Resistance to Geminivirus Mixed Infections in Mexican Wild Peppers

J.L. Anaya-López, I. Torres-Pacheco, M. González-Chavira, J.A. Garzon-Tiznado, and J.L. Pons-Hernandez
Unidad de Biotecnología del Bajío, Instituto Nacional de Investigaciones Forestales Agrícolas y Pecuarias-Campo Experimental Bajío, Apartado postal 112, Celaya, Gto, México

Departamento de Ingeniería Bioquímica, Instituto Tecnológico de Celaya, Apartado postal 57, Celaya, Gto, México

R.F. Rivera-Bustamante
Departamento de Ingeniería Genética, Centro de Investigación y de Estudios Avanzados del I.P.N. Unidad Irapuato, Apartado postal 629, Irapuato, Gto, México

S. Hernández-Verdugo
Facultad de Agronomía, Universidad Autónoma de Sinaloa; Código postal 80000, Culiacán, Sinaloa, México

Abstract. Screening for resistance to mixed infections with pepper huasteco virus (PHV) and pepper golden mosaic virus (PepGMV) was carried out on plants representing wild pepper accessions collected in different states of México. One accession collected in Yucatán (BG-3821) corresponded to Capsicum chinense Jacq., and three collected from Michoacán (BG-3818), Tamaulipas (BG-3820), and Sinaloa (BG-3819) were identified as C. annuum L. Forty-eight plants were initially inoculated with a 1:1 mix of PHV and PepGMV DNAs by a biolistic method. Those plants that did not show typical symptoms after the biolistic method, were inoculated by grafting. Half of the plants (24) were highly susceptible, while the other half expressed different degrees of resistance. Of the resistant individuals, eight plants were asymptomatic and viral DNA of both viruses was detected in low levels. Two individuals showed delayed symptoms 34 days after symptom expression in the control plants. This delay was correlated with an asymptomatic effect when plants became symptomatic. The remaining 14 plants showed symptom remission in newly developed leaves at 31 days postinoculation, and this asymptomatic effect was correlated diminished PHV DNA within the plants. Our results suggest that the resistance shown by some individuals to geminivirus mixed infections (PHV and PepGMV) is likely due to constraints in viral movement.

Pepper (Capsicum sp.) has been cultivated in México since prehispanic times; evidence reveals that Capsicum annuum L. and C. frutescens L. were domesticated in México (Loaiza-Figueroa et al., 1989; Pickersgill, 1984). México is considered one of the three main pepper producers worldwide (FAO, 1999). Seventeen geminiviruses have been reported in the western hemisphere, and five of these have been detected in México (Polston and Anderson, 1997; Torres-Pacheco et al., 1996). Two bipartite, whitefly-transmitted geminiviruses belonging to genus Begomovirus, are commonly detected in mixed infections affecting pepper and tomato (Lycopersicum esculentum Mill.) crops in México (Garzón-Tiznado et al., 1993; Torres-Pacheco et al., 1993, 1996; Vera-Aguado et al., 1999). These viruses are pepper huasteco virus (PHV) and pepper golden mosaic virus (PepGMV, formerly named as Texas pepper virus).

It is important that sources of resistance to these viruses be identified. The wild relatives of plants are an excellent source of resistance genes and interesting agronomic characteristics (Burdon and Juroz, 1989; Harlan, 1976; Hernández-Verdugo et al; 1998, 2001; Stalker, 1980; Williams, 1988). Several efforts have been carried out to identify resistance to single infections of geminiviruses in wild or cultivated tomato (Kasrawi et al., 1988; Pilowsky and Cohen, 1990; Piven and Uzcategui, 1995; Zakay et al., 1991) and pepper plants (Godínez-Hernández et al., 2001; Hernández-Verdugo et al., 2001), but there are no reports of efforts to identify plants with resistance to mixed infections of these viruses. The identification and characterization of resistance in pepper to mixed infections caused by PHV and PepGMV, may lead to an important advance for Mexican horticulture. Moreover, these materials may serve to study the genetics of resistance and develop materials for future biotechnological applications.

In this work, 48 individual pepper plants derived from four accessions collected in four different states of México were evaluated for resistance to mixed infections with PHV and PepGMV. Some aspects of the possible resistance mechanism detected in several individuals are discussed.

Materials and Methods

Plant material. The study consisted of 48 individual pepper plants representing four accessions of wild pepper collected in the Mexican states of Michoacán (accession BG-3818), Sinaloa (accession BG-3819), Tamaulipas (accession BG-3820), and Yucatán (accession BG-3821, formerly named UX SMH-1; Godínez-Hernández et al., 2001). The plants were produced by harvesting fruits from plants of at least 3 months old and planting the seed in pots (10 cm tall × 10 cm diameter) placed in a greenhouse. Seventeen plants were C. annuum (accessions from Michoacán, Sinaloa, and Tamaulipas). The remaining 31 were of the species C. chinense, and were collected in Yucatán. Cultivar Sonora Anaheim was used as an experimental control based on...
its high susceptibility to mixed infections of both PHV and PepGMV (Godínez-Hernández et al., 2001; Torres-Pacheco, 1997). This research was conducted on the individual plants and not the original accession. To corroborate the virus-free state of the plants used in our experiments, serological tests (ELISA kit detection: Agdia, Elkhart, Ind.) were conducted to detect viruses commonly found in Mexican pepper crops (tobacco mosaic virus, tobacco etch virus, and cucumber mosaic virus (Vera-Aguado et al., 1999)). All the accessions used in this work are available from Instituto Nacional de Investigaciones Forestales, Agropecuarias y Pecuarias (INIFAP), (Campo Experimental Bajío, carretera Celaya-San Miguel de Allende Km 6.5, Apartado Postal 112, Código Postal 38000, Celaya, Guanajuato, México).

**Virus inoculation.** Inoculation of both PHV and PepGMV (Tamaulipas strain; Torres-Pacheco et al., 1996) DNA was carried out using a biolistic procedure with a particle delivery system (model PDS 1000; Dupont, Wilmington, Del.). PHV and PepGMV genomes used in this work were originally isolated and cloned from infected pepper plants collected in Tamaulipas (Garzón-Tiznado et al., 1989, 1993). Before bombardments, PHV components A and B, and component B of PepGMV (1993) were excised from bluescript plasmid using HindIII. Component A of PepGMV was excised from the plasmid with enzyme EcoRI in order to increase the percentage of plants infected using the biolistic procedure (Bonilla-Ramírez et al., 1997). Bombardments used 5 μg of DNA from PHV and PepGMV (2.5 mg of genomic components A and B from each virus), this inoculum was divided up among six plants. These DNA samples were deposited on the surface of 3 mg of tungsten microparticles (Tungsten M-10; BIO-RAD, Hercules, Calif.). Bombardment conditions used a gap distance of 1.2 cm and 56.4 kg·cm–2 of pressure for each plant (Garzón-Tiznado et al., 1993; Godínez-Hernández et al., 2001). Plants were inoculated at the apical zone on plants at the four-leaf stage, which is the optimal stage for infecting pepper with geminiviruses using the biolistic procedure (Garzón-Tiznado et al., 1993). Inoculated plants were incubated at the apical zone on plants at the four-leaf stage, which is the optimal stage for infecting pepper with geminiviruses using the biolistic procedure (Garzón-Tiznado et al., 1993). Inoculated plants were incubated for 3 months in a greenhouse. Tissue samples from both lower (older) and upper (newly developed) leaves were randomly taken at 11-, 21-, 31-, 41-, 51-, and 90-d post-inoculation (dpi). Severity of pepper disease by geminiviruses was qualitatively measured using a scale reported elsewhere (Godínez-Hernández et al., 2001; Hernández-Verdugo et al., 1998; Torres-Pacheco, 1997), where: 0 = asymptomatic; 1 = slight crumpling on apical leaves and presence of yellow spots (≈1 mm in diameter) when exposing leaves to sunlight; 2 = presence of yellow spots in groups on apical leaves; 3 = groups of yellow spots coalesced forming a slight network on the base of apical leaves; 4 = network clearly visible; 5 = protuberances observable in the middle zone of apical leaves; 6 = slight leaf curling; 7 = severe leaf curling; 8 = complete apical leaf distortion; and 9 = leaf stunting. Plant DNA extractions from samples were carried out according to Dellaporta et al., (1983). Detection of PHV and PepGMV DNA was carried out using polymerase chain reaction (PCR) with specific oligonucleotides for each virus. For PHV detection, the oligonucleotides used were, 240 (‘5GGGTTTTTGT AATAAGAGGTTG3’ and 241 (‘5GAATT AAAGTGATCGGACCACCTT3’), which amplify 350 bp from the intergenic region of component A. For PepGMV detection, the oligonucleotides used were JM23 (‘5TGTTAGACTCTGACGAAGGTC3’ and JM24 (‘5TAGGCCCAC ACC TTG GTG ACC AAG 3’) that amplify the intergenic region of component A (211 bp). PCR reactions contained the following components: 0.75 μL each deoxynucleotides (2.5 mm), 2 μL of oligonucleotides (50 ng·μL–1), 0.5 μL of Taq DNA polymerase (6 U/μL), and μL of plant DNA (100 ng·μL–1) in a reaction volume of 50 μL. PCR conditions to amplify PHV were: 94 °C, 1 min; 55 °C, 1 min and 72 °C, 2 min; and for PepGMV: 94 °C, 1 min; 55 °C, 2 min and 72 °C, 2 min; in both cases for 35 cycles. PCR products were visualized on 1.5% agarose gels and densitometric values were obtained using a digital image system (1D Image Analysis Software, version 3.02; Kodak Digital System, Rochester, N.Y.).

**Table 1. Disease reaction, collection location, inoculation method, and *Capsicum* species of evaluated plants.**

<table>
<thead>
<tr>
<th>Disease reaction</th>
<th>Collection location</th>
<th>Inoculation method</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Susceptible</td>
<td></td>
<td></td>
<td>C. chinense Jacq.</td>
</tr>
<tr>
<td>16</td>
<td>Yucatán</td>
<td>Biologic</td>
<td>C. annuum L.</td>
</tr>
<tr>
<td>5</td>
<td>Sinaloa</td>
<td>Biologic</td>
<td>C. annuum L.</td>
</tr>
<tr>
<td>2</td>
<td>Michoacán</td>
<td>Biologic</td>
<td>C. annuum L.</td>
</tr>
<tr>
<td>1</td>
<td>Tamaulipas</td>
<td>Biologic</td>
<td>C. annuum L.</td>
</tr>
<tr>
<td>Symptom delayed</td>
<td></td>
<td></td>
<td>C. chinense Jacq.</td>
</tr>
<tr>
<td>1</td>
<td>Yucatán</td>
<td>Biologic/Grafting</td>
<td>C. annuum L.</td>
</tr>
<tr>
<td>1</td>
<td>Tamaulipas</td>
<td>Biologic/Grafting</td>
<td>C. chinense Jacq.</td>
</tr>
<tr>
<td>Symptom remission</td>
<td></td>
<td></td>
<td>C. annuum L.</td>
</tr>
<tr>
<td>12</td>
<td>Yucatán</td>
<td>Biologic</td>
<td>C. chinense Jacq.</td>
</tr>
<tr>
<td>1</td>
<td>Tamaulipas</td>
<td>Biologic</td>
<td>C. annuum L.</td>
</tr>
<tr>
<td>Asymptomatic</td>
<td></td>
<td></td>
<td>C. annuum L.</td>
</tr>
<tr>
<td>4</td>
<td>Tamaulipas</td>
<td>Biologic/Grafting</td>
<td>C. annuum L.</td>
</tr>
<tr>
<td>2</td>
<td>Yucatán</td>
<td>Biologic/Grafting</td>
<td>C. chinense Jacq.</td>
</tr>
<tr>
<td>1</td>
<td>Sinaloa</td>
<td>Biologic/Grafting</td>
<td>C. annuum L.</td>
</tr>
</tbody>
</table>

*The accessions are identified as BG-3818 (Michoacán), BG-3819 (Sinaloa), BG-3820 (Tamaulipas), and BG-3821 (Yucatán).
Table 2. Mean of viral DNA levels, and severity of symptoms in pepper plants expressing 4 disease responses to geminiviral mixed infections with PHV and PepGMV.

<table>
<thead>
<tr>
<th>Disease reaction</th>
<th>Mean(^1) of DNA levels</th>
<th>Total(^2)</th>
<th>Severity(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PHV</td>
<td>PepGMV</td>
<td>PHV</td>
</tr>
<tr>
<td>Susceptible</td>
<td>28.02 a(^4)</td>
<td>13.31 a</td>
<td>7.79 a</td>
</tr>
<tr>
<td>Delay in symptoms</td>
<td>5.69 ab</td>
<td>2.93 b</td>
<td>2.17 b</td>
</tr>
<tr>
<td>Remission of symptoms</td>
<td>5.91 ab</td>
<td>3.47 b</td>
<td>3.77 b</td>
</tr>
<tr>
<td>Asymptomatic plants</td>
<td>1.90 b</td>
<td>1.33 b</td>
<td>2.31 b</td>
</tr>
</tbody>
</table>

\(^1\) Mean of six observations.

\(^2\) Total DNA of PHV and PepGMV (upper plus lower leaves).

\(^3\) Mean of six observations. Values are according to a scale reported by Torres-Pacheco et al. (1996), 0 = asymptomatic; 1, slight crumpling in apical leaves and presence of yellow spots (1 mm in diameter) when exposed to sun light; 2, presence of yellow spots in groups on apical leaves; 3, groups of yellow spots coalesced forming a network on the base of apical leaves; 4, network clearly visible; 5, protuberences observable in the middle zone of apical leaves; 6, slight leaf curling; 7, severe leaf curling; 8, complete apical leaf distortion; 9, leaf stunting.

\(^4\) Values in each column followed by different letters (a, b, or c), indicates they are significantly different at \(P < 0.01\). The Tukey test was the method of mean separation used.

Fig. 2. Detection by PCR of PHV and PepGMV on upper and lower leaves of representative individuals of each phenotypical response. Panel a = susceptible; and d = remission in symptoms. Primers used in PCR detect specifically a fragment of the common region of each virus (350 bp for PHV and 200 bp for PepGMV, respectively). Lane 1 = molecular weight marker; lanes 3–8 = detection of upper leaves at 11, 21, 31, 54, 59, and 92 dpi; and lanes 9–13 = detection off lower leaves at 11, 21, 31, 45, and 59 dpi. Lanes 2 and 14 = positive and negative controls, respectively.

**Results and Discussion**

After viral inoculation with both methods, 24 plants were resistant at different degrees to PHV-PepGMV infection. Eight of these plants were asymptomatic, no symptoms were observed on either the four initially inoculated leaves using the biolistic procedure (four-leaf stage) or on the newly developed leaves (after biolistic and grafting inoculation). These plants were: four from Tamaulipas (BG-3820), two from Yucatán (BG-3821), and two from Sinaloa (BG-3819) (Table 1). Fourteen of the plants showed symptoms of remission. These plants were inoculated by the biolistic method and expressed symptoms on upper and lower leaves at 13–15 dpi, but a reduction in the severity of the symptoms was observed in the newly developed leaves (Table 1). The remaining two plants showed a delay in symptom appearance of 40 dpi. These plants were initially inoculated using biolistic and 30 d later by grafting and originated in Yucatán and Tamaulipas (Table 1). Fifty percent of the plants tested in this study were susceptible (Table 1). All of these plants were inoculated only by the biolistic procedure.

The relationship between viral DNA levels of PHV and PepGMV, and severity of symptoms in pepper plants are shown in Table 2. In regard to PHV, DNA levels in upper and lower leaves, were positively correlated to severity of symptoms \((r = 0.70\) and 0.78, respectively; \(P < 0.01\)). Statistical analysis suggested three groups for PHV DNA levels on upper leaves: group a = susceptible plants; ab = plants with remission or delay in symptom appearance; and b = asymptomatic plants (Table 2). However, two statistical groups were identified for PHV levels of lower leaves: a = susceptible; and b = remission, delayed in symptom appearance and asymptomatic plants (Table 2). Considering total PHV DNA levels (upper and lower leaves), three statistical groups were identified: a = susceptible; ab = remission; and b = delayed in symptoms and asymptomatics (data not shown). As for upper and lower leaves, total PHV DNA levels correlated with severity of symptoms \((r = 0.79, P < 0.01)\).

For PepGMV DNA levels, two statistical groups were detected on both upper and lower leaves: a = susceptible individuals; and b = remission, delayed in symptom appearance and asymptomatic plants (Table 2). Correlation analysis indicated a positive relationship between severity of disease and PepGMV levels on upper and lower leaves \((r = 0.68\) and 0.66, respectively; \(P < 0.01\)). Total PepGMV levels showed two statistical groups; a = susceptible; and b = remission, delayed in symptoms appearance and asymptomatic plants (data not shown). Besides, in this case,
pressed by the wild pepper accessions, DNA PepGMV for the four type of syndromes expressed by the wild pepper accessions, DNA levels of each virus and severity of disease was analyzed on a representative individual of each kind of syndrome during 90 d (Fig. 2, Tables 3 and 4). It is worth mentioning that data showed in Tables 3 and 4 are from the same representative individual plant of each syndrome. In order to simplify the analysis, only viral DNA levels on upper leaves were studied in this experiment. For the susceptible individual, DNA levels of both geminiviruses were high and similar from 11–90 dpi (Table 3), and severity of disease progressively increased (Table 4). In the plant with delay in symptom appearance, DNA levels of both viruses were low through 31 dpi, and severity of disease was null. Then at 45 dpi, a slight increase in DNA levels of PHV was observed and severity of disease was also increased (Tables 3 and 4). This severity increment appeared 15 d after grafting inoculation on this plant, suggesting that biotic inoculation introduced both viruses within the plant. Although they were innocuous until grafting was done, then the plant became affected. This result suggests that the inoculation method is an important variable to be considered while testing for virus resistance in plants. Furthermore, for the plant with remission in symptoms, DNA levels of both geminiviruses were low at 11 dpi and at 21 dpi. However, PHV levels increased and were correlated with a concomitant increase in symptom severity (Tables 3 and 4). Thereafter, PHV DNA levels decreased, and PepGMV remained low and severity of symptoms progressively diminished (Tables 3 and 4). Finally, in the case of the asymptomatic plants, DNA levels of both geminiviruses were low during the 90 d of incubation, and the severity of disease was null (Tables 3 and 4). The fact that PHV levels were significantly higher than those of PepGMV on either upper or lower leaves (Table 3), suggests that PHV is somehow more prone than PepGMV to infect these pepper plants. This suggestion is supported by the fact that in pepper plants, PHV is able to complement the movement of PepGMV component A, but this does not occur (or it occurs at an extremely low level) in the reciprocal combination (Torres-Pacheco, 1997). It was clear, that whatever the mechanism of resistance in the plants with no presence, remission or delay in symptoms, it appears to be affecting PHV in a lesser degree.

In a recent report on pepper plants resistant to PHV in single infections, it was demonstrated that the resistance mechanism did not affect viral replication, thus, it was concluded that the mechanism may have restricted viral movement (Godínez-Hernández et al., 2001). Thus, it is unlikely that replication of PHV and PepGMV is affected in some plants used in this study; then, a likely resistance mechanism to geminiviruses in the pepper plants evaluated in this work could somehow be affecting viral movement. Additionally, another explanation of the results should be considered. The problems in viral replication efficiency or translation of viral genes could be negatively affected by these plants and the resulting reduction in viral accumulation may provoke, indirectly, a reduced or delayed movement. Moreover, the fact that several plants displayed a delay in symptom appearance, also suggests that the movement of both PHV and PepGMV was affected. When the plant with delay in symptom appearance (Tables 3 and 4) was inoculated by biotic, both PHV and PepGMV were innocuous (perhaps because cell to cell movement was affected). However, when that plant was grafted, characteristic PHV and PepGMV symptoms were observed, which suggests that resistance was broken in this plant by the direct viral entrance into vascular bundles by grafting inoculation. Several reports indicate that viral movement is frequently affected in plants displaying resistance to a virus (Fraser, 1990; Hull, 1991). It has been demonstrated that viral replication is possible in protoplasts of nonhost plants; moreover, tissues of symptomless plants, support virus multiplication in a single or few directly inoculated cells (Fraser, 1990; Paje-Manalo and Lommel, 1989). The interaction in which pepper plants showed a remission in symptomatology suggests that initially, viral movement and replication were adequate to infect the plants, but later, a resistance mechanism was probably induced, causing a reduction in viral DNA levels and symptom severity. Plants showing symptom remission may be expressing a type of RNA-
mediated virus resistance or gene silencing in which a cellular, cytoplasmic activity is provoking a highly selected RNA elimination as demonstrated elsewhere (de Haan et al., 1992; Lindbo et al., 1993; Pang et al., 1993; Schiebel et al., 1998; van der Vlugt et al., 1992). Asymptomatic plants may be resistant at any one or combinations of the mechanisms mentioned. Experiments trying to characterize the resistance mechanism on the interactions found in this work are currently in progress in our laboratory. The individual resistant pepper plants identified in this work, may be useful candidates to study genetics and molecular biology of resistance to geminiviruses, and these individuals may serve as sources of resistance in pepper breeding programs.

Literature Cited


