Detection and Molecular Characterization of Two Little Leaf Phytoplasma Strains Associated with Pepper and Tomato Diseases in Guanajuato and Sinaloa, Mexico

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ABSTRACT

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Pepper (*Capsicum annuum*) and tomato (*Lycopersicon esculentum*) are important vegetable crops in Mexico. Recently, symptoms associated with phytoplasma diseases such as witches'-broom (shoot proliferation) and little leaf were observed in pepper and tomato fields in central and northwestern Mexico. DNA extracted from symptomatic and asymptomatic plants was used in nested polymerase chain reaction (PCR) assays with primers amplifying 16S rDNA sequences for phytoplasmas. Twenty-four percent of pepper and 49% of tomato samples yielded a nested rDNA product of 1.25 kb. Restriction fragment length polymorphism profiles and sequencing of PCR products allowed classification of the detected phytoplasmas with the aster yellows group (16SrI). Both phytoplasmas, pepper little leaf (PeLL) and tomato little leaf (ToLL), could be included as new members of the aster yellows group because *HaeIII* and *TaqI* restriction enzymes discriminated among these phytoplasmas and members of other 16SrI subgroups. PeLL and ToLL phytoplasma sequences were deposited and compared with those in GenBank, and the maximum identity was found with several isolates of '*Candidatus Phytoplasma asteris*'. The highest identity (99%) has been observed with tomatillo little leaf phytoplasma and ash witches'-broom phytoplasma. This is the first report of '*Ca. Phytoplasma asteris*' associated with pepper and tomato diseases in the Mexican states of Guanajuato and Sinaloa.

Additional keyword: RFLP-PCR

Pepper (*Capsicum annuum*) and tomato (*Lycopersicon esculentum* Mill.) are important vegetable crops in Mexico. The national production of pepper and tomato in 2005 to 2006 was 2.1 and 2.25 million metric tons with a value of US\$938 million and US\$944 million, respectively (34). Increased market demand has contributed to more intensive production of different pepper and tomato varieties. However, due to occurrence of insect pests and disease problems, the yield and quality of the fruit are lower than expected, causing heavy economic losses. Recently, symptoms associated with phytoplasma diseases such as witches'-broom (shoot

proliferation) and little leaf were observed in pepper and tomato crops.

Phytoplasma diseases of potato (*Solanum tuberosum*), tomato (*Lycopersicon esculentum*), tomatillo (*Physalis ixocarpa*), ornamental plants (*Catharanthus roseus*, *Opuntia* spp., *Alstroemeria* sp.), Cempazuchil (marigold, *Tagetes erecta*), Pigeon pea (*Cajanus cajan*), lima bean (*Phaseolus lunatus*), coconut palms (*Cocos nucifera*), and weeds (*Vinca minor*, *Trifolium* spp., *Amaranthus hybridus*) have been reported in Mexico (2,9,16,17,26–31).

Phytoplasmas are bacteria without cell walls that inhabit plant phloem. They are poorly understood plant pathogens because they cannot be cultured *in vitro*. Until recently, they were identified and classified based on symptomatology, host range, and vector specificity, but these methods are not suitable to reveal the genetic relatedness among different phytoplasmas (23). DNA-based molecular techniques such as polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) analysis of PCR products, and sequence analysis have been recently used to detect and differentiate phytoplasma

strains (15,23,27,33,36). To date, 18 groups and more than 40 subgroups of phytoplasmas have been delineated based on RFLP analysis of 16S rDNA sequences, and 26 *Candidatus Phytoplasma* species have been proposed (5,6,18,21,23,38). The '*Candidatus Phytoplasma asteris*' concept encompasses all known subgroups within group 16SrI (aster yellows group). It is the largest phylogenetic group, with approximately 15 subgroups. Subgroups 16SrI-A, 16SrI-B, and 16SrI-C are distributed worldwide and are associated with more than 80 plant species. Subgroups 16SrI-L and 16SrI-M appear to be restricted to Europe. The subgroup 16SrI-D is found only in Asia. Subgroup 16SrI-B represents the largest and most diverse strain cluster in the group (25). In Mexico, the phytoplasma causing the diseases "filodia del cempazuchil" and "permanent yellowing" of tomato belongs to subgroup 16SrI-B. The potato purple top phytoplasma associated with potato diseases is considered to be a new member of the aster yellows group based on RFLP and sequence analysis of the 16S rDNA (16,27,30).

Phytoplasma diseases of pepper have been reported in Spain (8), Australia (37), India (20), and Cuba (7). Phytoplasma diseases of tomato have been reported from several areas in the Mediterranean Basin (1,4,10,11,39,41), the United States (22,24,35), Australia (13,32), Bolivia (19), and Brazil (3). However, there is little information about phytoplasma diseases of pepper and tomato in Mexico. To our knowledge, this is the first report of '*Ca. Phytoplasma asteris*' associated with pepper and tomato diseases in the Mexican states of Guanajuato and Sinaloa.

MATERIALS AND METHODS

Plant samples. Pepper and tomato plants showing symptoms of witches'-broom (shoot proliferation) and little leaf were collected in the Mexican states of Guanajuato and Sinaloa. A total of 63 pepper samples and 101 tomato samples were collected during 2002 to 2005.

DNA extraction. A procedure modified from Zhang et al. (40) was used for DNA extraction from pepper and tomato tissues.

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Two hundred milligrams of actively growing fresh leaf tissue was ground in hot hexadecyltrimethylammonium bromide (CTAB) buffer (3% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0, 0.2% mercaptoethanol).

Phytoplasma detection by PCR. Two “universal” phytoplasma-specific nested primers were used for the detection of phytoplasma. The primers R16mF2/R16mR1 and R16F2n/R16R2 were used to amplify a portion of the 16S rDNA (14). Primers R16mF2/R16mR1 amplify a DNA fragment of 1.45 kb, whereas primers R16F2n/R16R2 amplify a fragment of 1.25 kb. PCR amplifications were conducted in an automated thermocycler (model Icyler, Bio-Rad, USA) with one unit of Taq DNA polymerase (Invitrogen Life Technologies, Brazil) in a volume of 25 μ l containing 100

ng of total DNA, 200 mM each dNTP, and 0.4 pmol/ μ l each primer. The reaction was conducted with one cycle of 4 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 60°C (55°C for R16F2n/R16R2), 3 min at 72°C, and an additional cycle of 5 min at 72°C. PCR products were analyzed by electrophoresis in a 1% agarose gel and visualized by staining with ethidium bromide and UV illumination. DNA samples extracted from pepper and tomato plants were used as template for PCR. DNA extracted from asymptomatic plants and sterile water were used as negative controls. Total genomic DNA from a previously characterized phytoplasma infecting *Opuntia* spp. was used as a positive control (26).

RFLP analysis of detected phytoplasma. Fragments of 16S rDNA from

pepper little leaf (PeLL) and tomato little leaf (ToLL) phytoplasmas amplified by nested PCR with the primer pairs R16mF2/R16mR1 and R16F2n/R16R2 from at least 10 different symptomatic leaf samples were digested with *AluI*, *HpaII*, *KpnI*, *HaeIII*, *HhaI*, *TaqI*, *MseI*, *RsaI*, and *BfaI* according to the manufacturer’s instructions (Invitrogen Life Technologies, USA). The digestion products were then fractionated by electrophoresis using 3% agarose gels and stained in ethidium bromide. RFLP patterns were compared with previous results (24).

Cloning of PCR products and sequencing of DNA. Two PCR products from PeLL phytoplasma and ToLL phytoplasma were purified using a Genclean III kit (Gibco-BRL) and ligated onto the pGEM-T vector (Promega Corporation). The ligation mixture was used to transform *Escherichia coli* DH5 α (Promega). Plasmid DNA from cultures of recombinant colonies was purified using QIAprep Spin Miniprep Kit (QIAGEN GmbH). Both strands of two different clones of each phytoplasma were sequenced completely in an ABI PRISM 377 sequencer using the Dye cycle sequencing kit (Applied Biosystems, Foster City, CA). The sequences of 16S rDNA from PeLL phytoplasma and ToLL phytoplasma have been assigned the GenBank accession numbers DQ092321 and DQ375238, respectively.

Sequence similarity analysis and *in silico* enzyme digestions. The 16S rDNA sequence similarities between strains were evaluated after alignments were generated

Fig. 1. Gel electrophoresis of products from polymerase chain reaction (PCR) performed on DNA extracted from pepper and tomato samples using nested-PCR with primers pairs R16mF2-R16mR1/R16F2n-R16R2. Lane M, 1 kb plus DNA ladder (Invitrogen Life Technologies); lanes 1 to 6, pepper samples; lanes 7 to 12, tomato samples; lane 13, negative control (water); lane 14, positive control.

Fig. 2. Restriction endonuclease digestion profiles of nested (R16mF2/R16mR1 and R16F2n/R16R2) polymerase chain reaction (PCR) products. Lanes 1 to 3, 7 to 9, 13 to 15, represent pepper little leaf, and lanes 4 to 6, 10 to 12, 16 to 18, tomato little leaf samples digested with *AluI*, *HpaII*, *KpnI* (A) and *HaeIII*, *HhaI*, *TaqI* (B) enzymes. Lane M, 1 kb plus DNA ladder (Invitrogen Life Technologies).

using the Clustal W method of the MegAlign program (DNASTAR Inc., Madison, WI). Virtual restriction fragment analysis was performed from the partial sequences of the 16S rDNA gene using the virtual gel plotting program pDRAW32 (AcaClone). Each aligned DNA fragment was digested *in silico* with nine distinct restriction enzymes (*AluI*, *HpaII*, *KpnI*, *HaeIII*, *HhaI*, *TaqI*, *MseI*, *RsaI*, and *BfaI*) that have been routinely used for phytoplasma 16S rRNA gene RFLP analysis.

Phylogenetic analysis. Nearly complete 16S rDNA sequences from PeLL and ToLL phytoplasmas, along with 19 known phytoplasmas and *Acholeplasma palmae*, were aligned using the Clustal X method of the MegAlign program. The phylogenetic tree was generated using the Njplot program from Clustal X. *A. palmae* was employed as the outgroup to root the tree. Bootstrapping was performed to estimate the stability and support for the branches.

RESULTS

Detection of phytoplasmas from pepper and tomato plants. Phytoplasmas

were detected by nested PCR with universal primer pairs R16F2n/R16R2 and R16mF2/R16mR1 in 15 out of 63 samples and 49 out of 101 samples from pepper and tomato, respectively. Typical bands of 1.25 kb were visualized in agarose gels for DNA extracted from pepper, tomato, and the positive control (Fig. 1). No amplification was observed when DNA from asymptomatic plants was used as template (data not shown).

RFLP analysis. To identify phytoplasma isolates infecting pepper and tomato plants, products from nested PCR (R16F2n/R16R2) amplification of 10 different plant samples were digested with *AluI*, *HpaII*, *KpnI*, *HaeIII*, *HhaI*, *TaqI*, *MseI*, *RsaI*, and *BfaI* (RFLP patterns obtained from *AluI*, *HpaII*, *KpnI*, *HaeIII*, *HhaI*, and *TaqI* of three pepper and tomato samples are shown in Figure 2). All the samples tested of each phytoplasma showed the same profile. The *KpnI* restriction patterns of DNA from phytoplasmas detected in tomato and pepper were identical to the predicted RFLP profiles of potato purple top phytoplasma (PPT) (type

strain of '*Ca. Phytoplasma asteris*') and other previously described phytoplasmas of the 16SrI group (24,27). *HaeIII* and *TaqI* digests of PeLL and ToLL fragments were identical, but were different from other members of the 16SrI group (24). *HhaI* digests of PeLL and ToLL fragments were identical to those predicted for members of the subgroup 16SrI-B (24). However, the ToLL phytoplasma was distinguished from the PeLL phytoplasma and other members of the 16SrI group by its *HpaII* digestion pattern (Fig. 2A).

Nucleotide sequence analysis and *in silico* enzyme digestions. The sequences of the 1.25-kb PCR fragment of the 16S rDNA of PeLL and ToLL phytoplasmas were compared with those in the NCBI/GenBank database, and the maximum identity was found with several isolates of the aster yellows, 16SrI, group ('*Ca. Phytoplasma asteris*'). The highest identity was observed with tomatillo little leaf phytoplasma (99.3 and 99.6%, respectively, GenBank accession number DQ987871) and ash witches'-broom (99.1 and 99.5%, respectively, GenBank acces-

Fig. 3. Virtual restriction fragment length polymorphism (RFLP) patterns from *in silico* digestions of 16S rRNA gene F2nR2 fragments from pepper and tomato little leaf phytoplasmas and seven phytoplasma strains representing different subgroups of aster yellows group (16SrI). Nine restriction enzymes were used in the digestions: *AluI*, *HpaII*, *KpnI*, *HaeIII*, *HhaI*, *TaqI*, *MseI*, *RsaI*, and *BfaI*. MW, ϕ X174DNA-*HaeIII* digestion. Virtual RFLP patterns were generated using the gel plotting program pDRAW32.

sion number AY566302). Ash witches'-broom belongs to the 16SrI group of phytoplasmas (aster yellows), subgroup B (24). Sequence identities of the PeLL and ToLL 16S rDNA were 99 and 99.4%, respectively, with different strains of aster yellows (16SrI, subgroups B, M, and N, GenBank accession numbers AY180943, AY265209, and AY265205, respectively); and less identity, 98.5 and 98.8%, respectively, was found with soybean purple top (16SrI, subgroup O, GenBank accession number AF268405) (24). The rDNA sequences of PeLL and ToLL phytoplasmas were distinct from those of potato purple top (PPT) associated with potato diseases in Mexico (98.4 and 98.8%, respectively) (27).

Virtual RFLP patterns (Fig. 3) revealed differences between PeLL and ToLL and others strains of '*Ca. Phytoplasma asteris*' used. The PeLL and ToLL phytoplasmas showed *TaqI* and *HaeIII* restriction patterns different from all the described members of the 16SrI group. *AluI*, *HaeIII*,

TaqI, *RsaI*, and *BfaI* restriction patterns of PeLL and ToLL were different from PPT phytoplasma. The PeLL and ToLL phytoplasmas can be differentiated by the restriction enzyme *HpaII*, and ToLL has a unique restriction pattern among phytoplasmas previously classified in the 16SrI group (24). Therefore, we designated PeLL and ToLL phytoplasmas as new subgroups S and T (16SrI-S and 16SrI-T, respectively) following criteria reported by Lee et al. (24).

Phylogenetic analysis. The 16S rDNA sequences of the PeLL and ToLL phytoplasmas compared with those of 18 other phytoplasmas and *A. palmae* yielding the consensus tree are illustrated in Figure 4. The bootstrapping values indicated strong support for most branches. PeLL and ToLL phytoplasmas give rise to new branches not seen in previous phylogenetic trees, indicating that these phytoplasmas may represent a new phytoplasma lineage, and supporting the designation of PeLL and ToLL phytoplasmas as representative of

new subgroups in group 16SrI (aster yellows group).

DISCUSSION

Phytoplasma diseases of pepper have been reported in Australia, Spain, India, and Cuba (7,8,20,37), and tomato in the United States, Australia, Italy, Jordan, Bolivia, and Brazil (3,4,11–13,19,22,24,32,35). In this communication, we describe the discovery in Mexico of two new members of the aster yellows phytoplasma group (16SrI).

This work to identify and classify the causal agents of little leaf of pepper and tomato in Mexico was primarily based on RFLP analysis of PCR amplified rDNA, using the restriction endonucleases *AluI*, *HpaII*, *KpnI*, *HaeIII*, *HhaI*, and *TaqI*. This approach proved suitable for phytoplasma differentiation and preliminary classification of PeLL and ToLL phytoplasmas. The RFLP profile results were consistent with the phylogenetic tree and the virtual restriction patterns constructed with the 16S rDNA gene sequence. We have placed the PeLL and ToLL phytoplasmas in new subgroups (I-S and I-T, respectively) of group 16SrI, indicating that they possess unique properties that are reflected in the results from enzymatic RFLP analyses, sequence similarities, and phylogenetic analysis based on 16SrRNA gene sequences. Other phytoplasma diseases detected in Mexico, potato purple top (PPT) and coconut lethal yellowing (LY), cause severe losses in potato and coconut crops (16,27,29). In this study, we found that PeLL and ToLL phytoplasmas have a very close relationship with tomatillo little leaf phytoplasma reported previously in Mexico (31).

Although phytoplasmas were for a long time assumed to be the causal agent of the disease known in Mexico as "permanente" of tomato, molecular identification of the causal phytoplasmas was not previously attempted. However, a recent study indicated that 85% of symptomatic plants from the Baja California Peninsula of Mexico were PCR-positive, and that the rDNA had restriction patterns typical of phytoplasmas belonging to the aster yellows subgroup B (16SrI-B) '*Ca. Phytoplasma asteris*' and 10% were associated with a phytoplasma related to the 16SrXIII-A Mexican periwinkle virescence group using the enzymes *AluI*, *MseI*, *HhaI*, and *HpaII* (17). The results of our studies indicated that phytoplasmas were present in 48% of the tomato samples tested and that these phytoplasmas belong to the 16SrI group.

On the basis of RFLP analysis, nucleotide sequence, and phylogenetic analysis, phytoplasmas associated with diseases of pepper and tomato in various countries have been classified in different 16S rDNA groups: I, II, III, V, VI, XII, and XIII (3,4,11,13,17,22,24,32,35). This indicates that phytoplasma diseases of pepper and tomato are genetically diverse. Therefore,

Fig. 4. Phylogenetic tree for the 16SrI rDNA group from 20 phytoplasmas. The tree was constructed using the neighbor-joining method. *Acholeplasma palmae* was used as the outgroup. The length of branches represents the distance between sequences. Bootstrap (confidence) values for 1,000 replications are shown on the branches. GenBank accession numbers for sequences are given in parentheses, followed by the phytoplasma subgroup.

DNA-based molecular techniques and computer analysis of the sequences obtained appear to be good methods for detecting and differentiating phytoplasma strains.

In Mexico, different phytoplasmas associated with plant diseases from 16S rDNA groups 16SrI (aster yellows) (27,30,31), 16SrII (peanut witches'-broom) (27), 16SrIV (16), and 16SrIX (pigeon pea witches'-broom) (28), have been characterized.

Identification of the causal agent of little leaf of pepper and tomato in Mexico should facilitate studies concerned with epidemic aspects of the disease, and could contribute to knowledge of the genetic diversity of phytoplasmas present in Mexico.

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