Genetic Composition of *Pepino mosaic virus* Population in North American Greenhouse Tomatoes

Kai-Shu Ling, U.S. Department of Agriculture-Agricultural Research Service, U.S. Vegetable Laboratory, 2700 Savannah Highway, Charleston, SC 29414; **William M. Wintermantel**, U.S. Department of Agriculture-Agricultural Research Service, 1636 E. Alisal Street, Salinas, CA 93905; and **Michael Bledsoe**, Village Farms, 400 International Parkway, Suite 130, Heathrow, FL 32746

ABSTRACT

Ling, K.-S., Wintermantel, W. M., and Bledsoe, M. 2008. Genetic composition of *Pepino mosaic virus* population in North American greenhouse tomatoes. *Plant Dis.* 92:1683-1688.

In just a few short years, pepino mosaic disease has quickly become endemic in greenhouse tomatoes around the world. Although three genotypes of *Pepino mosaic virus* (PepMV) were identified in the United States, genetic composition of PepMV in greenhouse tomato crops in North America has not been determined. In this study, genetic variability and population structure of PepMV were evaluated through nucleotide sequence comparison and phylogenetic analysis of two genomic regions (helicase domain and TGB2-3) derived from 91 cDNA clones that were derived from 31 field-collected samples. These samples were collected from several major greenhouse tomato facilities in five states in the United States and two provinces in Canada. All four major genotypes of PepMV (EU, US1, US2, and CH2) were found in North America. Three distinct genotypes (EU, US1, and US2) were found in mixed infection in samples collected from Arizona and Colorado, two genotypes (EU and CH2) in Texas, and a single genotype (EU) in Alabama and California and the provinces of British Columbia and Ontario in Canada. The complexity of population genetics of PepMV in the United States poses an additional challenge to the greenhouse tomato industry because a tomato cultivar with durable resistance to multiple genotypes of PepMV may be harder to develop.

Additional keywords: Potexvirus, *Solanum lycopersicum*, *Solanum muricatum*

Pepino mosaic virus (PepMV), genus *Potexvirus*, family *Flexiviridae*, is first described on pepino (*Solanum muricatum* Ait.) (7). In just a few years since its first discovery on tomato (*Solanum lycopersicum* L.) in 1999 (23), pepino mosaic disease has become widespread in greenhouse tomato production areas in Europe (1,3,8,12,14–17,19,23,24), North America (4,13), South America (9,12,20), and Asia (25). Thus far, five PepMV genotypes (EU, LP, CH2, US1, and US2) have been identified in the world (5). The EU genotype was used to group several original European tomato isolates that share >98% nucleotide sequence identity (1,3,14,24). The LP genotype was used to represent the Peruvian isolate, which shares 95% overall genome nucleotide sequence identity to the EU genotype (12,15). The US1 and US2 genotypes were used to represent two unique isolates characterized from tomato

in the United States, which shared ~80% nucleotide sequence identity to each other and to the EU genotype (13). The CH2 genotype was derived from an isolate from infected tomato seed in Chile, which shared 90% nucleotide sequence identity to US2 and only 80% similarity to the EU or US1 genotypes (9). Interestingly, sequence variation between CH2 and US2 was not evenly distributed throughout the genome. Whereas the 5' and 3' portions of CH2 and US2 genome were very much the same, the central one-third of the genome (from nt 2,307 to 4,738 of CH2) shared only 80% nucleotide sequence identity (9). Thus, CH2 is considered a distinct genotype of PepMV (5,9). A population genetic study on PepMV in 2005 in Spain showed that the EU genotype was prevalent with a much smaller proportion of natural population with the US2 genotype (15). In another similar study in Belgium (5), however, the CH2 emerged as the predominant genotype in Belgian greenhouse tomatoes. The CH2 genotype has also been detected in Poland (6,16).

In North America, PepMV was first identified in 2001 (4). Two unique isolates (US1 and US2) were initially characterized from tomato in Arizona (13). A third isolate (US3) identified from a tomato fruit in Maryland belonged to the EU genotype. However, genetic variability and popula-

tion structure of PepMV isolates in North American greenhouse-grown tomatoes have not been characterized. To assess the current status of the genetic diversity and geographical distribution of PepMV genotypes in North America, we conducted a survey in several major commercial greenhouse tomato-growing facilities in the United States and Canada. Understanding genetic diversity and population structure is important for the reliable deployment of a sequence-based virus detection method (11) and the successful development of durable disease resistance against different virus genotypes (10,21).

MATERIALS AND METHODS

Virus cultures. The origin of 31 assayed samples and their genetic background are given in Table 1. Samples were collected and supplied by the collaborating growers with proper USDA interstate or international permits during the growing season of 2006. In addition to the major survey in 2006, two additional samples, one collected in 2001 from Ft. Lupton, CO (CO1) and a second from Marfa, TX (TX8) in 2007, were analyzed. In total, 27 samples were collected in the United States, including one from Alabama (AL1), five from Arizona (AZ1 to AZ5), nine from California (CA1 to CA 9), four from Colorado (CO1 to CO4), and eight from Texas (TX1- TX8). In Canada, three samples were collected from British Columbia (BC1 to BC3) and one from Ontario (ON1). Once received, samples were inspected for symptom expression and tested for the presence of PepMV by enzyme-linked immunosorbent assay (ELISA) with an antibody prepared by Agdia (Elkhart, IN), following the manufacturer's instructions. In addition, real-time reverse transcription polymerase chain reaction (RT-PCR) was carried out to provide additional confirmation for the presence of PepMV sequence using conditions as described (11). Virus infectivity for each isolate was tested through mechanical inoculation of leaf tissue extract prepared in phosphate buffered saline (137 mM NaCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 2.7 mM KCl, and 7.9 mM Na₂SO₃; pH 7.0) onto tomato seedlings (cv. Horizon) and/or *Nicotiana benthamiana* plants. Inoculated plants were grown in a greenhouse maintained at 20 to

Corresponding author: Kai-Shu Ling
E-mail: kai.ling@ars.usda.gov

Accepted for publication 2 September 2008

doi:10.1094/PDIS-92-12-1683

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30°C with 12 to 14 h of natural sunlight for 4 weeks. Symptoms expressed on the inoculated tomato plants (cv. Horizon) were recorded and compared with the symptoms observed on samples when they were received (Table 1).

Primer selection. Accurate assessment of the genetic variation within a population requires the use of PCR primers that are capable of amplifying the targeted genomic region effectively and efficiently on all major genotypes of PepMV. Toward this goal, a primer pair (KL05-13, 5' GTC CTC ACC AAT AAA TTT AG, and KL05-14, 5' AGG AAA ACT TAA CCC GTT C) targeting the TGB2-3 region (nt 5,374 to 5,575) was developed with the consensus sequence of a multiple nucleotide sequence alignment of all major PepMV genotypes (Fig. 1B). Initial experiments demonstrated that this primer pair was capable of amplifying the expected RT-PCR product (202 bp) from each representative isolate for each PepMV genotype (EU, US1, US2, or CH2) (data not shown). Remarkably, the sequence data contained in this relatively small RT-PCR amplified product were sufficient to make a specific and accurate identification of three major genotypes (EU, US1, and US2). In the case

where genome sequence identity between the CH2 and US2 genotypes in this region (TGB2-3) was too high to achieve an unambiguous identification, an additional primer set (KL04-52, 5'GTG CTT ACA GTT CTG ACA TC, and KL05-20, 5'CTA GAA TTG GCA CTT TGC AC) targeting the CH2 sequence (9) was utilized (Fig. 1C). This CH2 genotype-specific primer set was initially used in RT-PCR to screen through all samples received for any possible presence of the CH2-like sequence. Once the expected RT-PCR product (642 bp) was obtained in a sample, then nucleotide sequence from the RT-PCR product was used to support the final genotype identification.

Viral RNA preparation and RT-PCR amplification. The template viral RNA, prepared either from immunocapture (11) or total plant RNA with the RNeasy Plant kit (Qiagen, Valencia, CA), was used in RT-PCR to amplify the targeted genomic segment with the Superscript III one-Step RT-PCR kit (Invitrogen, Carlsbad, CA) following the vendor's instructions. Briefly, the purified plant RNA (1.0 µl) or the immunocapture-prepared viral RNA was denatured at 94°C for 5 min on a thermocycler after the addition of 0.25 µl of

each primer (20 µM) in a final volume of 11.0 µl in diethyl pyrocarbonate (DEPC)-treated water. In each 25-µl RT-PCR reaction was added 12.5 µl of 2X Reaction mix (a buffer containing 0.4 mM of each dNTP, 2.4 mM MgSO₄), 1.0 µl of 5 mM MgSO₄ and 0.5 µl of RT/Platinum Taq Hifi mix. The cycling parameters were reverse transcription for 30 min at 50°C, denaturation for 2 min at 94°C, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 min with a final extension cycle of 10 min at 72°C. An aliquot of RT-PCR preparation (10 µl) from each reaction was applied onto a 1.5% agarose gel for electrophoresis and the RT-PCR products were visualized under a UV transilluminator after stained with 0.01% SyBr Safe (Invitrogen). The gel image was taken with a digital imaging system (ChemiDoc XRS; Bio-Rad, Hercules, CA).

Cloning, nucleotide sequencing, and phylogenetic analysis. For every isolate, once the expected RT-PCR product was confirmed on an agarose gel, its PCR product was subjected to purification with the QIAquick PCR Purification kit (Qiagen) and used directly for sequencing. To assess

Table 1. Survey of greenhouse tomatoes in the United States and Canada for the occurrence of *Pepino mosaic virus*

Sample	Location, year	Tomato cultivar ^a	Symptom ^v	ELISA ^w	Real-time RT-PCR (C _T) ^x	Genotype (no. of clones) ^y
AL1	Birmingham, AL, 2006	NA	ym	++	18.51	EU
AZ1	Wilcox, AZ, 2006	NA	Mild, ym	++	23.59	EU (2), US1 (3), US2 (5)
AZ2	Wilcox, AZ, 2006	NA	Mild, ym	++	17.94	EU (1), US2 (1)
AZ3	Wilcox, AZ, 2006	NA	Mild, ym	++	26.45	EU
AZ4	Snowflake, AZ, 2006	NA	Mild, ym	++	12.04	EU
AZ5	Snowflake, AZ, 2006	NA	Mild, ym	+	15.61	EU
BC1	Pitt Meadows, BC, Canada, 2006	Matrix	ym	+++	21.12	EU
BC2	Pitt Meadows, BC, Canada, 2006	Matrix	ym	++++	24.62	EU
BC3	Delta, BC, Canada, 2006	Beef Matrix	ym	++++	21.38	EU
CA1	Camarillo, CA, 2006	Tricia	Mild, ys	++	26.04	EU
CA2	Camarillo, CA, 2006	Tricia	Mild, ys	++	21.86	EU (7)
CA3	Camarillo, CA, 2006	Tricia	Mild, ys	++	25.00	EU (7)
CA4	Camarillo, CA, 2006	Tricia	Mild, ys	++	23.4	EU
CA5	Camarillo, CA, 2006	Tricia	Mild, ys	++	25.31	EU
CA6	Camarillo, CA, 2006	Ivonne	Mild, ys	++	24.94	EU (4)
CA7	Camarillo, CA, 2006	Ivonne	Mild, ys	++	26.29	EU
CA8	Camarillo, CA, 2006	Ivonne	Mild, ys	++	24.35	EU
CA9	Camarillo, CA, 2006	Ivonne	Mild, ys	++	26.14	EU
CO1	Ft. Lupton, CO, 2001	NA	...	++	20.46	EU
CO2	Golden, CO, 2006	NA	ym	++	30.01	EU (1), US1 (5)
CO3	Golden, CO, 2006	NA	nc	++	25.99	EU (5), US1 (3)
CO4	Golden, CO, 2006	NA	Mild, ym	++	22.19	EU (5), US2 (2)
ON1	Kingsville, ON, Canada, 2006	NA	ym	++++	24.04	EU
TX1	Fort Davis, TX, 2006	Bonvivant	Severe, ym	++	23.59	EU
TX2	Fort Davis, TX, 2006	Bonvivant	Severe, ym	++	24.19	EU
TX3	Marfa, TX, 2006	Clarence	cl	++	17.91	EU
TX4	Marfa, TX, 2006	Clarence	cl	++	20.14	EU (7)
TX5	Presidio, TX, 2006	Dundee	Ym, cl	++	21.87	EU (2)
TX6	Presidio, TX, 2006	Clarence	Ym, cl	++	20.06	EU
TX7	Presidio, TX, 2006	Clarence	Ym, cl	++	18.80	EU
TX8	Marfa, TX, 2007	NA	Mild, ym	++	17.99	EU (8), CH2 (3) ^z

^a NA = not available.

^v Cl = chlorosis; ym = yellow mosaic; ys = yellow spot on leaves; nc = necrosis on stem and petiole, and ... = not available.

^w Enzyme-linked immunosorbent assay (ELISA) absorbance = < 0.099; + = 0.100–0.999; ++ = 1.000–1.999; +++ = 2.000–2.999; and ++++ = >3.000.

^x Threshold cycle value (C_T) was determined using the immunocapture real-time reverse transcription-polymerase chain reaction (RT-PCR) conditions as described in Ling et al. (11). The C_T value for the nontemplate control (NTC) was 0 (below the detectable threshold level).

^y The identical sequences in the TGB2-3 segment in cDNA clones generated from the same isolate through immunocapture RT-PCR were pooled on the basis of sequence identity to the typical genotypes (EU, US1, or US2) and the number of clones belonging to that genotype was presented with a number in parenthesis.

^z A different primer set (KL04-52/KL05-20) was used that targeted specifically for the CH2 sequence.

the degree of genetic variation among PepMV isolates, the same RT-PCR product was also cloned into the pCR4-TOPO vector (Invitrogen). For every isolate, at least two cDNA clones were sequenced and their sequences were matched with the direct PCR sequence data. Only the consensus sequence from each alignment in every isolate was chosen for analysis. To determine the genetic composition of PepMV population in each location, two to three isolates from four states (Arizona, California, Colorado, and Texas) were chosen for in-depth analysis. Additional cDNA clones (between 7 and 11) from each isolate were randomly picked for sequencing. Nucleotide sequencing was performed with the GenomeLab DTCS-Quick Start kit and CEQ8000 Genetic Analyzer (Beckman Coulter, Fullerton, CA). Both forward and reverse cDNA strands were sequenced. Clones sharing the identical nucleotide sequences were pooled and presented only with a number following the isolate designation (e.g., AZ1_US1.3 represents 3 cDNA clones from the AZ1 sample with the US1 genotype). Only the representative sequences were used for phylogenetic analysis. All

sequence data were deposited in the GenBank database with accession nos. EU358789–EU358822 and EU442510–EU442512.

To assess the population genetic composition in PepMV samples collected in the United States and Canada, nucleotide sequences generated from cDNA clones of viral isolates were aligned with Clustal-X (22). Phylogenetic relationships were analyzed by the neighbor-joining method (18). One thousand bootstrap iterations were performed by the DNAMAN program package (Lynnon BioSoft, Quebec, Canada) to generate robust phylogenetic trees. In this study, all sequences from PepMV samples collected in North America were delineated into four major genotypes (EU, US1, US2, or CH2). cDNA clones with at least 95% nucleotide sequence identity to that of the representative genotype were considered in the same genotype.

RESULTS

The EU genotype is predominant in North American greenhouse tomatoes. Although foliar symptoms in the collected tomato samples varied greatly from one greenhouse to another (including leaf dis-

tortion, yellow mosaic, bright yellow spots or patches on leaves, and necrotic lesions on stems and petioles), the results from ELISA and real-time RT-PCR tests showed the presence of PepMV in all 31 samples collected in the United States and Canada (Table 1). Interestingly, symptoms expressed on the inoculated tomato plant (cv. Horizon) from each isolate matched well with the symptoms displayed on samples of diverse tomato cultivars received from the various growers. The surveyed greenhouse facilities, some of the largest in North America, represent a majority of total greenhouse tomato production areas in the United States and Canada. Although most of the surveyed greenhouse facilities in four states (Arizona, California, Colorado, and Texas) in the United States and two provinces (British Columbia and Ontario) in Canada had a history of PepMV infection (4,13), this was the first time PepMV was identified in the state of Alabama.

Initial attempts with direct sequencing of RT-PCR products did not produce sufficient high-quality sequence data to make an accurate assessment of genetic relationship, particularly on those isolates where

Fig. 1. A diagram depicting the relative positions of the polymerase chain reaction (PCR) primers in *Pepino mosaic virus* (PepMV) genome and the multiple sequence alignments of genome segments used for reverse transcription (RT)-PCR among several representatives of PepMV genotypes. **A**, Genome structure of *Pepino mosaic virus* (PepMV) and the two genomic regions targeted for the genetic variability analysis were identified with relative primer designations. **B**, Nucleotide sequence alignment of the representative PepMV genotypes (CH2, US1, US2, and EU) showing two PepMV generic primers (KL05-13 and KL05-14) selected for the amplification of genetic fragments (202 nt) in RT-PCR. The identical nucleotide sequences to the SP-13 (EU genotype) sequence were presented in dash lines. The sequence in KL05-14RC was reverse complement to KL05-14. **C**, Nucleotide sequence alignment of the representative PepMV genotypes (CH2, US1, US2, and EU) showing two additional primers (KL04-52 and KL05-20) selected for specific amplification of CH2-like sequence. Only the first and the last 107 nt of the 642-nt fragment are presented along with the two primers (KL04-52 and KL05-20). The identical nucleotide sequences to the CH2 sequences were presented in dash lines. The sequence in KL05-20RC was reverse complement to KL05-20.

mixed infection of different genotypes might have occurred. Upon cloning of the RT-PCR product to the TA cloning vector, a cDNA library was generated for each sample. To assess the genetic diversity of PepMV in samples collected in that state or province, initially two cDNA clones per

isolate were randomly selected and sequenced. To determine genetic composition of PepMV genotypes in four major greenhouse tomato-production facilities in the United States (Arizona, California, Colorado, and Texas), two to three samples in each state were selected for in-depth

analysis. For each sample, additional cDNA clones (as many as 10 clones) were randomly selected. To minimize sequence redundancy, identical sequences from cloned DNAs in each sample were pooled and only the representative sequences were used for phylogenetic analysis. In total, sequences from 91 cDNA clones from 31 samples were used for this purpose. Representative sequences from the four major genotypes (EU, US1, US2, and CH2) were also included (Fig. 2). Surprisingly, the EU genotype was identified in every sample from every surveyed greenhouse tomato-production facility (Fig. 2; Table 1). When sequences from all clones were analyzed, 69 of 91 clones belonged to the EU genotype, 11 to US1, 8 to US2, and 3 to CH2. These results indicated that the EU genotype was predominant in both countries. In addition to the EU genotype, both US1 and US2 were also detected in mixed infection in samples from Arizona (Fig. 3A) and Colorado (Fig. 3B). The CH2 and EU genotypes were identified in mixed infection from one sample (TX8) collected in Texas in 2007 (Fig. 3C), whereas all seven Texan samples collected in 2006 contained only one genotype (EU) (Table 1; Fig. 2).

The mixed infection of genotypes (EU, US1, and US2) was common in Arizona and Colorado greenhouse tomatoes. We considered individual samples collected from each greenhouse tomato-production facility to be a distinct population. Because the primers used in RT-PCR were generated from the consensus sequence of all major PepMV genotypes (EU, US1, US2, and CH2), the opportunity to be amplified by RT-PCR from each representative genotype in a viral mixture should be proportional to reflect the actual population composition. Thus, individual clones selected randomly from a cDNA library likely represented the genetic composition of PepMV genotypes in a population, particularly those with major representative sequences. However, because of a relatively small number of clones sequenced in the current study, some minor representative sequences in a population may not actually be detected. Nevertheless, the results showed that within-population genetic diversities were high in Arizona and Colorado. Although the EU-like sequence was identified from every sample collected from Arizona and Colorado, the US1 and/or US2-like sequences were also identified in mixed infection. From a total of 13 cDNA clones generated from three Arizona samples, four clones belonged to the EU genotype, three to US1, and six to US2 (Fig. 3A). Specifically, among 10 clones analyzed in the AZ1 isolate, two had the EU genotype (AZ1_EU.2), three had the US1 genotype (AZ1_US1.3), and five had the US2 genotype (AZ1_US2.5) (Fig. 3A). Similarly, three major PepMV genotypes (EU, US1, and US2) were also detected in Colorado samples (Fig. 3B).

Fig. 2. Predicted phylogenetic relationships of *Pepino mosaic virus* (PepMV) isolates in the United States and Canada (AL1, AZ1-AZ5, CA1-CA9, CO1-CO4, TX1-TX8, BC1-BC3, and ON1) showing the genetic diversity relative to the other known virus isolates currently in the GenBank including: Belgium (EF599605, nt 5,244-5,445); UK2 (AF340024, nt 2,571-2,772); France (AJ438767, nt 5,378-5,579); Hungary (AM491606, nt 5,378-5,579); Poland_PK (EF408821, nt 5,374-5,575); Poland_SW (EF408822, nt 1,482-1,683); US1 (AY509926, nt 5,376-5,577); US2 (AY509927, nt 5,372-5,573); US3 (AY508411, nt 39-240); SM.74 (AM109896, nt 5,376-5,577); SP-13 (AF484251, nt 5,378-5,579); Spain_MU (AM042007, nt 1,017-1,218); LE-2000 (AJ606359, nt 5,378-5,579); LE-2002 (AJ606360, nt 5,378-5,579); LP-2001 (AJ606361, nt 5,376-5,577); CH1 (DQ000984, nt 5,375-5,576); and CH2 (DQ000985, nt 5,374-5,575). Each PepMV isolate sequence used in this study was presented with the isolate name (see Table 1) followed by the identified genotype and the number of clones with that sequence (i.e., AZ1_EU.2 represents Arizona sample no. 1, with the EU genotype and two cDNA clones).

Three samples (CO2, CO3, and CO4) collected in 2006 contained three different genotypes (EU, US1, and US2). From a total of 22 cDNA clones analyzed from Colorado, 12 had the EU-like sequence, eight had the US1-like sequence, and two had the US2-like sequence. Specifically, in six clones analyzed in CO2, one clone was with EU (CO2_EU) and five were US1 (CO2_US1.5). In eight clones analyzed in the CO3 sample, five of them were EU (CO3_EU.5) and the other three were US1 (CO3_US1.3) (Fig. 3B). Similarly, in the sample CO4, five clones were EU (CO4_EU.5) and two were US2 (CO4_US2.2) (Fig. 3B).

The CH2 was in mixed infection with the EU genotype in Texas in 2007. The primers KL05-13 and KL05-14 were effective in amplifying all known PepMV genotypes in RT-PCR. However, the sequence polymorphism generated from this genome segment was capable only in differentiating three main PepMV genotypes (EU, US1, and US2), but not between the US2 and CH2. Therefore, additional primer pair (KL04-52 and KL05-20) that was designed to target specifically the CH2 sequence was used in RT-PCR to screen through all the assayed samples. The expected RT-PCR product (642 bp) was obtained only from the new sample (TX8) collected in 2007 (data not shown). Sequences from three cDNA clones generated from the amplified RT-PCR product confirmed the identity of the CH2 genotype (Fig. 3C). Although EU-like sequences were identified in all seven cDNA clones generated from the sample TX8 using the primer set (KL05-13 and KL05-14) (TX8_EU.7 in Figure 2), the CH2-like sequence was also detected when the CH2-specific primer set (KL04-52 and KL05-20) was used (Fig. 3C). These results indicated that in the TX8 sample a majority of the PepMV genetic population belonged to the EU genotype. A small proportion, however, was with CH2. It remained unknown how the CH2 genotype was introduced into this facility in Texas in 2007. Although an isolate with CH2 genotype was first identified in a commercial tomato seed lot imported to the United States from Chile (7), to our knowledge, this was the first instance where CH2 genotype was identified in a major greenhouse tomato-growing facility in North America. This finding added a new dimension to the already complicated situation of PepMV genetic composition in the United States, although the impact of this genotype on greenhouse tomato production in North America is still awaiting further investigation.

DISCUSSION

Greenhouse tomato production is a relatively young industry in North America. However, the industry has grown considerably in the last 15 years, generating an estimated \$400 million in annual sales in

the United States, occupying more than 37% market share for fresh tomatoes in 2005 (<http://www.ers.usda.gov/Publications/ERR2/>). Unlike field tomato production, the high-throughput greenhouse tomato-production system demands frequent hands-on activities, including planting, pruning, plant tying, de-leafing, spraying, and fruit picking. These normal production practices may potentially initiate a mechanical transmission of PepMV to healthy plants from a contaminated hand or tool. Pepino mosaic, first reported in 2001 (4), is now an endemic disease in North American greenhouse tomato-production systems.

Despite the remarkable progress achieved toward the understanding of genome sequences of PepMV isolates from various countries (1,3,9,12,16,20,24), the overall picture of the genetic diversity and population structure of PepMV is still lacking. Pagan et al. (15) reported the study on genetic variability of PepMV isolates in Spain. The authors concluded that despite a varying degree of disease symptoms exhibited on the PepMV-infected tomato plants, the genetic composition of the Spanish PepMV population was quite homogeneous. Even though a US2-like sequence was detected sporadically, the prevalent genotype of PepMV in Spain is the EU. Another similar study conducted in Belgium on its 2006 tomato crops detected two major genotypes, CH2 and EU in single or mixed infection (5). Contrary to the general belief of the EU genotype in European tomatoes, the CH2 was shown to be the prevalent genotype of PepMV in the Belgian greenhouse tomatoes (5). Interestingly, the CH2 genotype has also been detected in Poland (6,16).

Previous studies in the United States have shown the existence of PepMV genetic diversity in limited tissues in tomato (13) or in an imported commercial tomato seed (9). However, the genetic variability and population structure of PepMV in North American greenhouse tomatoes were not characterized until now. The current study involved careful analyses of sequences generated from 91 cloned cDNAs in addition to that of the RT-PCR products derived from 31 PepMV-infected tomato samples. These samples were collected from five states (Alabama, Arizona, California, Colorado, and Texas) in the United States and two provinces (British Columbia and Ontario) in Canada. This survey was facilitated through the support and assistance of the member-growers in the North American Greenhouse Tomato Growers Association. Using sequences generated from the targeted genome segments, phylogenetic relationships among PepMV isolates in major greenhouse tomato facilities in North America were evaluated along with representative isolates of all major PepMV genotypes in the world. Several important findings were made. First, unlike the relatively homogeneous genetic populations in Spain (15), all four major PepMV genotypes (EU, US1, US2, and CH2) were identified among North American greenhouse tomato facilities. Second, much like the situation in Spain, the predominant genotype of PepMV in North America was also the EU. Third, the US1 and US2 genotypes were only found in mixed infection in samples collected from Arizona and Colorado. Fourth, although all seven Texan isolates of PepMV were identified as the EU genotype in 2006, a new sample (TX8) col-

Fig. 3. Genetic composition of *Pepino mosaic virus* (PepMV) population in selected greenhouse facilities in the United States and Canada. **A**, Using sequences (202 bp) generated in the TGB2-3 region, phylogenetic relationships of PepMV isolates in Arizona samples (AZ1, AZ2, and AZ5) showing the presence of three different genotypes (US1, US2, and EU). **B**, Phylogenetic relationships of PepMV isolates in Colorado samples (CO1-CO4) using TGB2-3 region (202 bp), three different genotypes (US1, US2, and EU) were identified. In addition to the EU genotype identified in the Colorado samples, US1 genotype was also found in samples CO2 and CO3, and US2 genotype was shown in sample CO4. **C**, Using genome fragments in the helicase domain (nt 2,082 to 2,723 of CH2) generated with the CH2 specific primer set (KL04-52 and KL05-20), CH2 sequence was detected from three cDNA clones derived from the TX8 isolate in 2007.

lected in 2007, contained not only the EU but also the CH2 genotype in mixed infection. An isolate with the CH2 genotype was first characterized from an imported commercial tomato seed lot (9). Because of the seedborne nature of PepMV in tomato seed and the ease in mechanical transmission (K.-S. Ling, *unpublished data*), commercial tomato seed could potentially serve as an initial virus inoculum for PepMV (2,19). Identification of the CH2 genotype in a greenhouse tomato plant indicated the likelihood that transmission of PepMV-CH2 from contaminated seeds to plants had occurred. Therefore, it is important to plant certified PepMV-free commercial tomato seed, both of scion and rootstock cultivars.

Maroon-Lango et al. (13) first characterized two PepMV isolates (US1 and US2) with unique sequences in the United States. A third isolate (US3) was shown to have genome sequence that is similar to the EU genotype. Using an imported commercial tomato seed sample from Chile, Ling (9) characterized two new isolates (CH1 and CH2). CH1 had a high genome sequence identity with the US1. It was considered to be a US1 isolate. However, genome sequence in the CH2 isolate was unique. Although two-thirds of the CH2 virus genome had high sequence identity (98%) to the US2, the other one-third of the genome, made up of a part of replicase (helicase and RdRp domains) and TGB1 (from nt 2,307 to 4,738), had approximately 80% nucleotide sequence identity to the other three major genotypes of PepMV (EU, US1, or US2). Therefore, using primers that were unique to this region of CH2, any potential CH2-like sequence in a sample could be preferentially amplified by RT-PCR (9). Indeed, this was how we identified the presence of CH2 genotype in a sample collected in 2007 from Texas (TX8) (Fig. 3C).

Findings of the genetic diversity and population structure of PepMV in the United States and Canada in the current study are significant for designing an effective sequence-based detection method (11), as well as for the development of a tomato cultivar with durable disease resistance (10,21). In contrast to the general expectation that the predominant genotypes of PepMV are US1 and US2 (11), the EU genotype now appears to be prevalent in greenhouse tomato-production facilities in the United States and Canada and the US1, US2, and CH2 genotypes have only limited distributions (Table 1). For example, in Colorado, an isolate with only the EU genotype was detected in the 2001 sample. However, in 2006, two additional genotypes (US1 and US2) were also identified. Similarly, these same genotypes (EU, US1, and US2) were also identified in the Arizona samples. On the other hand, all seven samples collected in 2006 in

Texas belonged to the EU genotype, whereas an isolate with the CH2 genotype was identified for the first time in 2007 from this same facility. This latest finding likely represents the new introduction of a unique genotype of PepMV in North American greenhouse tomatoes. Furthermore, it is highly possible that a new genotype may be created through RNA recombination during virus replication in mixed infection of different genotypes. The CH2 genotype likely resulted from a recombination between US2 and another yet unidentified ancestor. The effect of mixed infection of several genotypes of PepMV on the severity of disease symptoms and its impact on yield and quality of tomato is still awaiting further investigations.

ACKNOWLEDGMENTS

We thank Jim Jacobi of C. Beaty Hanna Horticulture and Environmental Center, Birmingham, AL; John Van Etten and Michael VanZeyst of Village Farms at Marfa, TX; Karin Tift of EuroFresh at Wilcox, AZ; Dana Sfetcu of Houweling Nurseries at Camarillo, CA; Frek Knol of De Ruiter Seeds at Golden, CO; Jonathan Bos of Hothouse (now part of Village Farms) in British Columbia, Canada; and Guido E. H. van het Hof of Great Northern Hydroponics in Ontario, Canada for plant materials. We thank Andrea Gilliard and Emily Walters for their excellent technical assistance and W. Patrick Wechter and Amnon Levi for critical reading of the manuscript.

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