Characterization of Resistance to Pepper Huasteco Geminivirus in Chili Peppers from Yucatán, México

Yolanda Godínez-Hernández³, José Luis Anaya-López³,
Raúl Díaz-Plaza¹, Mario González-Chavira¹, and
Irineo Torres-Pacheco²

Instituto Nacional de Investigaciones Forestales y Agropecuarias (INIFAP),
Apartado Postal 112, Celaya, Gto, México

Rafael F. Rivera-Bustamante²

Departamento de Ingeniería Genética, Centro de Investigación y de Estudios
Avanzados del IPN, Unidad Irapuato, Apartado Postal 629, Irapuato, Gto,
México

Ramón G. Guevara-González⁴

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

Additional index words. *Capsicum chinense*, geminiviruses, pepper, resistance, virus
movement, replication

Abstract. Seven pepper (*Capsicum chinense* Jacq.) populations from the Yucatán Peninsula, México, that were selected from a field screening for viral diseases were tested for pepper huasteco geminivirus (PHV) resistance. Two populations (UX-SMH-1 and UX-SMH-24) displayed <50% of infection with PHV. Four plants did not show viral symptoms 3 months postinoculation using biolistic and grafting methods. When leaf tissue from these individuals was analyzed for PHV using quantitative PCR, it supported PHV replication, thus, the ineffective PHV infection in these symptomless individuals may be a result of restricted viral movement.

In México, pepper has been cultivated since prehispanic times. Macneish (1967) and Pickersgill (1969) have even suggested that pepper was the first domesticated plant in Mesoamérica. Currently, pepper is cultivated on ≈1 million ha worldwide, and México is one of the three main producers (Food and Agriculture Organization, 1999). In 1998, México cultivated 110,000 ha of pepper. Four species of *Capsicum* are cultivated in México (*C. annuum* L. var. *annuum*, *C. chinense*, *C. frutescens* L., and *C. pubescens*), of which *C. annuum* predominates (Pickersgill, 1984). *Capsicum chinense* is cultivated in the Yucatán peninsula of México and is part of a

complex that includes *C. annuum* and *C. frutescens*. These three species are sometimes considered identical, based upon qualitative and quantitative morphological characteristics (Eshbaugh, 1975; Pickersgill, 1991). Fertile F₁ hybrids have been recovered; however, fertility is higher when the crosses are made with wild plants (Eshbaugh, 1975; Pickersgill, 1971, 1988, 1991).

Low yield, low quality, and environmental pollution contribute to significant economic losses in Mexican pepper cultivation. Low yield is mainly due to viral diseases (Garzón-Tiznado et al., 1993); which cause economic losses of between 20% and 100%. Geminiviruses are the main viral group affecting pepper crops in México (Torres-Pacheco et al., 1996).

Geminiviruses are plant viruses with a twinned particle morphology, monopartite, or bipartite genomic structure, and are transmitted by insects of the order Homoptera (Hanley-Bowdoin et al., 1999; Lazarowitz et al., 1992). In México, two geminiviruses are considered to be the main pathogens in pepper: Pepper Huasteco Virus (PHV) and Pepper Golden Mosaic Virus (PepGMV, formerly called Texas Pepper Virus) (Torres-Pacheco et al., 1996). The former virus is a well-characterized bipartite, whitefly-transmitted geminivirus belonging to the begomovirus group (formerly subgroup III), and is the most widely distributed and important virus in pepper and tomato

(*Lycopersicon esculentum* Mill.) crops in México (Garzón-Tiznado et al., 1993; Torres-Pacheco et al., 1993, 1996; Vera-Aguado et al., 1999).

Alternative sources of resistance to PHV in Mexican pepper species are needed. One strategy to identify individuals with adequate viral resistance is by screening wild or cultivated plants, a methodology that has been successfully employed in the search for resistance to geminiviruses affecting tomato (Kasrawi et al., 1988; Pilowsky and Cohen, 1990; Piven and Uzcategui, 1995; Zakay et al., 1991). In this paper, we present the results from screening several *C. chinense* lines from Yucatán, México for resistance to PHV infection. Aspects of the resistance mechanism found in some symptomless *C. chinense* individuals are also described.

Materials and Methods

Plant material. Forty-nine wild populations of *C. chinense* were collected in several different areas of Yucatán. All these accessions were evaluated in the field for viral diseases and the best seven were selected for this work (Trujillo-Aguirre and Díaz-Plaza, 1995) and designated as UX-SMH-1, UX-SMH-5, UX-SMH-18, UX-SMH-22, UX-SMH-24, UX-SMH-26, and UX-SMH-55. Individual plants within an accession were used as an experimental unit. *Capsicum annuum* cv. Sonora Anaheim, which is highly susceptible to PHV, was used as the control (Torres-Pacheco, 1997). All the accessions used in this work are available from Instituto Nacional de Investigaciones Forestales y Agropecuarias (INIFAP) (Campo Experimental Bajío, carretera Celaya-San Miguel de Allende Km 6.5, apartado postal 112, código postal 38000, Celaya, Guanajuato, México).

Virus inoculation. Natural inoculum was used in all field experiments (Trujillo-Aguirre and Díaz-Plaza, 1995). For greenhouse experiments, one inoculation method was a biolistic procedure using a particle delivery system (model PDS 1000; Dupont), with 5 µg of DNA from PHV genomic components A and B deposited on the surface of 3 mg of tungsten microparticles (Sylvania No. 10), a gap distance of 1.2 cm and 56.4 kg·cm⁻² of pressure for each bombardment (Garzón-Tiznado et al., 1993). The apical zones of the plants were bombarded at the four-leaf stage; after inoculation, all plants were transferred to a greenhouse at 24 to 32 °C (Garzón-Tiznado et al., 1993). Cloned PHV genomic components A and B (Garzón-Tiznado et al., 1993) were excised from the plasmid by digestion with *Hind*III before inoculation in order to improve the efficiency of plant infection (Bonilla-Ramírez et al., 1997). Twenty plants of each population of *C. chinense* were inoculated by the biolistic method. Symptomless plants 4 weeks post-bombardment were inoculated via grafting using stem pieces from PHV-infected *C. annuum* plants, (cv. Sonora Anaheim). During the 3 months of greenhouse incubation, plant symptoms were measured as previously described (Torres-Pacheco et al.,

Received for publication 27 Oct. 1999. Accepted for publication 28 June 2000. This research was supported by a grant from Sistema de Investigación Miguel Hidalgo (SIHGO) CONACYT-México (1998-0201018), CONCYTEG (98-09-01-049), and CONACYT-México (K325-P9702 and J31638-B) to R.G. Guevara-González and I. Torres-Pacheco. Y. Godínez-Hernández and L.L. Anaya-López received fellowship support from COSNET. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked *advertisement* solely to indicate this fact.

¹Plant Breeder.

²Plant Pathologist.

³Graduate Students (Instituto Tecnológico de Celaya).

⁴Plant Virologist. To whom requests for reprints should be addressed. E-mail address: rgggon@hotmail.com

1996). A resistant (symptomless) individual showed neither symptoms nor virus detection during 3 months of plant incubation; tolerant individuals were plants displaying delayed symptoms (at ≈ 70 d) as compared with 'Sonora Anaheim'.

Evaluation of viral resistance and virus detection. During the 3 months of incubation, samples were taken at 14, 25, and 36 d post-inoculation (dpi) from the newly developed upper and lower leaves of biolistically inoculated plants, and at 27 and 44 dpi for grafted plants. The presence of PHV in the samples was detected by PCR analysis using specific primers for PHV (primer 240: 5'GGC TTATTTGTAATAAGAGAGGTGT3' and primer 241: 5'GAATTAAAGGTACAT GGACCACTT 3') to amplify a 350-bp fragment from the intergenic region of PHV component A (Torres-Pacheco et al., 1996). The procedure of Dellaporta et al. (1983) was used for extraction of DNA for PCR analysis. PCR reactions contained 2.5 mM each dNTP, 1 μ L of oligonucleotides (50 ng- μ L⁻¹), 1 μ L of Taq polymerase (5 U- μ L⁻¹), and 250 ng plant DNA in a reaction volume of 50 μ L. The PCR conditions were 94 °C for 2 min, 50 °C for 2 min and 72 °C for 2 min, for 35 cycles. The PCR products were analyzed on 0.8% agarose gels.

Determination of the resistance mechanism. Leaves from both susceptible and resistant plants were bombarded twice with 5 μ g of DNA from PHV genomic component A, using the same bombardment conditions as described above. Before bombardment, leaf tissue was incubated for 4 h at 25 °C on solid Murashige-Skoog medium (Ruíz-Medrano et al., 1999). Tissue samples (0.5 g) were taken at 24 and 72 h after bombardment and total DNA was extracted (Dellaporta et al., 1983). Total DNA was quantified and brought to a concentration of 50 ng- μ L⁻¹. An estimation of relative quantities of viral DNA was carried out using a quantitative PCR technique (Sykes et al., 1992). Serial dilutions of each sample and PCR amplifications with specific primers for PHV were carried out as described above. Agarose gels showing amplification products were analyzed using an IS-100 digital Image System (Alpha Innotech Corporation, San Leandro, Calif.), and integrated densitometry values were measured.

Results and Discussion

Seven populations of *C. chinense* with the lowest percentage of field-infected plants (Trujillo-Aguirre and Díaz-Plaza, 1995) were selected and inoculated with PHV by the biolistic method. Plants that did not show PHV symptoms 4 weeks postbombardment were inoculated by grafting, in order to determine whether they were resistant to PHV infection or were escapes. The original frequency of escapes using biolistic inoculation was 30% (Garzón-Tiznado et al., 1993); however, our biolistic method had only 5% escapes (Table 1). Moreover, our grafting method yielded no escapes when the scion survived (Table 1). The *C. chinense* accessions initially showed

high intrapopulation variation for percentage of PHV infected plants (40% to 90%, Table 2). This work used individual plants within an accession as the experimental unit. Two months after grafting, the UX-SMH-1 and UX-SMH-24 populations had the highest percentage of symptomless plants (Table 2). Although population UX-SMH-24 contained 43% infected plants, it had no symptomless individuals (Table 2). However, UX-SMH-22 had 60% infected plants, with one asymptomatic individual (Table 2). After biolistic and grafting inoculations, several tolerant individuals were detected in almost all populations. Four resistant/symptomless individuals were detected in populations UX-SMH-1 and UX-SMH-22 (Table 2).

Following biolistic inoculation, PHV was detected on upper or lower leaves of susceptible plants with symptoms (Fig. 1A), but was confined to symptomatic zones, suggesting that symptoms in *C. chinense* are determined by viral presence. Following biolistic inoculation, symptomless individuals did not possess detectable levels of viral DNA (Fig. 1B). Occasionally, viral DNA was detected in the earlier samplings from resistant plants, perhaps because DNA was introduced into leaf pieces by bombardment (Fig. 1B, lanes 1–4). Analysis by PCR confirmed that some

asymptomatic individuals from the biolistic inoculation were effectively resistant to PHV infection; no viral DNA was detected on upper leaves 44 d after inoculation by grafting (Fig. 1C, lanes 7–9). Three resistant individuals were identified in the UX-SMH-1 population and 1 in the UX-SMH-22 population (Table 2).

In susceptible plants, higher dilutions were needed to diminish PHV amplification via PCR at 72 h than at 24 h after bombardment (Table 3). Densitometric analyses of amplified DNA from serial dilutions from the same individuals indicated that samples were significantly stronger at 72 h than at 24 h (Table 3). This suggests that the amount of viral DNA increased between 24 and 72 h postinoculation. The result was the same in both resistant and susceptible plants (Table 3). These results suggested that PHV replication was not affected in resistant plants, and that resistance to PHV may affect viral movement. Resistance to viral infection is commonly the result of restricted viral movement within the plant (Hull, 1991). Several reports indicate that a nonhost virus can multiply in inoculated protoplasts of nonhost species (Fraser, 1990; Hull, 1991; Paje-Manalo and Lommel, 1989). Apparently, immune plants support virus multiplication in a few inocu-

Table 1. Evaluation of both biolistic and grafting PHV inoculation methods, on *Capsicum annuum* cv. 'Sonora Anaheim'.

Inoculation method	No. of plants			PHV ^z detection
	Inoculated	With symptoms	Asymptomatic	
Biolistic	34	32	2 ^y	32
Grafting	40 ^x	40	0	40

^zVirus detection by PCR, as indicated in materials and methods. In all cases, only symptomatic plants showed the presence of PHV.

^yThese plants were later inoculated by grafting, and are included in the account of total grafting inoculated plants.

^xThese plants were those in which the scion survived; originally 43 plants were inoculated by grafting.

Table 2. Percentage of infected plants, and severity and number of symptomless plants in *C. chinense* populations after PHV infection using both biolistic and grafting methods.

Population	Infected plants ^z (%)	Severity ^y	No. of symptomless plants
UX-SMH-1	40	8.0	3
UX-SMH-5	85	8.4	0
UX-SMH-18	50	8.0	0
UX-SMH-22	60	8.6	1
UX-SMH-24	43	8.0	0
UX-SMH-26	84	8.4	0
UX-SMH-55	90	8.1	0

^zPercentage with PHV symptoms 3 months after inoculation.

^ySeverity based on a scale from 1 (no symptoms) to 10 (severe symptoms).

Table 3. Relative quantitation of PHV using PCR analysis on serial dilutions from DNA samples extracted from bombarded pepper leaves. (Arithmetic mean = 276, and standard deviation = 184.6).

Sample	Time after inoculation		Dilution			
	(h)		1:10	1:100	1:1000	1:10,000
Susceptible plant	24		416 ^z	363	104	0
	72		624	468	156	104
Immune plant	24		410	368	90	0
	72		620	450	152	108

^zValues are densitometry readings expressed as integrated densitometry values (IDV).

Fig. 1. PCR amplifications of intergenic region of PHV component A from inoculated *C. chinense* plants. (A) Set of four typical PHV-susceptible plants that resulted after biolistic inoculation. Lanes 1–8, 9–16, and 17–24 are amplifications from DNA samples taken 14, 25, or 36 d postinoculation (dpi) respectively. (B) Set of four typical PHV-resistant plants that resulted after biolistic inoculation. Descriptions of lanes, but not the plants, are the same as in (A). (C) PCR amplifications of DNA samples from grafted plants. Lanes 1–6 and 7–12 correspond to samples taken 27 and 44 d after grafting, respectively. In the grafting experiment, only DNA samples from upper leaves were analyzed. The amplification of the intergenic region of PHV component A was primed using oligonucleotides 240/241, which amplify a 350-bp fragment (indicated by arrows on each panel). U = upper leaves, L = lower leaves, C+ = PCR amplification from DNA samples of PHV-infected plant from 'Sonora Anaheim', C- = PCR analysis from noninoculated plant from 'Sonora Anaheim', and M = molecular weight marker (1 Kb).

lated cells (Fraser, 1990; Paje-Manalo and Lommel, 1989).

Capsicum chinense plants immune to PHV infection are excellent resources with which to study the genetics of the resistance and for use in breeding programs.

Literature Cited

- Bonilla-Ramírez, G.M., R.G. Guevara-González, J.A. Garzón-Tiznado, J.T. Ascencio-Ibañez, I. Torres-Pacheco, and R.F. Rivera-Bustamante. 1997. Analysis of the infectivity of monomeric clones of pepper huasteco virus. *J. Gen. Virol.* 78:947–951.
- Dellaporta, S.L., J. Wood, and J.B. Hicks. 1983. A plant DNA miniprep: Version II. *Plant Mol. Biol. Rpt.* 1:19–21.
- Eshbaugh, W. 1975. Genetical and biochemical systematic studies of chili peppers (*Capsicum-Solanaceae*). *Bul. Torr. Bot. Club* 102:396–403.
- Food and Agriculture Organization. 1999. Agricultural production: Primary crops. http://apps.fao.org/lim500/Agri_db.pl
- Fraser, R.S.S. 1990. The genetics of resistance to plant viruses. *Annu. Rev. Phytopathol.* 28:179–200.
- Garzón-Tiznado, J.A., I. Torres-Pacheco, J.T. Ascencio-Ibañez, L. Herrera-Estrella, and R.F. Rivera-Bustamante. 1993. Inoculation of peppers with infectious clones of a new geminivirus by a biolistic procedure. *Phytopathology* 83:514–521.
- Hanley-Bowdoin, L., S.B. Settledge, B.M. Orozco, S. Nagar, and D. Robertson. 1999. Geminiviruses: Models for plant replication, transcription, and cell regulation. *Crit. Rev. Plant Sci.* 18:71–106.
- Kasrawi, M.A., M.A. Suwwan, and A. Mansour. 1988. Sources of resistance to tomato yellow leaf curl virus (TYLCV) in *Lycopersicon* species. *Euphytica* 37:61–64.
- Lazarowitz, S.G. 1992. Geminiviruses: Genome structure and gene function. *Crit. Rev. Plant Sci.* 11:327–349.
- Macneish, R.S. 1967. A summary of subsistence, p. 3–13. In: D. Byers. (eds.). *The prehistory of the Tehuacán Valley*. Univ. of Texas, Austin.
- Paje-Manalo, L.L. and S.A. Lommel. 1989. Independent replication of red clover necrotic mosaic virus RNA-1 in electroporated hosts and nonhost *Nicotiana* species protoplasts. *Phytopathology* 79:457–461.
- Pickersgill, B. 1969. The domestication of chili peppers, p. 443–450. In: P.J. Ucko and G.W. Dimbley (eds.). *The domestication and exploration of plants and animals*. Duckworth, London.
- Pickersgill, B. 1971. Relationships between weedy and cultivated forms in some species of species of chili peppers (genus *Capsicum*). *Evolution* 25:683–691.
- Pickersgill, B. 1984. Migration of chili peppers, *Capsicum* spp., in the Americas, p. 105–123. In: D. Stone (ed.). *Papers of the Peabody Museum of Archeology and Ethnology*. Harvard Univ. Press.
- Pickersgill, B. 1988. The genus *Capsicum*: A multidisciplinary approach to the taxonomy of cultivated and wild plants. *Biol. Zentralblatt* 107:381–389.
- Pickersgill, B. 1991. Cytogenetics and evolution of *Capsicum* L., p. 139–160. In: T. Tsuchiya and P.K. Gupta (eds.). *Chromosome engineering in plants: Genetics, breeding and evolution*. Elsevier, Amsterdam.
- Pilowsky, M.A. and S. Cohen. 1990. Tolerance to tomato yellow leaf curl virus derived from *Lycopersicon peruvianum*. *Plant Dis.* 74:248–250.
- Piven, N.M. and R. Uzcategui. 1995. Resistance to mosaic virus in species of *Lycopersicon*. *Plant Dis.* 79:590–594.
- Ruíz-Medrano, R., R.G. Guevara-González, G.R. Arguello-Astorga, Z. Monsalve Fonnegra, L.R. Herrera-Estrella, and R.F. Rivera-Bustamante. 1999. Identification of a sequence element involved in AC2-mediated transactivation of the pepper huasteco virus coat protein gene. *Virology* 253:162–169.
- Sykes, P.J., S.H. Neoh, M.J. Brisco, E. Hughes, J. Condon, and A.A. Morley. 1992. Quantitation of targets for PCR by use of limiting dilution. *Biotechniques* 13:444–449.
- Torres-Pacheco, I. 1997. Geminivirus involucrados en el rizado amarillo del chile: Interacciones entre PHV y TPV. PhD Diss., Centro de Investigación y de Estudios Avanzados del I.P.N., Unidad Irapuato, Mexico.
- Torres-Pacheco, I., J.A. Garzón-Tiznado, J.K. Brown, A. Becerra-Flora, and R.F. Rivera-Bustamante. 1996. Detection and distribution of geminiviruses in Mexico and Southern United States. *Phytopathology* 86:1186–1192.
- Torres-Pacheco, I., J.A. Garzón-Tiznado, L. Herrera-Estrella, and R.F. Rivera-Bustamante. 1993. Complete nucleotide sequence of pepper huasteco virus: Analysis and comparison with bipartite geminiviruses. *J. Gen. Virol.* 74:2225–2231.
- Trujillo-Aguirre, J. and R. Díaz-Plaza. 1995. Obtención de cultivares de chile habanero con resistencia a virosis transmitida por mosca blanca. IV. Taller latinoamericano sobre moscas blancas y geminivirus. Zamorano, Honduras.
- Vera-Aguado, M.G., R. Díaz-Plaza, M. González-Chavira, J.A. Garzón-Tiznado, R.F. Rivera-Bustamante, R.G. Guevara-González, and I. Torres-Pacheco. 1999. Detection of viruses in tomato (*Lycopersicon esculentum*), pepper (*Capsicum annuum*), and weeds in different environments in México. *Advances. VIII Congreso Nacional de Horticultura*, Manzanillo, Colima, México.
- Zakay, Y., N. Navot, M. Zeidan, N. Kedar, H. Rabinowitch, H. Czosnek, and D. Zamir. 1991. Screening *Lycopersicon* accessions for resistance to tomato yellow leaf curl virus: Presence of viral DNA and symptoms development. *Plant Dis.* 75:279–281.