infected cells. To differentiate between these two possibilities, tobacco protoplasts were transfected with different viral DNA combinations and RF DNA detected by Southern blot hybridization assays (Fig. 2B). Again, RF levels were elevated in protoplasts transfected with both viruses or with tripartite mixtures containing both DNA A components. These results indicate that the presence of both DNA A components (PHV and PepGMV) in the same cell increases, by a mechanism still unknown, the replication rate of both viruses.

PHV facilitates the long-distance movement of PepGMV DNA A. Another type of interaction between geminiviruses infecting the same plant could occur at the level of movement. During the analysis of field-collected pepper samples, it was noticed that, in some cases, it was relatively easy to detect three distinct viral DNAs (PHV A and B and PepGMV DNA A), whereas PepGMV DNA B was either absent or at very low concentrations. Similar results with a field sample of tomato infected with PHV and an uncharacterized DNA A of PepGMV (reported therein as TPV) recently were communicated (32). This suggested that some type of movement complementation between PHV and PepGMV is possible. To investigate the ability of one virus to facilitate the long-distance movement of the DNA A of the second virus (movement complementation), pepper plants were inoculated with the respective tripartite mixture (PHV and PepGMV DNA A or PepGMV and PHV DNA A) by particle bombardment. The inoculated plants were evaluated for symptom appearance and for the presence of both types of viral DNAs (PHV A and PepGMV A) using Southern blots and specific probes. In the case of the plants inoculated with the mixture PHV + PepGMV DNA A, the symptoms induced were stronger than the ones induced by PHV alone, suggesting the presence of PepGMV DNA A in the

systemically infected plants. Indeed, Southern blot hybridization analysis of uninoculated, newly developed leaves revealed the presence of PepGMV DNA A. This demonstrated that this viral DNA was able to move systemically using viral movement factors provided by PHV (Fig. 3). In contrast, when pepper plants were inoculated with the reciprocal tripartite mixture PepGMV and

Fig. 2. *Pepper huasteco virus* (PHV) and *Pepper golden mosaic virus* (PepGMV) accumulate to higher levels in mixed-infected plants and protoplasts. **A**, Tobacco plants were inoculated with either PHV (line 1), PepGMV (line 2), or the mixture PHV/PepGMV (lines 3 and 4). After symptom appearance (10 to 12 days postinoculation), DNA was extracted from apical tissue and analyzed by Southern hybridization using specific probes for PHV (1 and 2) or PepGMV (3 and 4). Plants inoculated with the mixture showed a higher concentration of viral DNA than the one found in single infections. **B**, Protoplasts were transfected with the following viral DNAs: Line 1 = mock, 2 = PHV, 3 = PHV + PepGMV, 4 = PHV A + PepGMV, 5 = PHV + PepGMV A. After 48 h of incubation, DNA was extracted and analyzed by Southern hybridization using a PHV specific probe. **C**, Similar assay as in B but with the following DNAs: Line 1 = mock, 2 = PepGMV, 3 = PHV, 4 = PHV + PepGMV, 5 = PepGMV A + PHV, and a probe specific for PepGMV. Arrows indicate the main viral DNA forms; top = supercoiled double-stranded (ds)DNA, middle = relaxed dsDNA, and bottom = single-stranded DNA. Gel electrophoresis in panel A was carried out using a large electrophoresis chamber; thus, a better separation was obtained.

PHV DNA A, only PepGMV was detected in the newly developed leaves, suggesting that PHV DNA A was not able to move systemically in this combination (Fig. 3). In other words, an asymmetric movement complementation between PHV and PepGMV was observed in pepper plants because PHV facilitated the systemic movement of PepGMV DNA A, whereas the PepGMV did not facilitate the movement of PHV DNA A.

To verify if the asymmetric movement complementation between PHV and PepGMV was a host-specific event, similar experiments were carried out in *N. benthamiana*. To facilitate the monitoring of virus movement, two chimeric viral DNA A components (PHV A-GFP and PepGMV A-GFP) were constructed by replacing the CP gene with that encoding the GFP from the jelly fish *Aequorea victoria*. *N. benthamiana* plants were inoculated with the proper tripartite mixture (PHV and PepGMV A-GFP or PepGMV and PHV A-GFP) by particle bombardment. In this case, the symptoms induced by both tripartite mixtures were stronger than those induced by PHV alone and similar to those induced by the mixture of both viruses (four components) (Fig. 1). Two types of leaves were analyzed in these experiments: inoculated leaves, which were analyzed for viral replication and GFP expression to ascertain the infectivity nature of the chimeric viruses; and young, uninoculated leaves, which were analyzed to verify viral systemic movement. After bombardment, leaves inoculated with either tripartite mixture were removed from the plant and examined by confocal laser-scanning microscopy for GFP expression (Fig. 4). In our conditions, GFP expression was clearly detected at 4 dpi in both cases, mainly in epidermal cells (Fig. 4B) or as irregular, small fluorescent clusters (Fig. 4A). Southern blot and PCR analysis confirmed that the chimeric DNA A-GFP constructs were able to replicate in their respective inoculated leaves (data not shown).

To analyze the systemic spread of the viruses and, therefore, the possible movement complementation, young, uninoculated leaves from systemically infected plants were observed up to 26 dpi with a fluorescence microscope for GFP expression. In most of the plants co-inoculated with PepGMV A-GFP and PHV, patches of bright fluorescence were observed between secondary veins of the newly developed leaves (Fig. 4C). In some leaves, GFP fluorescence was also found along the main vein (Fig. 4D). It is important to note that, after an extensive analysis of leaves, petioles, and stems, GFP expression was observed only as discontinuous,

well-separated, hard-to-find patches of fluorescence. In the experiments with the reciprocal mixture, PHV A-GFP and PepGMV, none of the inoculated plants showed GFP fluorescence in the upper, younger leaves, indirectly indicating the absence of this viral DNA (PHV A-GFP) in those tissues. Southern blot or PCR analysis confirmed those observations (data not shown). These results suggest that PHV A-GFP (co-inoculated with PepGMV) is able to replicate at the initial sites of inoculation and, perhaps, to show some cell-to-cell movement (because of the size of the fluorescent clusters). However, it cannot move systemically, suggesting that PepGMV, as previously demonstrated in pepper, does not complement the long-distance movement of PHV A. Therefore, the asymmetric *trans*-complementation in movement between PHV and PepGMV is not host-specific.

Heterologous transactivation between PHV and PepGMV.

Transactivation of the CP promoter by TrAP protein has been reported for several geminiviruses, including PHV (28). In addition, it also was reported that TrAPs from several viruses were able to transactivate the CP promoter of TGMV, suggesting a high conservation of TrAP function and mode of action (35). To evaluate the possibility of heterologous transactivation between PHV and PepGMV, the GUS reporter gene was cloned downstream of the CP promoters from both viruses to generate PHV CP-GUS and PepGMV CP-GUS constructions that were evaluated in transactivation assays as described earlier (28). The chimeric GUS constructions were co-bombarded into tobacco leaf tissue (or a layer of cells from a cell suspension culture) with either PHV DNA A or PepGMV DNA A as source of TrAP. As a control, the same constructs were co-bombarded with pBluescript (pBS) DNA. As described earlier (28), the level of transactivation is defined as the difference in the values of GUS expression obtained in the presence (PHV DNA A or PepGMV DNA A) or absence (pBS) of a source of TrAP.

The results of the transactivation assays are shown in Figure 5. First, both CP promoters (PHV CP-GUS and PepGMV CP-GUS) were transactivated when co-bombarded with either virus, therefore showing homologous and heterologous transactivation. Second, it also was consistently noticed that the overall expression of PHV CP promoter, observed as GUS activity, was higher than that observed from the PepGMV promoter, independent of which virus was being used as a source of TrAP. Even the background transcription of PHV CP promoter (when co-bombarded with pBS; no TrAP) is higher than the one observed with PepGMV CP promoter under the same conditions, suggesting a stronger promoter activity. Overall, these results demonstrate that virus interactions in a mixed infection also could occur at the level of gene expression by altering regulation mechanisms.

DISCUSSION

Although natural mixed infections are common in all agricultural areas, most studies focus on single infections, perhaps due to the complexity in addressing mixed infections. With the development of tools that permit the precise identification of a virus (or variant), facilitate monitoring of recombination events, and allow ectopic expression of viral genes to study their effect on viral infection, the number of reports addressing complex diseases has increased.

After their initial detection in northeastern Mexico in pepper fields, PHV and PepGMV have been spreading throughout the country infecting, in addition to pepper, other important crops such as tomato, tobacco, and tomatillo (2,12,21,36). An analysis of the dynamics of their distribution shows that their rate of spread has been different. For example, although both viruses are currently present in all horticultural areas, PHV was detected earlier than PepGMV in most cases (e.g., in Sinaloa and Morelos). In addition, the number of plants infected with PHV is usually higher than the number of plants infected with PepGMV.

Fig. 3. Asymmetric movement complementation between *Pepper huasteco virus* (PHV) and *Pepper golden mosaic virus* (PepGMV). Pepper plants were inoculated with the proper tripartite mixture (PHV + PepGMV A or PHV A + PepGMV). After symptom appearance, DNA was extracted from the apical leaves and analyzed by Southern hybridization using specific probes. **A**, Line 1 = PepGMV-infected plant, line 2 = PHV-infected plant, line 3 = plant inoculated with PHV + PepGMV A. **B**, Line 1 = PepGMV-infected plant, line 2 = plant inoculated with PepGMV + PHV A, line 3 = PHV cloned DNA as hybridization control. PHV-specific probe; arrows indicate the main viral DNA forms; top = supercoiled double-stranded (ds)DNA, middle = relaxed dsDNA, and bottom = single-stranded DNA. Both membranes were regenerated and hybridized with the reciprocal probe to verify the presence of the second viral DNA (data not shown).

From an agronomical and practical point of view, probably the most important aspect of a mixed infection is the alteration of symptoms. Disease complexes showing stronger, more severe symptoms than those induced by single infections may be economically more important. The results reported here show that, in the case of the PHV/PepGMV mixture, the alteration of the symptoms is host-dependent because an antagonistic effect was observed in pepper, whereas a synergism was obtained in tobacco and *N. benthamiana* plants. Nevertheless, the reduction in the symptoms observed in pepper plants is probably more meaningful because this crop is the most common host for both viruses in nature. It is thought that viruses that are lethal or that induce very strong symptoms will have reduced opportunities to spread in nature. It is possible that one of the beneficial effects of the mixture (at least for PepGMV) is that PHV reduces the severity of the symptoms caused by PepGMV, therefore favoring its exposure to insect vectors and facilitating its plant-to-plant spread.

It has been suggested that pseudorecombination and recombination processes are evolutionary mechanisms that might facilitate the emergence of new geminiviruses (or variants) that are more fit than the parental viruses for a specific niche or factor (e.g., new vector, new hosts). Direct evidence for this ability has been reported recently in natural mixed infections (8,26,41). On the other hand, most attempts to produce pseudorecombinant viruses by reassortment of genomic components have supported the hypothesis that the iteron elements found in the intergenic region are important factors that determine replication specificity and, therefore, only viruses (or strains) that share iterons with similar sequences are able to produce a viable pseudorecombination event (1,6,13, 39). However, it has been shown that, despite the low identity of their common region, some viruses can produce viable pseudorecombinants (11,26). In this report, it was shown that an exchange of genomic components between PHV and PepGMV (PHV A + PepGMV B or PHV B + PepGMV A) did not generate infectious pseudorecombinant viruses when inoculated into pepper plants. It also was confirmed that neither PHV nor PepGMV DNA A could *trans*-replicate, in protoplast assays, their heterogenomic component DNA B, indicating a genomic incompatibility. The experimental data presented here corroborates the theoretical prediction that PHV and PepGMV could not form viable pseudorecombinant viruses because of the different iteron sequences (1). On the other hand, a careful analysis of PepGMV sequence suggests that perhaps this virus is already a recombinant entity. When only the sequence of component A is used for phylogeny studies, PepGMV is clustered in a separated group along with *Squash leaf curl virus* (SqLCV), *Bean calico mosaic virus* (BCaMV), and *Cabbage leaf curl virus* (CabLCV). This grouping also agrees with the analysis of the organization of the iterative elements in the intergenic region (1). However, when the sequence of the component B is used for the analysis, PepGMV is clustered together with PHV and the rest of the American begomoviruses. Indeed, at the amino acid level, BC1 and BV1 from PepGMV (Accession No. AF499442) showed the highest percentage of identity with the equivalent proteins from PHV. It is possible that the current component B of PepGMV emerged from a recombination event, involving PHV and PepGMV, similar to the one described in laboratory experiments for ToMoV and BDMV (17). This event probably occurred some time ago because the percentage of identity between the intergenic regions of PHV and PepGMV DNA B is rather low.

Synergism at the level of replication probably has been studied best in the interaction between *Potato virus X* (PVX) and *Potato virus Y* (PVY), where PVX replication is increased by a mechanism involving PVY protein HC-Pro (27,38). In the case of the PHV/PepGMV mixture, the RF levels from both viruses are increased in the infected plant. Similar observations have been recently communicated with a tripartite mixture of PHV and an uncharacterized TPV (PepGMV) A (32). As mentioned above, this increase can be accomplished in two ways. One is to increase, by

whatever mechanism, the replication rate of both viruses in every infected cell. The second way is to increase the number of infected cells. The fact that similar results were obtained in protoplast assays indicates that the replication rate of both viruses is, indeed, increased in the double infection. A third alternative combines both options.

Asymmetric movement complementation has been previously reported (9,17) for artificial geminivirus mixtures infecting the experimental host *N. benthamiana*. In that report, it was demonstrated that ACMV could mediate the systemic movement of TGMV and *Abutilon mosaic virus* (AbMV) DNA A components; whereas, in the reciprocal experiment, neither TGMV nor AbMV could mediate systemic movement of ACMV DNA A. The evolutionary divergence between New and Old World geminiviruses was suggested to explain those results (9). For the results reported here, however, other possibilities have to be considered. First, phylogenetic analyses suggest that both viruses are indigenous to America. A differential host adaptation also is unlikely because the host range, which is very similar for both viruses, includes natural infections of both pepper and tobacco. The possibility that

Fig. 4. *Pepper huasteco virus* (PHV) can complement the movement of *Pepper golden mosaic virus* (PepGMV) DNA A in *Nicotiana benthamiana*. *N. benthamiana* plants were inoculated with either a tripartite mixture, PHV + PepGMV A-GFP, or PepGMV + PHV A-GFP. Both **A** and **B**, inoculated (local expression) and **C** to **F**, newly developed leaves (systemic expression) were analyzed for green fluorescent protein (GFP) expression. Local expression: leaves inoculated with mixtures **A**, PepGMV + PHV A-GFP or **B**, PHV + PepGMV A-GFP showed strong fluorescence in epidermal cells. In the case of **B**, some individual fluorescent cells often were detected. Systemic expression: only plants inoculated with the mixture PHV + PepGMV A-GFP showed fluorescence in the apical leaves at 26 days postinoculation. Fluorescence was detected in varied forms. In some cases (e.g., young, developing leaves) clusters of cells from were visualized (**C** and **D**, bright field image of **C**). In other cases (e.g., leaves a few days older than in **C**), fluorescence was visualized in cells near the main vein of the leaf, mainly in the nuclei and cell wall. Fluorescence also was detected in some trichomas (**E** and **F**, combination of UV exposure and bright field image of **E**). **A** and **B**, Olympus confocal microscope. **C** to **F**, Nikon fluorescence microscope, 1600 ISO film.

PepGMV movement proteins do not efficiently recognize PHV DNA components is also unlikely because of their high percentage of sequence identity. Therefore, other biological differences between the viruses have to be considered, and one such difference is tissue tropism. Several results support the hypothesis that PHV may be restricted to the vascular tissue (14,28). On the other hand, PepGMV seems to have a less restricted tissue tropism (33; R. F. Rivera-Bustamante, *unpublished data*). It is possible that, during its systemic movement, PHV is replicated preferentially in vascular tissue-associated cells, whereas PepGMV replicates, in addition to vascular tissue, in a secondary type of cells (e.g., mesophyll cells). Thus, when the tripartite mixture PepGMV and PHV A passes (following the PepGMV normal route) through these non-vascular cells, PHV A will replicate very inefficiently in these cells and will be left behind. In the case of TGMV, it has been recently determined that its tissue tropism is genetically determined and three elements were involved, including a regulatory, non-coding region (23). It is possible that, despite the fact that PHV and PepGMV movement proteins present a high percentage of identity, they are regulated in a different manner, presenting different tissue specificities, such as in the case of CP promoters.

The fact that both CP promoters were able to show heterologous transactivation is not surprising, given the closeness of the viruses. Although some PepGMV strains do not contain a typical CLE motif, it has been shown recently that a CP promoter from a virus without CLE is functionally equivalent to a CLE-containing CP promoter (18). This suggests that, in the former case, there are some other elements involved in TrAP-mediated transactivation. The difference in CP promoter strength between PHV and PepGMV CP observed in Figure 5 has been recently confirmed after the analysis of tobacco transgenic lines containing the PepGMV CP-GUS fusion (J. C. Vaca-Vaca and R. F. Rivera-Bustamante, *unpublished data*). The expression of viral genes usually is well coordinated; therefore, the presence of viral proteins from a second virus (that is, following its own coordination) might have an impact on the regulation of the virus infection cycle.

Fig. 5. Heterologous transactivation between *Pepper huasteco virus* (PHV) and *Pepper golden mosaic virus* (PepGMV). Coat protein promoters from both viruses were fused to the *uidA* reporter gene (GUS). The constructs were bombarded into tobacco leaf tissue in the presence of a source of TrAP. Tissue was stained for GUS activity and the blue spots per area were counted. Each bar represents the average of three independent bombardments (individual values also are shown as dots). As control (no TrAP), the GUS fusions also were co-bombarded with pBluescript DNA (pBS). Transactivation is observed as the increase in GUS expression obtained when the constructs are co-bombarded with a source of TrAP (26). Similar results were obtained after bombarding a layer of cells from a tobacco cell suspension culture.

An important question addressed in this study by using a natural virus mixture is the biological significance of virus interactions. Do the interactions reported here provide some special, advantageous characteristics to the PHV/PepGMV mixture? The answer probably is yes. For example, its epidemiology is different from that shown by other geminiviruses reported in Mexico. A typical situation is represented by the first geminivirus reported in Mexico, *Chino del tomate virus* (CdTV), which, 20 years after its initial detection in Sinaloa, still is located mostly in northwestern Mexico (36). Similar situations can be mentioned for other viruses (10,36). In most cases, they seem to show little or no spread from the original detection point. The fact that both PHV and PepGMV are distributed throughout Mexico and often are found in mixtures affecting a variety of crops, suggests that this mixture might offer some advantages compared with individual infections. The interactions studied here, and others not yet addressed, have to be studied in more detail to determine the precise nature of those advantages. Similarly, future research also should focus on how two viral genomes, instead of one, interact with the plant genome during mixed infections.

ACKNOWLEDGMENTS

This research was supported by grants from CONACYT-México (26331N) and UCMex-CONACYT to R. F. Rivera-Bustamante. We thank CONACYT-México for fellowship support to J. Méndez-Lozano and I. Torres-Pacheco, and IRD-France for support for J. Méndez-Lozano.

LITERATURE CITED

- Argüello-Astorga, G. R., Guevara-González, R. G., Herrera-Estrella, L. R., and Rivera-Bustamante, R. F. 1994. Geminivirus replication origins have a group-specific organization of iterative elements: A model for replication. *Virology* 203:90-100.
- Ascencio-Ibañez, J. T., Argüello-Astorga, G. R., Méndez-Lozano, J., and Rivera-Bustamante, R. F. 2002. First report of Rhynchosia golden mosaic virus (RhGMV) infecting tobacco in Chiapas, Mexico. *Plant Dis.* 88:692.
- Atabekov, J. G., and Taliansky, M. E. 1990. Expression of a plant virus-coded transport function by different viral genomes. *Adv. Virus Res.* 38:201-248.
- Berrie, L. C., Rybicki, E. P., and Rey, M. E. 2001. Complete nucleotide sequence and host range of South African cassava mosaic virus: Further evidence for recombination amongst begomoviruses. *J. Gen. Virol.* 82:53-58.
- Carr, R. J., and Kim, K. S. 1983. Evidence that bean golden mosaic virus invades non-phloem tissue in double infections with tobacco mosaic virus. *J. Gen. Virol.* 64:2489-2492.
- Faria, J. C., Gilbertson, R. L., Hanson, S. F., Morales, F. J., Ahlquist, P., Loniello, A. O., and Maxwell, D. P. 1994. Bean golden mosaic geminivirus type II isolates from the Dominican Republic and Guatemala: Nucleotide sequences, infectious pseudorecombinants and phylogenetic relationships. *Phytopathology* 84:321-329.
- Fedorkin, O. N., Merits, A., Luccesi, J., Solovyev, A. G., Saarma, M., Morozov, S. Y., and Mäkinen, K. 2000. Complementation of movement-deficient mutations in potato virus X: Potyvirus coat protein mediates cell-to-cell trafficking of C-terminal truncation but not deletion mutant of potyvirus at protein. *Virology* 270:31-42.
- Fondong, V. N., Pita, J. S., Rey, M. E., de Kochko, A., Beachy, R. N., and Fauquet, C. M. 2000. Evidence of synergism between African cassava mosaic virus and a new double-recombinant geminivirus infecting cassava in Cameroon. *J. Gen. Virol.* 81:287-297.
- Frischmuth, T., Roberts, S., Von, A. A., and Stanley, J. 1993. Specificity of bipartite geminivirus movement proteins. *Virology* 196:666-673.
- Garrido-Ramírez, E. R., and Gilbertson, R. L. 1998. First report of Tomato mottle geminivirus infecting tomatoes in Yucatan, Mexico. *Plant Dis.* 85:592.
- Garrido-Ramírez, E. R., Sudarshana, M. R., and Gilbertson, R. L. 2000. *Bean golden yellow mosaic virus* from Chiapas, Mexico: Characterization, pseudorecombination with other bean-infecting geminiviruses and germ plasm screening. *Phytopathology* 90:1224-1232.
- Garzón-Tiznado, J. A., Torres-Pacheco, I., Ascencio-Ibañez, J. T., Herrera-Estrella, L., and Rivera-Bustamante, R. F. 1993. Inoculation of peppers with infectious clones of a new geminivirus by a biolistic procedure. *Phytopathology* 83:514-521.

13. Gilbertson, R. L., Hidayat, S. H., Paplomatas, E. J., Rojas, M. R., Hou, Y. M., and Maxwell, D. P. 1993. Pseudorecombination between infectious cloned DNA components of tomato mottle and bean dwarf mosaic geminiviruses. *J. Gen. Virol.* 74:23-31.
14. Guevara-González, R. G., Ramos, P. L., and Rivera-Bustamante, R. F. 1999. Complementation of coat protein mutants of pepper huasteco geminivirus in transgenic tobacco plants. *Phytopathology* 89:540-545.
15. Harrison, B. D., Zhou, X., Otim-Nape, G. W., Liu, Y., and Robinson, D. 1997. Role of a novel type of double infection in the geminivirus-induced epidemic of severe cassava mosaic in Uganda. *Ann. Appl. Biol.* 131:437-448.
16. Heim, R., Cubitt, A. B., and Tsien, R. Y. 1995. Improved green fluorescence. *Nature* 373:663-664.
17. Hou, Y. M., and Gilbertson, R. L. 1996. Increased pathogenicity in a pseudorecombinant bipartite geminivirus correlates with intermolecular recombination. *J. Virol.* 70:5430-5436.
18. Hung, H. C., and Petty, I. T. 2001. Functional equivalence of late gene promoters in bean golden mosaic virus with those in tomato golden mosaic virus. *J. Gen. Virol.* 82:667-672.
19. Kamei, T., Goto, T., and Matsui, R. 1969. Reduced turnip mosaic virus multiplication in leaves infected with cauliflower mosaic virus. *Phytopathology* 59:1513-1516.
20. Malishenko, S. I., Kondakova, O. A., Talianky, M. E., and Atabekov, J. G. 1989. Plant virus transport function: Complementation by helper viruses is non-specific. *J. Gen. Virol.* 70:2751-2757.
21. Méndez-Lozano, J., Rivera-Bustamante, R. F., Fauquet, C. M., and De la Torre-Almaraz, R. 2001. *Pepper huasteco virus* and *Pepper golden mosaic virus* are geminiviruses affecting tomatillo (*Physalis ixocarpa*) crops in Mexico. *Plant Dis.* 85:1291.
22. Mettler, I. J. 1987. A simple and rapid method for miniprep of DNA from tissue cultured plant cells. *Plant Mol. Biol. Rep.* 5:346-349.
23. Morra, M. R., and Petty, I. T. D. 2000. Tissue specificity of geminivirus infection is genetically determined. *Plant Cell* 12:2259-2270.
24. Murphy, F. A., Fauquet, C. M., Bishop, D. H. L., Ghabrial, S. A., Jarvis, A. W., Martelli, G. P., Mayo, M. A., and Summers, M. D. 1995. Classification and nomenclature of viruses. Sixth report of the International Committee on Taxonomy of Viruses. *Arch. Virol. Suppl.* 10.
25. Petty, I. T. D., Miller, C. G., Meade-Hash, T. J., and Schaffer, R. L. 1995. Complementary and noncomplementary host adaptation defects in bipartite geminiviruses. *Virology* 212:263-267.
26. Pita, J. S., Fondong, V. N., Sangare, A., Otim-Nape, G. W., Ogwal, S., and Fauquet, C. M. 2001. Recombination, pseudorecombination and synergism of geminiviruses are determinant keys to the epidemic of severe cassava mosaic disease in Uganda. *J. Gen. Virol.* 82:655-665.
27. Pruss, G., Ge, X., Shi, X. M., Carrington, J. C., and Vance, V. B. 1997. Plant viral synergism: The potyviral genome encodes a broad range pathogenicity enhancer that transactivates replication of heterologous viruses. *Plant Cell* 9:859-868.
28. Ruiz-Medrano, R., Guevara-Gonzalez, R. G., Arguello-Astorga, G. R., Monsalve-Fonnegrá, Z., Herrera-Estrella, L. R., and Rivera-Bustamante, R. F. 1999. Identification of a sequence element involved in AC2-mediated transactivation of the pepper huasteco virus coat protein gene. *Virology* 253:162-169.
29. Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual*. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
30. Sanz, A. I., Fraile, A., García-Arenal, F., Zhou, X., Robinson, D. J., Khalid, S., Butt, T., and Harrison, B. D. 2000. Multiple infection, recombination and genome relationship among begomovirus isolates found in cotton and others plants in Pakistan. *J. Gen. Virol.* 81:1839-1849.
31. Saunders, K., Bedford, I. D., and Stanley, J. 2001. Pathogenicity of a natural recombinant associated with ageratum yellow vein disease: Implications for geminivirus evolution and disease etiology. *Virology* 282:38-47.
32. Sharp, L. P., Hou, Y. M., Garrido-Ramirez, E. R., Guzman, P., and Gilbertson, R. L. 1999. A synergistic interaction between geminivirus DNA components results in increased symptom severity and viral DNA levels in plants. (Abstr.) *Phytopathology* 89(suppl.):S71.
33. Stenger, D. C., Duffus, J. E., and Villalon, B. 1990. Biological and genomic properties of a geminivirus isolated from pepper. *Phytopathology* 80:704-709.
34. Sung, Y. K., and Coutts, R. H. 1995. Pseudorecombination and complementation between potato yellow mosaic geminivirus and tomato golden mosaic geminivirus. *J. Gen. Virol.* 76:2809-2815.
35. Sunter, G., Stenger, D. C., and Bisaro, D. M. 1994. Heterologous complementation by geminivirus AL2 and AL3 genes. *Virology* 203:203-210.
36. Torres-Pacheco, I., Garzón-Tiznado, J. A., Brown, J. K., Becerra-Flora, A., and Rivera-Bustamante, R. F. 1996. Detection and Distribution of Geminiviruses in Mexico and Southern United States. *Phytopathology* 86:1186-1192.
37. Torres-Pacheco, I., Garzón-Tiznado, J. A., Herrera-Estrella, L., and Rivera-Bustamante, R. F. 1993. Complete nucleotide sequence of pepper huasteco virus: Analysis and comparison with bipartite geminiviruses. *J. Gen. Virol.* 74:2225-2231.
38. Vance, V. B., Berger, P. H., Carrington, J. C., Hunt, A. G., and Shi, X. M. 1995. 5' Proximal potyviral sequence mediates potato virus X/potyviral synergistic disease in transgenic tobacco. *Virology* 206:583-590.
39. Von Arnim, A., and Stanley, J. 1992. Determinants of tomato golden mosaic virus symptom development located on DNA B. *Virology* 186:286-293.
40. Watanabe, Y., Meshi, T., and Okada, Y. 1987. Infection of tobacco protoplasts with *in vitro* transcribed tobacco mosaic virus RNA using an improved electroporation method. *FEBS Lett.* 219:65-69.
41. Zhou, X., Liu, Y., Calvert, L., Munoz, C., Otim-Nape, G. W., Robinson, D. J., and Harrison, B. D. 1997. Evidence that DNA-A of a geminivirus associated with severe cassava mosaic disease in Uganda has arisen by interspecific recombination. *J. Gen. Virol.* 78:2101-2111.