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Epidemiología y variabilidad patogénica del virus del mosaico del pepino dulce (*Pepino mosaic virus*). Nuevas enfermedades asociadas a su presencia (torrao o cribado)

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HACE CONSTAR: que Dña. Ana O. Alfaro Fernández, ha realizado bajo su dirección en dicho Departamento, el trabajo que con título "Epidemiología y variabilidad patogénica del virus del mosaico del pepino dulce (*Pepino mosaic virus*). Nuevas enfermedades emergentes asociadas a su presencia (torrao o cribado)" presenta para optar al grado de Doctor Ingeniero Agrónomo.

Para que así conste a los efectos oportunos, firma la presente en Valencia a 17 de noviembre de 2009.

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Resumen

El virus del mosaico del pepino dulce (*Pepino mosaic virus*, PepMV) es uno de los principales virus que afectan al cultivo del tomate y está ampliamente extendido por las diferentes zonas productoras del mundo, donde provoca importantes pérdidas económicas. Su eficiente transmisión mecánica hace que se trate de un virus de rápida dispersión y difícil control. La gran variabilidad sintomatológica unida a una gran diversidad molecular, dificulta su diagnóstico. Por el momento existen cinco genotipos caracterizados, aunque aún no se conoce que región del genoma del virus se encarga de la expresión de los síntomas. La construcción de clones infecciosos y su marcaje con proteínas fluorescentes es una herramienta eficaz para profundizar en el conocimiento de la biología molecular de los virus vegetales. Del mismo modo, la interacción del PepMV con otros agentes como el hongo *Olpidium brassicae* sensu lato (sl) se ha relacionado con el síndrome del "colapso", pero aún se desconoce si dicha relación lleva asociada la transmisión del virus por este hongo vector.

Por tanto, se desarrolló un método de diagnóstico que permitía la identificación y detección simultánea de los cinco genotipos del PepMV descritos por el momento: Europeo (EU), Peruano (PE), Chileno 1/US1 (CH1/US1), Chileno 2 (CH2) y US2. El método diseñado consistía, en primer lugar en la realización de una multiplex RT-PCR en un paso, con una mezcla de seis de cebadores específicos que amplifican una zona del gen de la RNA polimerasa RNA dependiente del virus, incluyendo un control interno, separándose así tres grupos de genotipos diferentes: EU/PE, CH1/US1 y CH2/US2. Para diferenciar el genotipo presente en la muestra entre los cinco determinados, se llevó a cabo un análisis de restricción con el enzima *SacI* con el producto de PCR obtenido. El método presentó un límite de detección y sensibilidad mayor que otras técnicas de diagnóstico comúnmente empleadas.

Por otro lado, se construyó un clon infeccioso de PepMV a partir del genoma completo de un aislado del genotipo CH2 de PepMV, consiguiendo transcritos *in vitro* capaces de reproducir la misma sintomatología que el aislado original tras la inoculación mecánica a *Nicotiana occidentalis* H-H Wheeler.

Posteriormente, se obtuvo un clon mutante de ese mismo aislado con el gen de la proteína fluorescente verde GFP, incluido en la región intergénica existente entre la ORF 4 y 5 del PepMV.

Para verificar la capacidad de *O. brassicae* sl (concretamente la especie *Olpidium virulentus* (Sahtiyanci) Karting) para transmitir el PepMV, se realizaron dos experimentos en cámara de cultivo donde se consiguió la transmisión del virus a planta de tomate sana mediante el riego de ésta última con el percolado obtenido tras el riego de plantas infectadas con PepMV que contenían un cultivo de *O. virulentus* en sus raíces, procedente de tomate.

En otro orden de cosas, en 2007 se identificó como agente causal de la enfermedad conocida como "torrao", al nuevo virus Tomato torrado virus (ToTV). Este hecho determinó la necesidad de conocer diversos aspectos de su epidemiología, diagnóstico, variabilidad y posible efecto sinérgico de éste con otras virosis que afectan habitualmente al cultivo del tomate en España. Para ello, en primer lugar se estudiaron la incidencia y distribución de la enfermedad del "torrao", realizando muestreos en las principales zonas productoras de España desde 2001 a 2008. Se recogieron 451 muestras en 92 invernaderos, se clasificaron por su sintomatología y se analizaron frente a diversos virus. Más del 75% de las muestras analizadas resultaron positivas a ToTV, de las cuales la mayoría mostraban los síntomas típicos de la enfermedad, sin embargo, algunas mostraban otras manifestaciones de necrosis y otras incluso resultaron asintomáticas. El 60,5% de las plantas analizadas presentaron co-infección de ToTV y PepMV, y una pequeña proporción resultaron infectadas con otras virosis del tomate. Asimismo, se demostró que la detección de ToTV mediante hibridación molecular con improntas de de tejido sobre la membrana es una técnica eficaz para el diagnóstico rápido de este nuevo virus. Adicionalmente, se muestrearon y analizaron frente a ToTV diferentes especies de la flora arvense encontradas en invernaderos de tomate de Murcia, Gran Canaria y Tenerife, de las cuales 22 pertenecientes a diversos géneros botánicos resultaron positivas al virus.

Una vez analizadas las muestras de campo, se eligieron aquellas que presentaron infecciones simples con ToTV, así como otras muestras que estaban

infectadas con este virus y otros encontrados habitualmente afectando al cultivo de tomate. Estas muestras se observaron mediante microscopía electrónica de transmisión para estudiar los efectos citopatológicos inducidos por el ToTV y evaluar el posible efecto sinérgico de la co-infección de éste con otras entidades virales a nivel ultraestructural. Las hojas infectadas únicamente con ToTV no presentaron grandes alteraciones celulares, a pesar de que se observaron cuerpos de inclusión conteniendo partículas virales isométricas en las células parenquimáticas del floema. Los tejidos con infecciones dobles de ToTV y PepMV, o ToTV y *Tomato chlorosis virus* (ToCV) manifestaron mayores alteraciones. Sin embargo, el efecto más perjudicial para los tejidos fue el causado por la infección triple de ToCV, PepMV y *Tomato spotted wilt virus* (TSWV) donde se observó un claro engrosamiento de la pared celular acompañado de una gran cantidad de células necrosadas.

Finalmente, se estudió la variabilidad genética de 19 aislados españoles de ToTV recogidos en diversas áreas productoras de tomate del país a lo largo de 9 años. En el estudio también se incluyeron tres aislados húngaros del virus. Se estudiaron cinco regiones del genoma del virus: proteasa-cofactor, la RNA polimerasa RNA dependiente, la proteína de movimiento y dos subunidades de la proteína de la cápside (Vp35 y Vp23). Todos los aislados presentaron una gran similitud en todas las zonas estudiadas. Los análisis filogenéticos revelaron un único grupo que englobaba a todos los aislados del ToTV estudiados separándolos de otros virus del nuevo género propuesto Torradovirus. Sin embargo, se observó un subgrupo en las dos subunidades de la CP estudiadas constituido por los aislados de tomate recogidos en Gran Canaria y un aislado procedente de *S. nigrum* recogido en Tenerife. Las regiones estudiadas estaban bajo una fuerte presión de selección negativa, encontrándose nueve nucleótidos bajo presión de selección negativa repartidos entre diversas zonas del genoma, frente a uno en la zona de la proteasa-cofactor bajo presión de selección positiva.

Abstract

Pepino mosaic virus (PepMV) is an important tomato-infecting virus which is widely spread throughout the tomato crops worldwide, where produces important economical losses. PepMV has an efficient mechanical transmission which leds to its quick spread and makes difficult the control of the disease. The high variability in symptom expression and large genetic diversity of this virus complicates the diagnosis. Nowadays five genotypes of PepMV are characterized although the genome region which is responsible of symptom expression remains unknown. Full-length infectious clones of the virus and the constructions of mutant clones marked with fluorescent proteins have been demonstrated as an efficient tool for studying the molecular biology of RNA viruses. Likewise, the interaction of PepMV with the fungal vector *Olpidium brassicae* (Wor.) Dang sensu lato was associated with the disease referred to as "tomato collapse", however the possible transmission of the virus by this fungal vector was never studied.

Therefore, a diagnosis method to allow the identification and simultaneous detection of the five described genotypes of PepMV: European (EU), Peruvian (PE), Chilean 1/US1 (CH1/US1), Chilean 2 (CH2) and US2 was developed. The method consisted in firstly perform a one step multiplex RT-PCR with a cocktail of six specific primers which amplified a fragment of the RNA-dependent RNA-polymerase, plus an internal control, differentiating three groups of genotypes: EU/PE, CH1/US1 y CH2/US2. To identify the concrete genotype within the groups a restriction analysis with the endonuclease *SacI* was carried out to the obtained PCR product. The detection limit and sensitivity of this method was higher when compared to DAS-ELISA and molecular hybridization, common techniques used for virus detection.

On the other hand, a full-length clone of an isolate of the CH2 genotype of PepMV was constructed. The derived *in vitro* RNA transcripts of this clone were inoculated to *Nicotiana occidentalis* H-H Wheeler resulting in virus infection and causing indistinguishable symptoms from those of the wild-type isolate. Later, a mutant clone of this isolate was constructed with the green

fluorescent protein (GFP) gene inserted in the intergenic region between ORF 4 and 5 of the PepMV genome.

To verify the possible transmission of PepMV by the fungal vector *O. brassicae* sl (specifically *O. virulentus* (Sahtiyanci) Karting), two experiments were perfomed in a growth chamber. PepMV was transmitted to healthy tomato plants by irrigation with the drainage water obtained from PepMV-infected plants and whose roots contained the fungal vector *O. virulentus* isolated originally from tomato.

Furthermore, in 2007 a new virus named Tomato torrado virus was identified as the causal agent of the "torrado" disease. Therefore, some aspects of the epidemiology, diagnostic methods, variability and possible synergic effects with other viruses which commonly affect Spanish tomato crops should be clarified.

Firstly, to determine the occurrence and distribution of the torrado disease in the main Spanish tomato growing areas from 2001 to 2008, a total of 584 samples from symptomatic and asymptomatic plants were collected from 92 greenhouses of different areas were infected with ToTV. Samples were classified by symptoms and analysed to different viruses. More than 75% of the collected samples were infected with ToTV. The majority of the positive samples showed typical symptoms of the disease; however plants showing different symptoms of necrosis or even asymptomatic plants were infected with the virus. Co-infection of ToTV with *Pepino mosaic virus* (PepMV) occurred in a large number of samples (60.5%), and several samples were infected with other tomato-infecting viruses. Moreover, the tissue-printing hybridization is a reliable technique which could facilitate the routine diagnosis and large-scale analysis of ToTV. Additionally, weed species found in the surrounding area of the tomato crops from Murcia, Tenerife and Gran Canaria were sampled and analysed against ToTV. Twenty-two samples from different botanic genera were positive to the virus.

Later, samples showing single-infection with ToTV and mixed-infected with other tomato-infecting viruses found in plants affected by torrado disease were selected. Optical and electron microscopy studies were conducted to know

the cytopathology induced in tomato leaves single-infected by ToTV and the effect of the co-infection of ToTV with other viruses which commonly infect tomato crops. Ultra-thin sections of ToTV-infected tomato leaves did not present a strong cellular alteration. However, crystalline arrays of isometric virus-like particles (VLPs) of 20-30 nm in the inclusion bodies were observed in phloem parenchyma cells of the infected tissues. Tissues double-infected by ToTV and *Tomato chlorosis virus* (ToCV) or *Pepino mosaic virus* (PepMV) presented more severe cellular alterations. The most deleterious consequences for tomato cells were found in triple infections of ToTV, PepMV and *Tomato spotted wit virus* (TSWV), where characteristic cell wall overgrowth was distinguishable, together with a large amount of necrotic cells.

Finally, the population structure and genetic variation of Tomato torrado virus (ToTV) were estimated from 19 Spanish isolates collected from 2001 to 2009 in different tomato production areas by analyses of the partial nucleotide sequences of five regions of the virus genome: the protease-cofactor and the RNA-dependent RNA-polymerase, the movement protein and two subunits of the coat protein (Vp35 and Vp23). In the analysis, three Hungarian isolates of the virus were also included. All the ToTV isolates clustered together in the phylogenetic analysis of the nucleotide sequences of the different regions. A high similarity was observed among all the isolates studied. However, some genetic diversity was observed in case of subunits of the CP studied among the Gran Canary isolates and the rest of analysed isolates of ToTV that grouped together. Studying the genetic distances between pairs of sequences, the ratio between nonsynonymous and synonymous substitutions was low, indicating a strong negative selective pressure in the studied regions. Nine negatively selected sites distributed in different areas of the genome and just one site in the protease-cofactor under positive selection for all the genome regions studied were found.

Resum

El virus del mosaic del cogombre dolç (PepMV) és un dels principals virus que afecten al cultiu de la tomaca i es troba àmpliament estés per les diferents zones productores del món, provocant importants pèrdues econòmiques. La seua eficient transmissió mecànica fa que es tracte d'un virus de ràpida dispersió i difícil control. La gran variabilitat simptomatològica unida amd la seua gran diversitat molecular, dificulta el seu diagnòstic. Fins ara han sigut caracteritzats cinc genotipus distints, encara que no es conega la regió del genoma del virus encarregada de l'expressió dels símptomes. La construcció de clons infecciosos i el seu marcatge amb proteïnes fluorescents és una eina eficaç per aprofundir en el coneixement de la biología molecular dels virus vegetals. De la mateixa manera, l'interacció del PepMV amb altres agents com el fonc *Olpidium brassicae* sensu lato s'ha relacionat amb el síndrome del "col·lapse", encara que es desconeix si es produeix la transmissió del virus per aquest fonc vector.

Per tant, es va desenvolupar un mètode de diagnòstic que va permetre l'identificació i detecció simultània dels cinc genotipus del PepMV descrits: Europeu (EU), Peruà (PE), Xilè 1/US1 (CH1/US1), Xilè 2 (CH2) i US2. El mètode dissenyat va consistir, en primer lloc, en la realització d'una multiplex RT-PCR en un pas, amb una barreja de sis cebadors específics que amplifiquen una zona del gen de la RNA polimerasa RNA dependent del virus, incloent un control intern, separant així tres grups de genotipus diferents: EU/PE, CH1/US1 i CH2/US2. Per a poder diferenciar quin dels cinc aïllats està present en la mostra, es va efectuar un anàlisi de restricció amb l'enzim *SacI* del producte obtés. El mètode va presentar un límit de detecció i sensibilitat major que altres tècniques de diagnòstic comúnment empleades.

D'altra banda, es va sintetitzar un clon infecciós de PepMV a partir del genoma complet d'un aïllat del genotipus CH2 de PepMV, aconseguint transcrits *in vitro* capaços de reproduir la mateixa simptomatología del aïllat original després de l'inoculació mecànica a *Nicotiana occidentalis* H-H Wheeler. Posteriorment, es va obtindre un clon mutant d'eixe mateix aïllat amb el gen de

la proteïna fluorescent verd (GFP), inclós en la regió intergènica existent entre la ORF 4 i 5 del PepMV.

Per a verificar la capacitat de *O. Brassicae* sl (concretament la espècie *Olpidium virulentus* (Sahtiyanci) Karting) de transmetre el PepMV, es van realitzar dos experiments en càmera de cultiu on es va lograr la transmissió de PepMV a planta de tomaca sana mitjançant el seu rec amb el residu filtrat obtingut del rec de plantes infectades amb PepMV que contenien un cultiu de *O. virulentus* en les seues arrels, procedent de tomaca.

Així doncs en 2007 es va identificar com agent causal de la malaltia coneguda com "torrao" al nou virus Tomato torrado virus (ToTV). Aquest fet va determinar la necessitat de conèixer diverses aspectes de la seua epidemiología, diagnòstic, variabilitat i possible efecte sinèrgic d'aquest amb altres virus que afecten habitualment al cultiu del tomaca en Espanya.

Es van estudiar l'incidència i distribució de la malaltia del"torrao", prenent mostres de les principals zones productores d'Espanya des de 2001 a 2008. Es van agafar 451 mostres en 92 hivernacles, classificant-se segons la seua símptomatología i analitzant-se front a diversos virus. Més del 75% de les mostres analitzades van resultar positives a ToTV, de les quals, la majoria mostraven els símptomes típics de la malaltia. No obstant, altres plantes van mostrar altres manifestacions de necrosi, i altres resultaren asimptomàtiques. El 60,5% de les plantes analitzades van presentar co-infecció de ToTV i PepMV, i una petita proporció van ser infectades amb altres virus de la tomaca. Tanmateix, es va demostrar que la detecció de ToTV mitjançant hibridació molecular amb empremtes de teixit sobre la membrana és una tècnica eficient per al diagnòstic ràpid d'aquest nou virus. Adicionalment, es van prendre mostres i es van analitzar front a ToTV diferents espècies de la flora arvense trobades en hivernacles de Múrcia, Gran Canària i Tenerife, de les quals 22 pertanyents a diversos gèneres botànics van resultar positives.

Una vegada analitzades les mostres, es van triar les que presentaven infeccions simples amb ToTV, o infeccions mixtes d'aquést amb altres virus trobats habitualment al cultiu de la tomaca. Aquestes mostres es van observar mitjançant microscopia electrònica de transmissió per a estudiar els efectes

citopatològics induïts pel ToTV i avaluar el possible efecte sinèrgic de la co-infecció d'aquest amb altres entitats virals a nivel ultraestructural. Les fulles infectades únicament amb ToTV no van presentar grans alteracions cel·lulars, malgrat que es van a observar cossos d'inclusió contenint partícules virals isomètriques a les fulles parenquimàtiques del floema. Els teixits amb infeccions dobles de ToTV i PepMV, o ToTV i *Tomato chlorosis virus* (ToCV) presentaven majors alteracions. No obstant, l'efecte més perjudicial per als teixits va ser el causat per l'infecció triple de ToCV, PepMV i *Tomato spotted wilt virus* (TSWV), on es va observar un engrossiment clar de la paret cel·lular acompanyat d'una gran quantitat de cèl·lules necrosades.

Finalment, es va estudiar la variabilitat genètica de denou aïllats espanyols de ToTV recollits en diverses àrees productores de tomaca del país, al llarg de nou anys. Al estudi també es van incluir tres aïllats hongaresos del virus. Es van estudiar cinc regions del genoma del virus: proteasa-cofactor, la RNA polimerasa RNA dependent, la proteïna del moviment i dos subunitats de la proteïna de la càpside (Vp35 y Vp23). Tots els aïllats van presentar una gran similitud a totes les zones estudiades. Els aïllats filogenètics van revelar un únic grup que englobava a tots els aïllats estudiats del virus, separant-los d'altres virus del nou gènere proposat (Torradovirus). En canvi, a les dos subunitats de la CP es va observar un subgrup constituït per aïllats de Gran Canària i un aïllat procedent de *S. nigrum* recollit a Tenerife. Les regions estudiades eren sota una forta pressió de selecció negativa, havent-hi nou nucleòtids sota pressió de selecció negativa repartits entre diverses zones del genoma, i tan sols un a la zona de la proteasa-cofactor sota pressió de selecció positiva.

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Abreviaturas

Virus

ACDV: Artichoke curly dwarf virus
ACLSV: *Apple chlorotic leaf spot virus*
ALSV: *Apple latent spherical virus*
AltMV: *Alternanthera mosaic virus*
AMV: *Alfalfa mosaic virus*
APLV: *Andean potato latent virus*
ArMV: *Arabis mosaic virus*
ASGV: *Apple stem grooving virus*
ASPV: *Apple stem pitting virus*
AV-3: *Asparagus virus 3*
BaMV: *Bamboo mosaic virus*
BanMMV: *Banana mild mosaic virus*
BanVX: Banana virus X
BarV-BI: Barley virus BI
BBWV-2: *Broad bean wilt virus 2*
BCTV: *Beet-curly top virus*
BNYVV: *Beet necrotic yellow vein virus*
BolVX: Boletus virus X
BRNV: Black raspberry necrosis virus
BRSV: *Beet ringspot virus*
BRV: *Blackcurrant reversion virus*
BYV: *Beet yellow virus*
CenMV: Centrosema mosaic virus
CGMMV: *Cucumber green mottle mosaic virus*
CGRMV: *Cherry green ring mottle virus*
CLBV: *Citrus leaf blotch virus*
CLV: *Carnation latent virus*
ClYMV: *Clover yellow mosaic virus*
CMV: *Cucumber mosaic virus*
CNRMV: *Cherry necrotic rusty mottle virus*
ComVX: *Commelina virus X*
CPMMV: *Cowpea mild mottle virus*
CPMV: *Cowpea mosaic virus*
CRLV: *Cherry rasp leaf virus*
CsCMV: *Cassava common mosaic virus*
CSNV: *Chrysantemum stem necrosis virus*
CsVX: *Cassava virus X*
CTV: *Citrus tristeza virus*
CVX: *Cactus virus X*
CVB: *Chrysantemum virus B*
CVYV: *Cucumber vein yellowing virus*
CYMMV: *Cymbidium mosaic virus*
CYSD: *Cucurbit yellow stunting disorder virus*
DLV: Dioscorea latent virus
DVX: *Daphne virus X*
EGMV: *Eggplant green mosaic virus*
EMV: *Eggplant mosaic virus*
FoMV: *Foxtail mosaic virus*
GFLV: *Grapevine fanleaf virus*
GRSV: *Groundnut ringspot virus*
GVA: *Grapevine virus A*
HdRSV: *Hydrangea ringspot virus*
HVX: *Hosta virus X*
ICRSV: *Indean citrus ringspot virus*
LBBV: *Lettuce big-vein virus*
LEV: *Lucerne enation virus*
LIYV: *Lettuce infectious yellows virus*
LRNV: *Lettuce ring necrosis virus*
LVX: *Lily virus X*
LysSLV: Lychnis symptomless virus
MCDV: *Maize chlorotic dwarf virus*
MiLV: *Mirafiori lettuce virus*
MNSV: *Melon necrotic spot virus*
MVNV: Malva veinal necrosis virus
NaMV: Nandina mosaic virus
NeCMV: Negro coffee mosaic virus
NIMV: *Navel infectious mosaic virus*
NMV: *Narcissus mosaic virus*
NVX: *Nerine virus X*
PAMV: *Potato aucuba mosaic virus*
PapMV: *Papaya mosaic virus*
ParV-3: Parsnip virus 3
ParV-5: Parsnip virus 5

PatVX: Patchouli virus X
PaV-5: Parsley virus 5
PepMoV: Pepper mottle virus
PepMV: *Pepino mosaic virus*
PepRSV: *Pepper ringspot virus*
PeVMV: *Pepper veinal mottle virus*
PFBV: *Pelargonium flower break virus*
PHV: *Pepper huasteco virus*
PIVX: *Plantain virus X*
PlAMV: *Plantago asiatica mosaic virus*
PLRV: *Potato leafroll virus*
PlSMOV: *Plantago severe mottle virus*
PMoV: Parietaria mottle virus
PRSV: *Papaya ringspot virus*
PTV: *Peru tomato mosaic virus*
PVS: *Potato virus S*
PVT: *Potato virus T*
PVX: *Potato virus X*
PVY: *Potato virus Y*
PYDV: *Potato yellow dwarf virus*
PYFV: *Parnsip yellow fleck virus*
PZSV: *Pelargonium zonate spot virus*
RoNRSV: Rhododendron necrotic ringspot virus
RpRSV: *Raspberry ringspot virus*
RTSV: *Rice tungro spherical virus*
RV-1: Rhubarb virus 1
ScaVX: *Scallion virus X*
SCSMaV: *Sugarcane striate mosaic-associate virus*
SDV: *Satsuma dwarf virus*
SGMV: Pepper golden mosaic virus
ShVX: *Shallot virus X*
SLRSV: *Strawberry latent ringspot virus*
SmiLV: Smithiantha latent virus
SMoV: *Strawberry mottle virus*
SMYEV: *Strawberry mild yellow edge virus*
SPMMV: *Sweet potato mild mottle virus*
SqNV: *Squash necrosis virus*
SToLCV: *Tomato leaf curl Sinaloa virus*
StPV: *Stocky prunne virus*
TAV: *Tomato aspermy virus*
TBRV: *Tomato black ring virus*
TBSV: *Tomato bushy stunt virus*
TCSV: *Tomato chlorotic spot virus*
TEV: *Tobacco etch virus*
TGMV: *Tomato golden mosaic virus*
TICV: *Tomato infectious chlorosis virus*
TLCrV: *Tomato leaf crumple virus*
TLCV: *Tobacco leaf curl virus*
TMoV: *Tomato mottle virus*
TMGMV: *Tobacco mild green mosaic virus*
TMV: *Tobacco mosaic virus*
TNDV: Tomato necrotic dwarf virus
TNV: *Tobacco necrosis virus*
TuYMV: *Turnip yellow mosaic virus*
ToANV: Tomato apex necrosis virus
ToCV: *Tomato chlorosis virus*
ToDLCV: *Tomato dwarf leaf curl virus*
ToMarV: Tomato marchitez virus
ToMV: *Tomato mosaic virus*
ToRSV: Tomato ringspot virus
ToTNV: *Tomato top necrosis virus*
ToTV: Tomato torrado virus
ToYMV: *Tomato yellow mosaic virus*
ToYTV: *Tomato yellow top virus*
ToYVSV: *Tomato yellow vein streak virus*
TPCTV: *Tomato pseudo curly top virus*
TRMV: *Tamus red mosaic virus*
TRSV: *Tobacco ringspot virus*
TSV: *Tobacco streak virus*
TSWV: *Tomato spotted wilt virus*
TTMV: *Tomato mottle Taino virus*
TuYMV: *Turnip yellow mosaic virus*
TVX: *Tulip virus X*
TVYV: Tomato vein-yellowing (strain of *Eggplant mottle dwarf virus*)
TYDV: *Tobacco yellow dwarf virus*
TYLCV: *Tomato yellow leaf curl virus*
TYNV: *Tomato yellow net virus*
VMOV: Viola mottle virus
WCIMV: *White clover mosaic virus*
WMV: *Watermelon mosaic virus*
ZSLV: Zygocactus symptomless virus
ZYMV: *Zucchini yellow mosaic virus*

Otras abreviaturas

aa: amino acid (aminoácido)
BLAST: basic local alignments search tool
bp: base pairs (pares de bases)
BS: bundle sheath (célula de la vaina)
ca: callose (calosa)
CC: companion cells (células de compañía)
cDNA: complementary DNA (DNA complementario)
Ch: chloroplasts (cloroplastos)
CH1: chilean 1 genotype (genotipo chileno 1)
CH2: chilean 2 genotype (genotipo chileno 2)
CP: coat protein (proteína de cubierta o proteína de la cápsida)
cv : cultivar
DAS-ELISA: double antibody sandwich-enzyme linked immunosorbent assay
DIECA : diethyk dithiocarbamin acid (dietilditiocarbamato)
d_{NS} : nonsynonymous substitutions (sustituciones no sinónimas)
dpi: days post inoculation (días post inoculación)
d_S : synonymous substitutions (sustituciones sinónimas)
DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
dsRNA: doble strand RNA (RNAs doble cadena)
E.Z.N.A.: EaZy Nucleic Acid Isolation
EC: Epidermal cell (célula epidérmica)
EDTA: ethylenediaminetetraacetic acid (ácido etilendiaminotetraacético)
EM : Electron microscopy (microscopía electrónica)
EPPO: European and Mediterranean Plant Protection Organization
EtBr: etidium bromide (bromuro de etidio)
ER: endoplasmic reticulum (retículo endoplásmatico)
EU: european genotype (genotipo europeo)
EU: European Union (UE, Unión Europea)
FAO: Food and Agriculture Organization (Organización de las Naciones Unidas para la Agricultura y la Alimentación)
FEL: fixed-effects likelihood
g: gram (gramo)
GFP: green fluorescent protein (proteína de fluorescencia verde)
gRNA: genomic RNA (RNA genómico)
H: helicase (helicase)
h: hour (hora)
H: hypertrophy (hipertrofia)
Ha: hectárea
IB: inclusion body (cuerpos de inclusión)

ICTV: International Committee on Taxonomy of Viruses (Comité Internacional de Taxonomía de Virus)
IFEL: internal fixed-effects likelihood
IR: intergenic region (región intergénica)
ISEM: Immunosorbent electronic microscopy
kb: kilobase
kDa: kilodalton
L: litro
LP: Peruvian strain (raza peruana)
M: metiltransferasa
m: Mitochondria
M: molararidad
MARM: Ministerio de Medio Ambiente Rural y Marino
MatGAT: Matrix Global Alignment Tool
MEGA: Molecular Evolutionary Genetics Analysis
mg mL−1: miligramo por mililitro
µg µL−1: micrograms per microliter (microgramo por microlitro)
µg : microgram (microgramo)
MH; molecular hybridization
min: minuto
mL: mililitro
µL: microlitre (microlitro)
µm: micrómetre (micrometro)
µM: micromolar
mm: milímetro
mM: milimolar
MMT: million metric tons
MP: movement protein (proteína de movimiento)
mRNA: messenger RNA (RNA mensajero=
Mt: miles de toneladas
N: nucleus (núcleo)
NC: necrotic cell (célula necrótica)
NCBI: National Center of Biotechnology Information.
NJ: neighbor-joining method (método de union de vecinos)
nm: nanómetro
nt: nucleotide (nucleótido)
OG: Osmiophilic globules (Cuerpos osmiofilos)
ORF: open reading frame (pauta de lectura abierta)
P: proliferation/increase in number
PCR: polymerase chain reaction (reacción en cadena de la polimerasa)
PE: peruvian genotype (genotipo peruano)
PhP: phloem parenchyma cell (célula parenquimática del floema)
pmol: picomol

Pro-co: protease-cofactor (cofactor proteasa)
Pro-Pol: protease-polymerase (proteasa-polimerasa)
r.p.m: revolutions per minute (revoluciones por minuto)
Rbcl gene: 1.5-biphosphate carboxylase chloroplast gene
RdRp: RNA dependent RNA polimerase (RNA polimerasa RNA dependiente)
RFLP: Restriction fragment lenght polymorphism (polimorfismos en la longitud de los fragmentos de restricción)
rs: resting spores (esporas de resistencia)
RT-PCR: reverse transcription-polymerase chain reaction (transcripción reversa de la reacción en cadena de la polimerasa)
s: second (segundo)
SE: sieve element (elementos cribosos)
sgRNA: subgenomic RNA (RNA subgenómico)
sl: sensu lato
sp.: species (especie)
spp.: species (especies)
ssRNA: single-stranded RNA (RNA de cadena simple)
t: ton (tonelada)
TAE: Tris-acetate-EDTA (tris acetato EDTA)
TBE: Tris-brorate-EDTA (tris borato EDTA)
TGB: Triple gene block (triple bloque de genes)
TGBp: triple gene block protein (proteína del bloque triple de genes)
TP: tissue printing (impronta de tejido)
U: unit (unidad)
UK: United Kingdom (Reino Unido)
UTR: unstranslated region (región no codificante)
UV: ultraviolet (ultravioleta)
VLP virus-like particles
wt/v : weight per volum (peso por volumen)
zs: zoosporangia (zoosporangios)

Capítulo 1

Introducción general

1. IMPORTANCIA ECONÓMICA DEL TOMATE

El cultivo del tomate (*Solanum lycopersicum* L., familia *Solanaceae*) tiene gran importancia económica a nivel mundial, habiéndose incrementado tanto su superficie cultivada como la producción obtenida en los últimos diez años. En la Unión Europea, a pesar de la entrada de nuevos países miembros, la superficie destinada a este cultivo se ha mantenido más o menos estable en los últimos años mientras que la producción se ha reducido ligeramente (Figura 1.1).

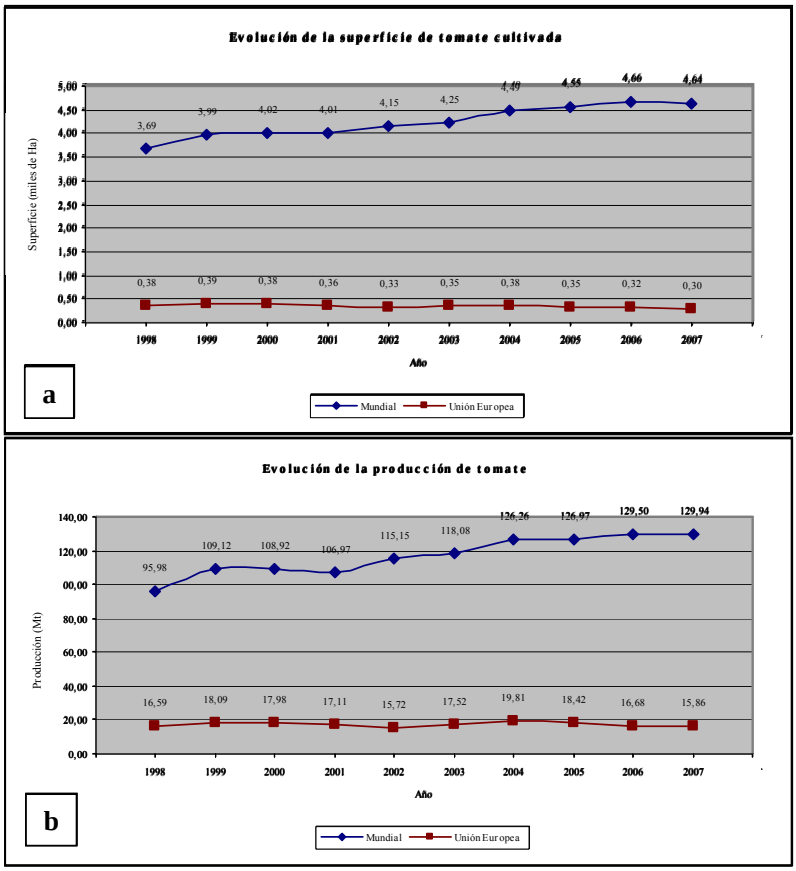


Figura 1.1. Evolución de la superficie cultivada (**a**) y la producción obtenida (**b**) de tomate a nivel mundial y en la Unión Europea desde 1998 hasta 2007 (FAO, 2009).

Según los últimos datos publicados, en el año 2007 se cultivaron en el mundo 4.643.957 Ha, habiéndose obtenido una producción de 129.942.416 t. En la Unión Europea se produjeron 15.860.034 t en ese mismo año (FAO, 2009). En España se cultivó el 3% del total del tomate producido en el mundo en 2007, situándose como el octavo país productor, y el segundo entre los 27 países miembros de la Unión Europea, por detrás de Italia (Figura 1.2).

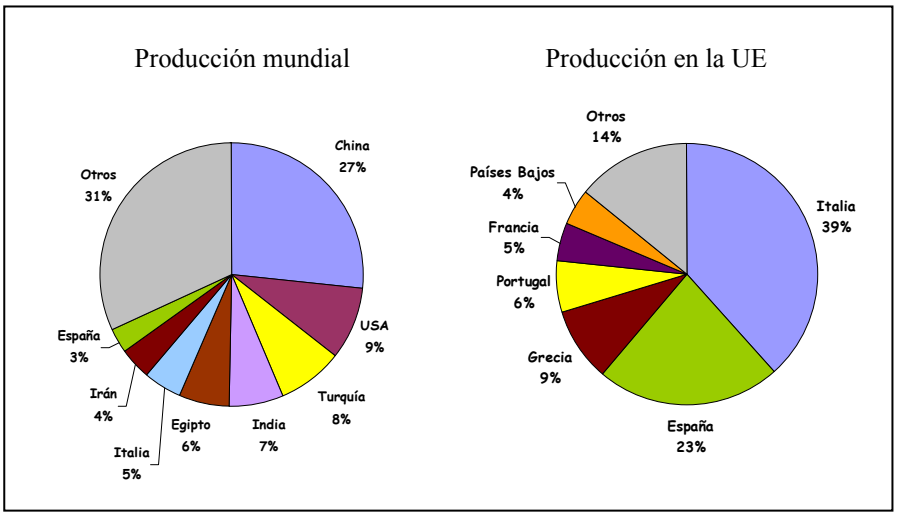


Figura 1.2. Porcentaje de toneladas de tomate producidas por diferentes países a nivel mundial y en la Unión Europea durante 2007 (FAO, 2009).

La evolución de la superficie de tomate cultivada en España también ha sufrido una ligera reducción en los últimos años (Figura 1.3a), aunque la producción se ha mantenido dentro de unos niveles más o menos estables, salvo algunos años donde fue más elevada (Figura 1.3b). En 2007, se cultivaron en España un total de 53.297 Ha, habiéndose obtenido un total de 4.081.477 t. Sin embargo, los datos provisionales de 2008 indican que en este último año se ha incrementado la superficie destinada a este cultivo (57.000 Ha), estimándose por el contrario una disminución del número de toneladas (3.922.500 t). De la producción obtenida en 2007, un 22% (884.244 t) se destinó a la exportación,

fundamentalmente a países miembros de la UE, recibiendo Alemania, Reino Unido, Países Bajos y Francia más del 75% del tomate exportado por nuestro país. En cambio, únicamente se importaron 246.202 t de tomate, procendente mayoritariamente de Portugal (MARM, 2009).

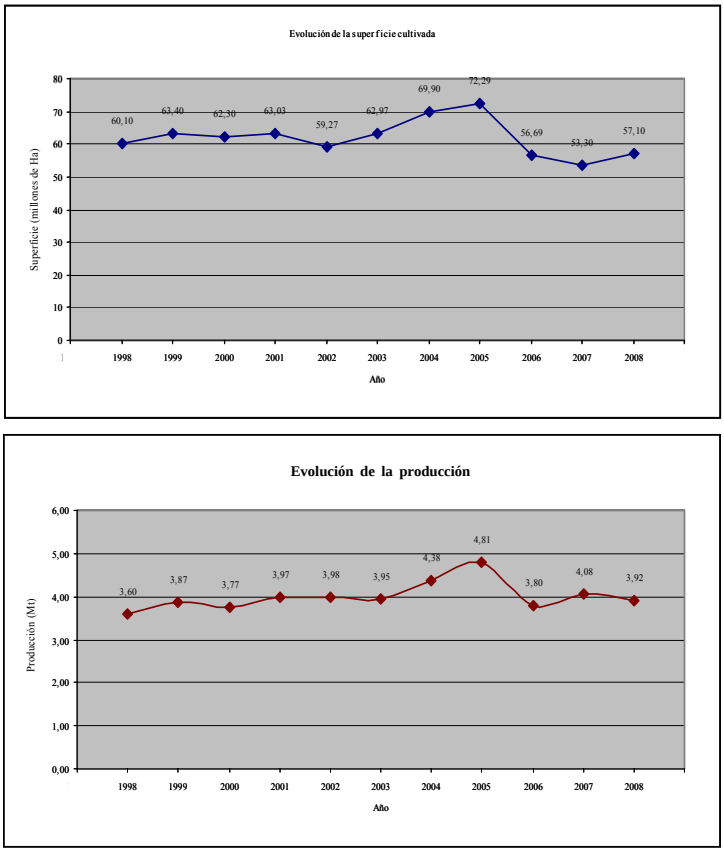


Figura 1.3. Evolución de la superficie cultivada (**a**) y la producción obtenida (**b**) de tomate en España desde 1998 hasta 2008 (MARM, 2009).

En España el tomate se cultiva principalmente en regadío al aire libre o en superficie protegida, siendo las comunidades autónomas con mayor producción Andalucía, Extremadura, Región de Murcia y Canarias. Las provincias de Almería, Murcia, Las Palmas, Granada y Málaga acaparan la

mayor superficie de cultivo de tomate protegido, siendo por otra parte Extremadura, la Comunidad Autónoma donde se concentra la mayor superficie de cultivo al aire libre. En España se realizan tres campañas de recolección de tomate al año; la primera comprende desde enero hasta finales de mayo, la segunda abarca desde junio hasta septiembre y la última desde principios de octubre hasta finales de diciembre, siendo la segunda de ellas la que mayor producción de tomate obtiene (MARM, 2009).

2. VIROSIS QUE AFECTAN AL TOMATE

El cultivo del tomate puede verse afectado por un gran número de enfermedades causadas por diversos agentes: hongos, bacterias, fitoplasmas, virus y viroides, que provocan importantes pérdidas económicas. Las enfermedades de etiología viral son difíciles de controlar y causan pérdidas substanciales en el cultivo, llegando a ser uno de los factores limitantes en la producción intensiva de tomate protegido (Jones *et al.*, 1991). Según la bibliografía consultada, se han descrito un gran número de virus que afectan a éste cultivo, alguno de los cuáles son considerados patógenos de cuarentena por la EPPO (*European and Mediterranean Plant Protection Organization*) (Tabla 1.1).

Tabla 1.1. Principales virus descritos que afectan al cultivo del tomate a nivel mundial[a].

Virus		Familia	Género	Transmisión
Alfalfa mosaic virus[b]	AMV	*Bromoviridae*	*Alfamovirus*	Áfidos, no persistente, semilla (no en tomate)
Andean potato latent virus	APLV	*Tymoviridae*	*Tymovirus*	Por contacto, crisomélidos *Epitrix* sp.
Arabis mosaic virus	ArMV	*Comoviridae*	*Nepovirus*	Nematodos *Xiphinema* o *Longidorus* sp.
Beet-curly top virus[b]	BCTV	*Geminiviridae*	*Curtovirus*	Cicadélidos (persistente, no propagativa)
Chrysantemum stem necrosis virus[b]	CSNV	*Bunyaviridae*	*Tospovirus*	Thrips
Cowpea mild mottle virus[b]	CPMMV	*Flexiviridae*	*Carlavirus*	Semilla, *Bemisia tabaci* (Gennadius)
Cucumber mosaic virus	CMV	*Bromoviridae*	*Cucumovirus*	Áfidos (no persistente)
Eggplant green mosaic virus	EGMV	*Potyviridae*	*Potyvirus*	Vector desconocido
Eggplant mosaic virus[b]	EMV	*Tymoviridae*	*Tymovirus*	Por contacto, crisomélidos *Epitrix* sp.
Lucerne enation virus[b]	LEV	*Rhabdoviridae*	-	Injerto, Áfidos (persistente)
Pelargonium zonate spot virus	PZSV	-	-	Vector desconocido, semilla, polen
Pepino mosaic virus[b]	PepMV	*Flexiviridae*	*Potexvirus*	Mecánica, por semilla
Pepper golden mosaic virus[b]	PepGMV	*Geminiviridae*	*Begomovirus*	*B. tabaci* (persistente)
Pepper huasteco virus[b]	PHV	*Geminiviridae*	*Begomovirus*	*B. tabaci*
Pepper ringspot virus	PepRSV	-	*Tobravirus*	Nemátodos, semilla
Pepper veinal mottle virus	PeVMV	*Potyviridae*	*Potyvirus*	Áfidos (no persistente)
Peru tomato mosaic virus	PTV	*Potyviridae*	*Potyvirus*	Áfidos (no persistente)
Potato aucuba mosaic virus	PAMV	*Flexiviridae*	*Potexvirus*	Mecánica, áfidos
Potato leafroll virus	PLRV	*Luteoviridae*	*Polerovirus*	Afidos (persistente, no propagativa)
Potato virus S	PVS	*Flexiviridae*	*Carlavirus*	Mecánica, áfidos (persistente)
Potato virus X	PVX	*Flexiviridae*	*Potexvirus*	Mecánica, semilla (no tomate)
Potato virus Y	PVY	*Potyviridae*	*Potyvirus*	Áfidos (no persistente)
Potato yellow dwarf virus[b]	PYDV	*Rhabdoviridae*	*Núcleo-rhabdovirus*	Cicadélidos (persistente, no propagativa)
Raspberry ringspot virus[b]	RpRSV	*Comoviridae*	*Nepovirus*	Nematodos *Xiphinema* o *Longidorus* sp.
Sweet potato mild mottle virus	SPMMV	*Potyviridae*	*Ipomovirus*	*B. tabaci* (persistente)
Strawberry latent ringspot virus	SLRSV	*Comoviridae*	*Nepovirus*[d]	Nematodos *Xiphinema* o *Longidorus* sp.
Tobacco etch virus	TEV	*Potyviridae*	*Potyvirus*	Áfidos (no persistente)
Tobacco leaf curl virus	TLCV	*Geminiviridae*	*Begomovirus*	*B. tabaci* (persistente, circulativa, no propagativa)
Tobacco necrosis virus	TNV	*Tombusviridae*	*Necrovirus*	Hongos de suelo (*Olpidium brassicae* (Wor.) Dang)
Tobacco mosaic virus	TMV	-	*Tobamovirus*	Mecánica, por semilla, restos
Tobacco ringspot virus[b]	TRSV	*Comoviridae*	*Nepovirus*	Nematodos *Xiphinema* o *Longidorus* sp.

Tabla 1.1. *(continuación)*

Virus		Familia	Género	Transmisión
Tobacco streak virus[b]	TSV	*Bromoviridae*	*Ilarvirus*	Thrips, transportado por polen
Tobacco yellow dwarf virus	TYDV	*Geminiviridae*	*Mastrevirus*	Cicadélidos (*Orosius argentatus*)
Tomato apex nerosis virus[c]	ToANV	Secoviridae	Torradovirus	Desconocida
Tomato aspermy virus	TAV	*Bromoviridae*	*Cucumovirus*	Áfidos (no persistente)
Tomato black ring virus[b]	TBRV	*Comoviridae*	*Nepovirus*	Nematodos *Xiphinema* o *Longidorus* sp.
Tomato bushy stunt virus[b]	TBSV	*Tombusviridae*	*Tombusvirus*	Mecánica, semilla, suelo , agua
Tomato chlorosis virus	ToCV	*Closteroviridae*	*Crinivirus*	*Trialeurodes vaporariorum* (Westwood), *B. tabaci*
Tomato double-virus streak (ToMV+PVX)	-	-	-	Mecánica
Tomato dwarf leaf curl virus[b]	ToDLCV	*Geminiviridae*	*Begomovirus*	*B. tabaci*
Tomato golden mosaic virus[b]	TGMV	*Geminiviridae*	*Begomovirus*	*B. tabaci* (persistente)
Tomato infectious chlorosis virus[b]	TICV	*Closteroviridae*	*Crinivirus*	*T. vaporariorum*
Tomato leaf crumple virus[b]	TLCrV	*Geminiviridae*	*Begomovirus*	*B. tabaci*
Tomato leaf curl Sinaloa virus[b]	SToLCV	*Geminiviridae*	*Begomovirus*	*B. tabaci*
Tomato marchitez virus	ToMarV	Secoviridae	Torradovirus	Desconocida
Tomato mosaic virus	ToMV	-	*Tobamovirus*	Mecánica, por semilla, restos
Tomato mottle Taino virus[b]	TTMV	*Geminiviridae*	*Begomovirus*	*B. tabaci*
Tomato mottle virus[b]	TMoV	*Geminiviridae*	*Begomovirus*	*B. tabaci*
Tomato necrotic dwarf virus	TNDV	-	-	*B. tabaci* (persistente)
Tomato pseudo curly top virus	TPCTV	*Geminiviridae*	*Topocuvirus*	Membrácido *Micrutallis malleifera* (peristente)
Tomato ringspot virus[b]	ToRSV	*Comoviridae*	*Nepovirus*	Nematodo *Xiphinema americanum*
Tomato spotted wilt virus[b]	TSWV	*Bunyaviridae*	*Tospovirus*	Trips (persistente)
Tomato top necrosis virus	ToTNV	*Comoviridae*	*Nepovirus*[d]	Nematodos *Xiphinema* o *Longidorus* sp.
Tomato torrado virus	ToTV	Secoviridae	Torradovirus	*T. vaporariorum*, *B. tabaci* (experimental)
Tomato vein-yellowing (strain of *Eggplant mottle dwarf virus*)	TVYV	*Rhabdoviridae*	*Núcleo-rhabdovirus*	Vector desconocido
Tomato yellow leaf curl virus[b]	TYLCV	*Geminiviridae*	*Begomovirus*	*B. tabaci* (persistente, circulativa)
Tomato yellow mosaic virus[b]	ToYMV	*Geminiviridae*	*Begomovirus*[d]	*B. tabaci*
Tomato yellow net virus	TYNV	*Luteoviridae*	*Luteovirus*[d]	Áfido *Myzus persicae* (persistente)
Tomato yellow top virus	ToYTV	*Luteoviridae*	*Polerovirus*	Áfidos, (persistente, circulativa)
Tomato yellow vein streak virus[b]	ToYVSV	*Geminiviridae*	*Begomovirus*	*B. tabaci*

[a]Fuente: Adaptado de Jones *et al.* (1991), Jordá (1998), Blancard (1990), ICTV 8[th] report (Fauquet *et al.*, 2005). Los virus que no aparecen en cursiva aún no han sido aceptados como especie por la ICTV.
[b]Virus considerado como patógeno de cuarentena por la EPPO (EPPO PQR v 4.6., 2007).
cVirus considerado actualmente como una cepa (*strain*) dentro de la especie ToMarV.
[d]Posibles especies dentro del género.

3. VIRUS DEL MOSAICO DEL PEPINO DULCE (*Pepino mosaic virus*, PepMV)

3.1. Antecedentes históricos y distribución geográfica

El virus del mosaico del pepino dulce, *Pepino mosaic virus* (PepMV) fue detectado por primera vez en 1974 en Perú afectando a pepino dulce (*Solanum muricatum* Ait.). Las plantas afectadas presentaban un mosaico amarillo en hojas jóvenes, y algunas de ellas mostraban a su vez enaciones o abullonados de color verde oscuro en el envés (Jones *et al.,* 1980). El estudio del comportamiento biológico del virus demostró la capacidad de éste de infectar diferentes especies de la familia *Solanaceae*, entre las que se encontraba el tomate, que a pesar de resultar afectado sistémicamente por el PepMV no manifestaba síntomas de ningún tipo (Jones *et al.,* 1980). No fue hasta veinticinco años después, a principios de 1999, cuando se detectó por primera vez una infección natural en tomate, identificándose en plantas que mostraban síntomas de mosaico y manchas amarillas aisladas en invernaderos de los Países Bajos (van der Vlugt *et al.,* 2000). En ese mismo año, se encontraron también tomates afectados por el virus en Reino Unido (Wright y Mumford, 1999) y en Alemania (Leserman *et al.,* 2000). En el año 2000, se detectó el PepMV en cultivos de tomate de algunos estados de Canadá y Estados Unidos (French *et al.,* 2001), así como en diferentes regiones de España (Jordá *et al.,* 2001a). Posteriormente, se ha determinado su presencia en cultivos de tomate de diferentes países como Italia, concretamente en las islas de Cerdeña (Roggero *et al.,* 2001), y Sicilia (Davino *et al.,* 2006), Francia (Cotillon *et al.,* 2002), Austria, Bélgica, Noruega, Finlandia, Ucrania (Verhoeven *et al.,* 2003), Chile (Ramírez y Bustamante, 2001; Muñoz *et al.,* 2002), Hungría (Forray *et al.,* 2004), China (Zhang *et al.,* 2003), Perú (Soler *et al.,* 2002), Suecia, Dinamarca, Polonia, Bulgaria, Eslovaquia, Marruecos, Guatemala, Ecuador e Irlanda (EPPO, 2009).

En España, a pesar de detectarse el virus por primera vez en plantas de tomate recogidas a principios del año 2000, los síntomas de la enfermedad ya habían sido observados en campañas de años anteriores, concretamente en la Región de Murcia (Jordá *et al.,* 2000a). Actualmente el virus está extendido por las diferentes zonas productoras del país; en concreto, se ha detectado en las provincias de Albacete, Alicante, Almería, Asturias, Badajoz, Islas Baleares, Barcelona, Castellón, Granada, Guadalajara, Las Palmas, Murcia, Navarra, Pontevedra, Santa Cruz de Tenerife, Toledo y Valencia, aunque con incidencia y difusión desiguales.

3.2. Taxonomía

El PepMV pertenece al género *Potexvirus* dentro de la familia *Flexiviridae*.

3.2.1. Familia *Flexiviridae*

Esta familia está compuesta por 8 géneros definitivos y uno pendiente de ser aprobado como tal por el Comité Internacional de Taxonomía de Virus (ICTV, *Internacional Committe on Taxonomy of Viruses*), además de algunas especies no asignadas a ningún género concreto, siendo éstas las siguientes: *Banana mild mosaic virus* (BanMMV), *Cherry green ring mottle virus* (CGRMV), *Cherry necrotic rusty mottle virus* (CNRMV), *Potato virus T* (PVT), *Sugarcane striate mosaic-associate virus* (SCSMaV) y Banana virus X (BanVX) (ICTV 8th report, Fauquet *et al.,* 2005; Martelli *et al.,* 2007; Tabla 1.2). Se debe indicar que en el presente trabajo los nombres de especies virales actualmente aprobadas por la ICTV como tales se encuentran escritas en letra cursiva, al contrario de aquellas que están pendientes de ello, como es el caso de BanVX, que se han escrito con letra nomal. La base principal para establecer la pertenencia de los diferentes géneros y especies a esta familia es la fuerte

relación filogenética existente entre las proteínas estructurales y replicativas, así como distintas características en la organización genómica. Algunas de las características que comparten los miembros de la familia son (Adams *et al.,* 2004; Martelli *et al.,* 2007):

- Los viriones presentan una morfología flexuosa de unos 12–13 nm de diámetro.
- Su genoma es monopartido, de cadena simple y polaridad positiva con una cola poliadenilada en su extremo 3´.
- Alguna de sus pautas de lectura abierta (ORFs, *open reading frames*) son traducidas mediante mRNAs subgenómicos.
- Presentan de 2 a 6 ORFs, organizándose en sentido 5´-3´ de la siguiente manera: la primera ORF que es a su vez la más larga, codifica para la replicasa viral con sus dominios característicos para la metiltransferasa (M), la helicasa (H) y la RNA polimerasa RNA dependiente (RdRp); a continuación se encuentra, una o más ORFs que codifican para proteínas de movimiento (MP); y por último la ORF que codifica para la proteína de la cápside (CP) de 22 a 44 kDa.

Además del rango de hospedantes y del modo de transmisión, existen diferencias entre los géneros en cuanto a las características del virión, su estructura y su organización genómica (Tabla 1.2). Por otro lado, esta familia engloba géneros que tienen dos organizaciones distintas en la codificación de su proteína de movimiento. Algunos géneros poseen tres ORFs parcialmente solapadas que codifican para tres proteínas encargadas del movimiento célula a célula, denominádose triple bloque de genes (TGB, *triple gen block*), en cambio, también pertenecen a esta familia otros géneros que poseen una ORF que codifica para una única proteína de movimiento de aproximadamente 30 kDa (Adams *et al.,* 2004; Martelli *et al.,* 2007). Estos últimos géneros se han

identificado como pertentecientes a la "supefamilia 30K" que engloba a un gran número de virus pertenecientes a diferentes géneros y familias (Melcher, 2000). Como puede verse en la Figura 1.4, aunque la organización genómica presenta características comunes descritas anteriormente, el genoma de los diferentes géneros presenta divergencias en algunos dominios dentro de la replicasa, así como variabilidad en el número de ORFs del genoma y otras proteínas (Martelli *et al.*, 2007).

Los análisis filogenéticos de las secuencias de aminoácidos que codifican el gen de la replicasa determinan que la familia *Flexiviridae* se divide en dos grupos (*clusters*), uno que engloba los géneros *Potex*-, *Allexi*- y *Mandarivirus,* y el segundo que contiene los géneros *Carla*-, *Viti*-, *Campillo*-, *Fovea*- y *Trichovirus*. En cambio, al realizar los análisis con las secuencias pertenecientes al fragmento que codifica la CP de los diferentes miembros de la familia, se revela igualmente la existencia de dos grupos, aunque los géneros englobados en este caso difieren respecto a los de la otra zona del genoma, agrupándose por un lado *Potex*-, *Allexi*-, *Mandari*-, *Carla*-, *Fovea*- y *Citrivirus*, y por otro *Capillo*-, *Tricho*-, y *Vitivirus* (Martelli *et al.*, 2007).

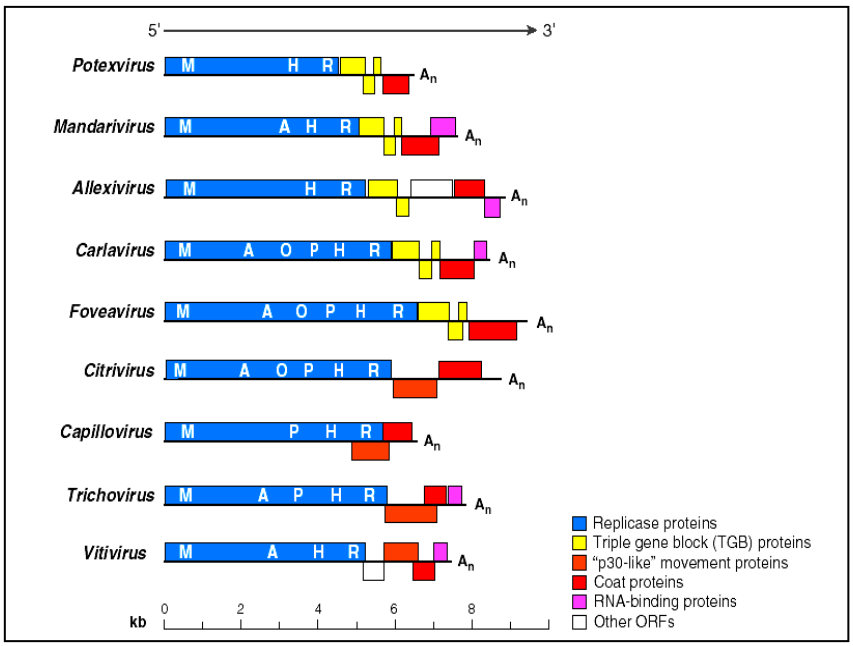


Tabla 1.2. Propiedades de los distintos géneros que constituyen la familia *Flexiviridae*.

Género	Miembro tipo	Abrevia-tura	Hospe-dantes[a]	Modo de transmisión	Longitud virión (nm)	Genoma (kb)	CP (kDa)	ORFs	Tipo MP	Rep (kDa)
Potexvirus	*Potato virus X*	PVX	PH	Mecánica por contacto	470-580	5,9-7,0	22-27	5	TGB	146-191
Mandarivirus	*Indian citrus ringspot virus*	ICRSV	PL	Injerto, material de propagación	650	7,5	36	6	TGB	187
Allexivirus	*Shallot virus X*	ShVX	PH	Material de propagación, ácaros	≈ 800	8,1-8,8	26-28	6	TGB	175-194
Carlavirus	*Carnation latent virus*	CLV	PH	Material de propagación, áfidos[b], por semilla[b], mosca blanca[b]	610-700	7,4-8,5	31-40	6	TGB	226-238
Foveavirus	*Apple stem pitting virus*	ASPV	PL	Injerto, material de propagación	723-800	8,7-9,3	28-45	5-6	TGB	244-247
Capillovirus	*Apple stem grooving virus*	ASGV	PL	Injerto, material de propagación	640-700	6,5-7,4	25-27	2-3	30 K	214-241
Vitivirus	*Grapevine virus A*	GVA	PL	Injerto, material de propagación, pseudocóccidos[b], áfidos[b]	725-285	7,4-7,6	21-23	5	30 K	195-196
Trichovirus	*Apple chlorotic leaf spot virus*	ACLSV	PL	Injero, material de propagación, transmisión por semilla[a], ácaros eriófidos[a]	640-760	7,5-8,1	21-22	3-4	30 K	216-217
Citrivirus[c]	*Citrus leaf blotch virus*	CLBV	PL	Injerto, material de propagación	960	8,7	41	3	30 K	227

Fuente: adaptada de Adams *et al.,* 2004; Martelli *et al.,* 2007.
[a]PH= plantas herbáceas, PL= Plantas leñosas.
[b]Modo de transmisión de algunas especies del género.
[c]Género pendiente de aprobación para ser incluido en la familia por el *Internacional Committee of Virus Taxonomy* (ICTV).

Figura 1.4. Organización genómica de los diferentes géneros de la familia *Flexiviridae*. M: metiltransferasa, A: dominio denominado AlkB, O: peptidasas del tipo OTU, P: proteasa tipo papain, H: RNA helicasa, R: RdRp. Los rectángulos representan las diferentes ORFs (Martelli *et al.,* 2007).

3.2.1.1. Género *Potexvirus*

El género *Potexvirus*, cuyo miembro tipo es el virus X de la patata (*Potato virus X*, PVX), incluye 47 especies, de las cuales 29 están formalmente incluidas en el género y el resto se encuentran de manera tentativa (Tabla 1.3; ICTV 8th report, Fauquet *et al.,* 2005). La gran mayoría de especies contenidas en este género se transmite fácilmente de forma mecánica y en el material de propagación como tubérculos. La transmisión por semilla es inusual, pero existe en algunas especies como *White clover mosaic virus* (WCIMV), *Cymbidium mosaic virus* (CIYMV), *Foxtail mosaic virus* (FoMV) y PepMV (Córdoba-Sellés *et al.,* 2007b). Se ha descrito la transmisión por vector en el caso de algunas especies, como mediante áfidos en el caso de Centrosema mosaic virus (CenMV) o WCIMV, y otros insectos como saltamontes o chinches del género *Nysius* en el caso de PVX o CenMV. Algunos estudios afirman que la transmisión de PVX se produce a través del hongo quitridial *Synchitrium endobioticum* (Schilbersky) Percival o mediante suelo infestado, aunque existe cierta controversia y no se conocen los mecanismos concretos (Purcifull y Edwardson, 1981).

Tal y como se indica en la Tabla 1.2, las partículas de los *Potexvirus* son flexuosas con longitudes que oscilan entre 470 y 580 nm, y su genoma consiste en un RNA de cadena simple (ssRNA) y polaridad positiva, con un peso molecular de 2,1-2,6 x 10^6, que constituye alrededor de un 5-7% del peso de la partícula. El genoma se organiza en cinco ORFs flanqueadas por dos extremos no codificantes (UTR), aunque algunas especies presentan una sexta ORF más pequeña que se encuentra localizada en el interior de la ORF5 como es el caso de CsCMV, NMV, SMYEV y WCIMV. La ORF1 se traduce a partir del RNA genómico (gRNA), sin embargo las ORFs 2, 3 y 4, y la ORF5 lo hacen a partir de RNA subgenómicos (sgRNA) (Purcifull y Edwardson, 1981; Hull, 2002).

Tabla 1.3. Lista de especies virales incluidas en el género *Potexvirus*.

Especie	Abreviatura
Alternanthera mosaic virus	AltMV
Asparagus virus 3	AV-3
Bamboo mosaic virus	BaMV
Cactus virus X	CVX
Cassava common mosaic virus	CsCMV
Cassava virus X	CsVX
Clover yellow mosaic virus	ClYMV
Commelina virus X	ComVX
Cymbidium mosaic virus	CYMMV
Daphne virus X	DVX
Foxtail mosaic virus	FoMV
Hosta virus X	HVX
Hydrangea ringspot virus	HdRSV
Lily virus X	LVX
Narcissus mosaic virus	NMV
Nerine virus X	NVX
Papaya mosaic virus	PapMV
Pepino mosaic virus	PepMV
Plantago asiatica mosaic virus	PlAMV
Plantago severe mottle virus	PlSMOV
Plantain virus X	PlVX
Potato aucuba mosaic virus	PAMV
Potato virus X	PVX
Scallion virus X	ScaVX
Strawberry mild yellow edge virus	SMYEV
Tamus red mosaic virus	TRMV
Tulip virus X	TVX
White clover mosaic virus	WClMV

Especie tentativa de ser incluida en el género[a]	Abreviatura
Artichoke curly dwarf virus	ACDV
Barley virus BI	BarV-Bl
Boletus virus X	BolVX
Centrosema mosaic virus	CenMV
Dioscorea latent virus	DLV
Lychnis symptomless virus	LycSLV
Malva veinal necrosis virus	MVNV
Nandina mosaic virus	NaMV
Negro coffee mosaic virus	NeCMV
Parsley virus 5	PaV-5
Parsnip virus 3	ParV-3
Parsnip virus 5	ParV-5
Patchouli virus X	PatVX
Rhododendron necrotic ringspot virus	RoNRSV
Rhubarb virus 1	RV-1
Smithiantha latent virus	SmiLV
Viola mottle virus	VMOV
Zygocactus symptomless virus	ZSLV

Fuente: ICTV 8th report (Fauquet *et al.*, 2005).
[a]Las especies pendientes de ser aprobadas por ICTV no están escritas en letra cursiva.

La proteína de movimiento de los virus de este género está constituida por el triple bloque de genes (TGB) que está compuesto por tres ORFs solapadas que codifican para tres proteínas distintas: TGBp1 (o TGB1), TGBp2 (o TGB2) y TGBp3 (o TGB3). Recientemente, gracias a los numerosos estudios con la especie tipo PVX a nivel molecular, se ha determinado que las regiones UTR de los extremos 5´y 3´regulan la síntesis de los RNAs genómico y subgenómicos. El TGBp1 regula la traducción del virus, así como la supresión del silenciamiento del RNA, abriendo los plasmodesmos. Por otro lado, los TGBp2 y TGBp3 son proteínas asociadas al retículo endoplásmico del virus necesarias para el movimiento viral (Verchot-Lubicz *et al.,* 2007). A pesar de que la CP no induce la apertura de los plasmodesmos como ocurre en otros virus, es necesaria para el movimiento célula a célula (Oparka *et al.,* 1996).

3.3. Estructura de la partícula viral y organización genómica

La partícula viral del PepMV es filamentosa alcanzando 508 nm de longitud y 12,5 nm de anchura, con simetría helicoidal (Figura 1.5). En el interior de los tejidos infectados se producen agregados laminares en el citoplasma, formando asimismo inclusiones que contienen partículas filamentosas bandeadas. En ocasiones, el interior de las mitocondrias de tejidos infectados presenta una matriz densa de electrones (Jones *et al.,* 1980).

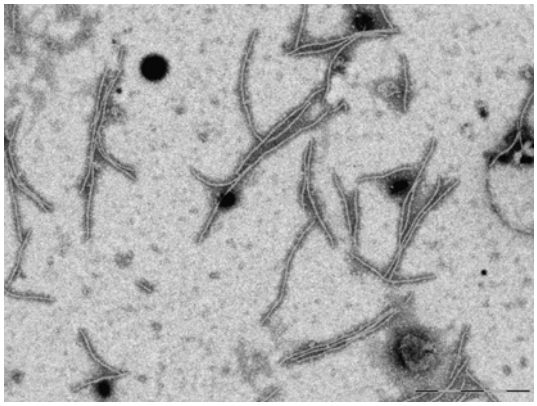


Figura 1.5. Morfología típica de las partículas de PepMV observadas mediante el microscopio electrónico de transmisión.

La organización genómica del PepMV es similar a las de otros *Potexvirus*. Se trata de un virus de RNA de cadena simple (ssRNA) y polaridad positiva. Su genoma completo se compone de aproximadamente 6.440 nucleótidos (nt), organizados en cinco ORF (Figura 1.6).

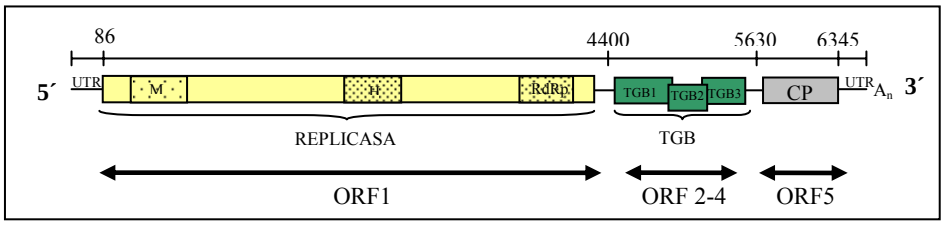


Figura 1.6. Representación esquemática del genoma del PepMV.

En su extremo 5´ se encuentra una región no codificante (*untranslated region*, UTR) de unos 85 nucleótidos, que contiene el motivo conservado 5´GAAAACAAA 3´, importante para la replicación viral o traducción de proteínas, así como para la unión con los ribosomas. Esta región también contiene una estructura metilada o CAP (m7GpppG). La ORF1, más próxima al extremo 5´, codifica una proteína de unos 164 kDa compuesta por 1.439 aminoácidos (aa) que está implicada en la replicación viral. Esta proteína contiene los tres dominios característicos de la replicasa: la metiltransferasa (M), la NPTasa/helicasa (H), que presenta el motivo característico de NTP-binding (5´GCGGSGKSVVIFDD 3´) y la RNA-polimerasa RNA-dependiente (RdRp) con su motivo conservado 5´ SGEGPTFDANT(22aa)GDD 3´. Tras una región intercistrónica de unos 25 nucleótidos (región intergénica entre la replicasa y la ORF2), se encuentran las ORF 2, 3 y 4. Estos tres genes solapados, el triple bloque de genes (TGB), codifican para tres proteínas implicadas en el movimiento del virus de célula a célula. Las ORF2 (TGBp1), ORF3 (TGBp2) y ORF 4 (TGBp3) codifican para tres proteínas de 26 kDa (234 aa), 14 kDa (123 aa) y 9 kDa (84 aa), respectivamente. En el gen del TGBp1 se observa un

dominio NTPasa/helicasa, mientras que los otros dos presentan motivos conservados típicos de los *Potexvirus*. Tras unos 38 nt que no se traducen (región intergénica entre el TGB y la ORF5), se encuentra la última región del genoma del virus, que codifica para la proteína de la cápside (CP) de 25 kDa, y en la cual se encuentra la secuencia conservada de naturaleza antifática 5´KFAAFDFFDGVT 3´, responsable posiblemente de la unión del RNA viral a la CP mediante interacciones hidrofóbicas. En su extremo 3´, tras una serie de nucleótidos que no se traducen (3´UTR), se encuentra la cola poli-A (Aguilar *et al.,* 2002; Cotillon *et al.,* 2002; Maroon-Lango *et al.,* 2005; Ling, 2007; Hasiów *et al.,* 2007).

En la región 3´UTR, se encuentra el motivo conservado 5´ ACUUAA 3´, involucrado en la síntesis de las hebras positiva y negativa del RNA. El genoma del PepMV presenta asimismo la señal de poli-adenilación (AAUAAA) en el extremo 3´ del mismo, y según el aislado del virus las últimas adeninas de esta señal pueden o no pertenecer a la cola poli-A (Cotillon *et al.,* 2002; Maroon-Lango *et al.,* 2005; Hasiów *et al.,* 2007). En un aislado francés del virus, se indica la presencia de la secuencia 5´AC/GGGGUUAAGUUUUCC(7nt)GAAA 3´, que se encuentra localizada tanto 44 nucleótidos por encima del codón de inicio de la CP, como en el extremo 5´ del TGBp1, y se supone como la secuencia promotora de la síntesis de los RNA subgenómicos (Cotillon *et al.,* 2002).

3.4. Aspectos epidemiológicos

3.4.1. Sintomatología

Como se ha indicado anteriormente el PepMV en su hospedante original, *S. muricatum*, produce un mosaico amarillo en hojas jóvenes, acompañado por un abullonado de la hoja únicamente observable en algunas plantas. En cambio,

las plantas de tomate inoculadas mecánicamente con dicho aislado resultan asintomáticas (Jones *et al.,* 1980).

Los aislados del virus descritos posteriormente afectando a tomate producen una sintomatología muy variada en las plantas infectadas (van der Vlugt *et al.,* 2000; Wright y Muford, 1999; Jordá *et al.,* 2000a). Los síntomas más característicos de la enfermedad son mosaicos en las hojas que presentan coloraciones desde verde-claro hasta verde-oscuro (Figura 1.7a, b), en ocasiones suelen ir acompañados de abullonados (Figura 1.7c, g). Algunas plantas manifiestan un mosaico dorado más o menos distribuido por toda la parte aérea (Figura 1.7d). En ocasiones, las hojas únicamente presentan manchas amarillas aisladas en mayor o menor número (Figura 1.7e). Las hojas pueden aparecer deformadas, recortándose el limbo foliar en sus bordes que le da un aspecto rizado al foliolo (Figura 1.7c, g), fenómeno que al ocurrir en la parte apical de la planta se denomina "nettle-head". Este recorte confiere en algunos casos un aspecto apuntado a los foliolos, llegando a filiformismos muy acusados (Figura 1.7f), que pueden confundirse con daños por exceso de hormonas o por infección con CMV. Asimismo se han descrito, síntomas de acorchado en los márgenes de los foliolos, causados realmente por una deficiente traslocación del calcio y un descenso rápido de la humedad de los invernaderos, pero cuyo efecto se ve agravado por la infección viral, la cual provoca una mala adaptación de las plantas infectadas a estas situaciones de estrés (Spence *et al.,* 2006). Ocasionalmente, los sépalos (Figura 1.7h) y los tallos (Figura 1.7j) presentan estrías cloróticas longitudinales, observándose también en algunos casos necrosis en los sépalos (Figura 1.7i). Sin embargo, el síntoma más preocupante se da en frutos afectados, en los que aparecen mosaicos o jaspeados en fruto maduro (Figura 1.8a) debido a una mala distribución del licopeno, o incluso presentan maduración irregular (Figura 1.8b). Estudios realizados en Reino Unido determinaron que, aunque el nivel de producción no se ve

significativamente alterado por la infección de las plantas con PepMV, existía un efecto negativo del virus sobre la calidad comercial de los frutos, que se reduce significativamente (Spence *et al.,* 2006).

A lo largo de los años, dentro de esta gran variabilidad sintomatológica, han ido apareciendo diversas manifestaciones de la infección viral, algunas de ellas más persistentes y severas. Por ejemplo, se han descrito aislados del virus que producen diversos grados de necrosis en hojas (Verhoeven *et al.,* 2003; Hasiów-Jaroszewska *et al.,* 2009b), e incluso en frutos (Figura 1.8c; Córdoba-Sellés *et al.,* 2007a). Además, paralelamente a la observación de síntomas típicos de PepMV, aparece en 1998 una nueva enfermedad denominada "colapso" del tomate, en diversas zonas productoras españolas. Las plantas afectadas presentaban un marchitamiento de la parte aérea en las horas centrales del día (Figura 1.8d), pudiendo llegar a ser irreversible provocando así la muerte de la planta. Los diversos ensayos realizados, llevaron a asociar esta manifestación con la interacción entre la infección con PepMV de la parte aérea de la planta con la presencia del hongo vector *O. brassicae sensu lato* (sl) en sus raíces, acompañada de una fluctuación en la temperatura (Córdoba *et al.,* 2004b). Hay que destacar que la especie tradicionalmente identificada como *O. brassicae* ha sido recientemente revisada, y se acuña el término *sensu lato* para referirse tanto a *O. brassicae*, especie heterotálica que afecta a las crucíferas, como a *Olpidium virulentus* (Sahtiyanci) Karting, especie homotálica que no afecta a las crucíferas, siendo esta última altamente polífaga (Koganezawa *et al.*, 2005). En el caso del tomate, sería *O. virulentus* la especie del hongo causante de la infección.

En general, la sintomatología del virus depende tanto de las condiciones ambientales, fundamentalmente luz y temperatura, como de la variedad de tomate infectado. Se ha observado que la agresividad del virus disminuye al elevarse la temperatura e incrementarse el nivel de luminosidad (Jordá *et al.,* 2000a; Fletcher, 2000; Lacasa *et al.,* 2000; Lacasa y Jordá, 2002).

Aparentemente, el momento de infección es otro factor influyente en la incidencia del daño que produce el virus, observándose que inoculaciones tardías con PepMV presentaron una mayor incidencia de síntomas en fruto, causantes de la reducción de la calidad de la cosecha, frente a infecciones tempranas de la planta (Spence *et al.,* 2006). Estos datos, obtenidos en un ensayo de invernadero, contrastan con las observaciones realizadas en invernaderos comerciales de tomate, donde el daño al cultivo parece ser mayor cuando las plantas son jóvenes, siempre y cuando no aparezca el síntoma del jaspeado en fruto (Lacasa *et al.,* 2000). Los síntomas en hojas disminuyen a medida que la planta se va desarrollando; de este modo, plantas adultas muestran con frecuencia síntomas muy suaves que pueden pasar desapercibidos (Spence *et al.,* 2006). No obstante, no cabe duda que el factor más determinante en el desarrollo de los síntomas en plantas infectadas es el propio aislado viral, aunque por el momento se desconocen la región o regiones del genoma que están involucradas en la expresión de dichos síntomas (Pagán *et al.,* 2006; Hanssen *et al.,* 2009b). En general, se ha señalado que los síntomas manifestados en plantas afectadas por virus derivan de las interacciones específicas entre el virus y los compornentes del hospedante (Culver y Padmanabhan, 2007).

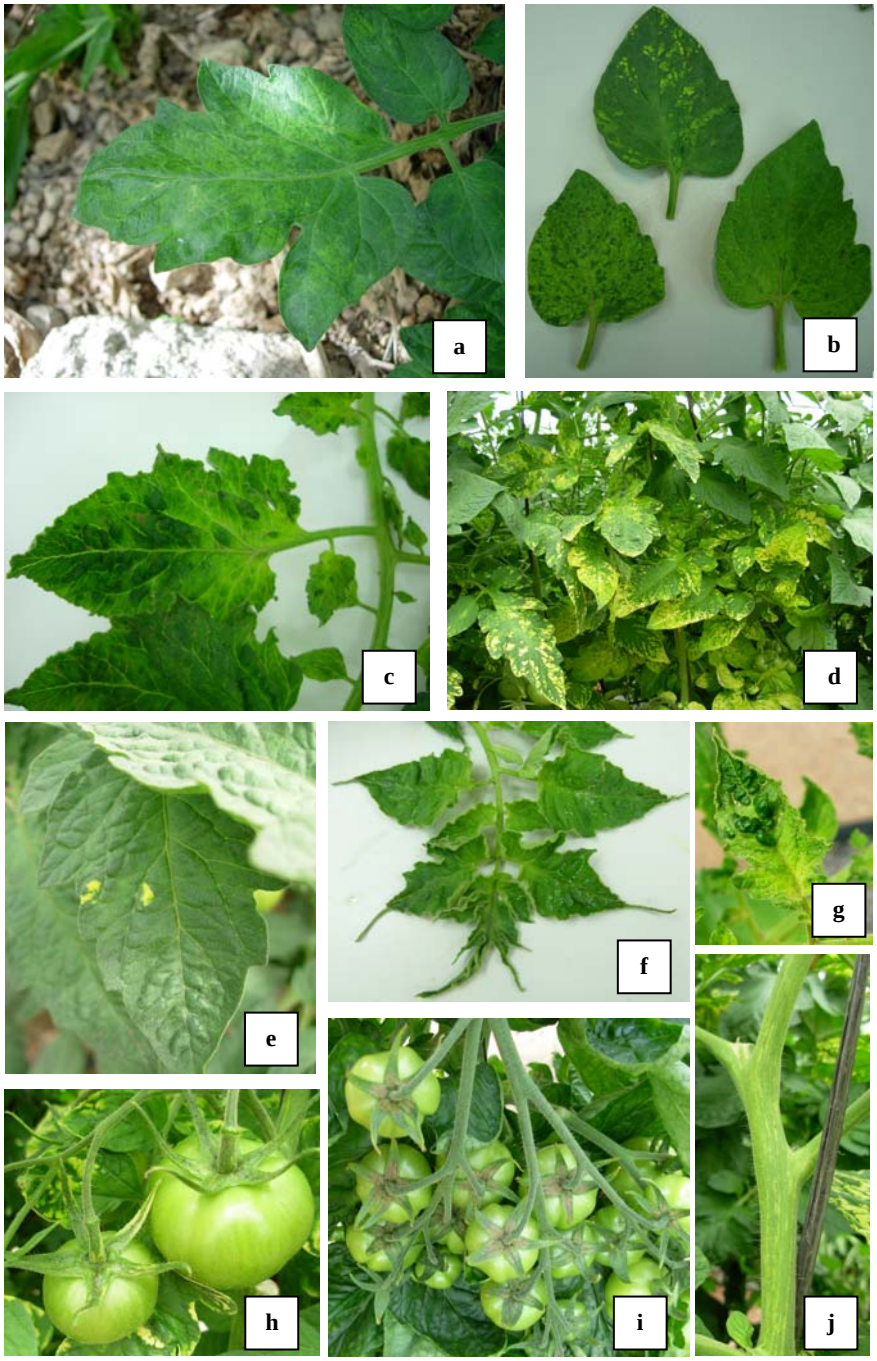


Figura 1.7. Síntomas de PepMV en plantas de tomate. Típico mosaico en hoja (**a**), que puede presentar diversas coloraciones (**b**). Abullonado y recortado de los foliolos (**c**). Mosaico dorado (**d**). Mancha amarilla aislada (**e**). Filiformismo (**f**). Apuntamiento, abullonado y rizado (**g**). Estrías cloróticas en sépalos (**h**). Manchas necróticas en sépalos (**i**). Estrías cloróticas longitudinales en tallo (**j**).

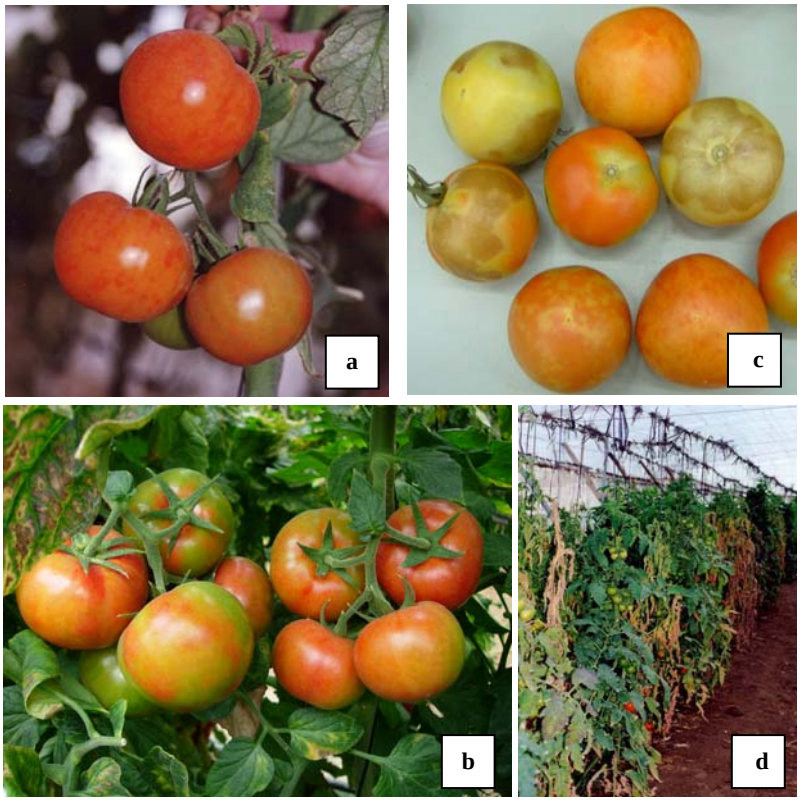


Figura 1.8. Síntomas de jaspeado (**a**), maduración irregular (**b**) y necrosis (**c**) en frutos de tomate infectados con PepMV. (**d**) Aspecto de un invernadero de tomate afectado por la enfermedad denominada "colapso" del tomate.

3.4.2. Transmisión

La principal forma de transmisión del PepMV es mecánica extendiéndose por contacto entre plantas, injerto, por el roce de manos, herramientas, bandejas o la misma ropa de los trabajadores (Wright y Mumford, 1999). De esta forma, es frecuente que la enfermedad avance en los invernaderos siguiendo el sentido del paso y trabajo de los operarios (Fletcher, 2000), llegando a contaminarse hasta seis plantas consecutivas o alternas tras una única manipulación de una planta infectada durante algunas de las labores propias del cultivo como el entutorado y el desbrotado (Lacasa *et al.,* 2001). Asimismo, se

debe tener en cuenta que el virus permanece en los restos vegetales contaminados hasta cuatro semanas después del arranque (Lacasa *et al.,* 2000).

El virus no se transmite por los insectos vectores típicos de otros virus vegetales como son el áfido *Myzus persicae* (Sulzer) o la especie de mosca blanca *Trialeurodes vaporariorum* (Westwood) (Jones *et al.,* 1980; Loomans *et al.,* 2000). En cambio, se ha demostrado la diseminación del virus a través de los abejorros polinizadores *Bombus terrestris* L., *B. canariensis* Pérez y *B. impatiens* (Cresson) (Lacasa *et al.,* 2003; Shipp *et al.,* 2008). En este modo de transmisión, la infección del virus parece producirse en las flores cuando son polinizadas por los abejorros portadores de partículas virales, y de allí se distribuye por el resto de la planta, existiendo dos posibles vías de transmisión. La primera de ellas consistiría en la infección por las partículas virales adheridas al cuerpo de los abejorros o el mismo polen infectado, que penetraría en la flor debido al daño directo que producen los abejorros al visitar las flores, ya que las agitan y muerden sus anteras provocándoles heridas. La otra posible vía de transmisión se produciría en el momento de la fertilización del óvulo con polen infectado por el virus, que habría sido transportado previamente por los abejorros (Shipp *et al.,* 2008). Aún no se ha demostrado cual de ambas es la que se produce, aunque en los estudios realizados se determinó que tanto los abejorros portadores de polen en sus patas (Lacasa *et al.,* 2003), como aquellos que no presentaban polen adherido fueron positivos al virus, aunque no se sabe si estos últimos son transmisores o no (Shipp *et al.,* 2008).

Entre los mecanismos para la diseminación del virus a larga distancia se encontrarían la transferencia de plántulas afectadas desde los semilleros hasta los agricultores, así como la recientemente demostrada transmisión por semilla (Córdoba-Sellés *et al.,* 2007b). Existen opiniones encontradas sobre este último tipo de transmisión. En 2001, Krinkels en colaboración con una empresa de semillas determina la existencia de una tasa de transmisión de entre 0,06 y

0,03% en plantas procedentes de semillas extraídas y tratadas mediante fermentación natural y posterior secado. Salomone y Roggero (2002) determinaron que no existía transmisión por semilla, al analizar 52 plántulas de tomate cv. Camone, germinadas a partir de semilla procedente de frutos infectados. No obstante, años más tarde, Cordoba-Sellés *et al.* (2007b) constataron que el porcentaje de transmisión del PepMV a plántulas crecidas a partir de semilla de fruto infectado sin ningún tratamiento previo, era de 1,84%. Esta forma de transmisión supone un grave riesgo de dispersión del virus debido a su, ya de por sí, elevada capacidad de transmisión mecánica. Posteriormente, Ling (2008) en nuevos ensayos de transmisión realizados con semillas extraídas de frutos infectados, no encontró transmisión tras el análisis de aproximadamente 10.000 plántulas. Estos últimos resultados contrastan con los obtenidos dentro del proyecto Europeo Pepeira (*Pepino mosaic virus: epidemiology, economic impact and pest risk analisis*. www.pepeira.wur.nl), donde se obtuvo una tasa de transmisión que oscilaba entre el 0,005% y el 0,057% tras el análisis de casi 90.000 plántulas procedentes de semillas extraídas de frutos infectados mediante un tratamiento ácido-enzimático (Hanssen *et al.,* 2009a). Así pues, se trata de un tema controvertido, aunque los investigadores sí coinciden en la detección reiteradas veces del virus en la semilla procedente de fruto infectado (Córdoba-Sellés *et al.,* 2007b; Ling, 2008; Hanssen *et al.,* 2009a), localizándose éste en la cubierta seminal y resultando dichas partículas virales infectivas tras reinoculación mecánica a *Nicotiana benthamiana* Domin (Ling, 2008) o a *Nicotiana occidentalis* H.-H. Wheeler (Hanssen *et al.,* 2009a).

Por último, se ha observado la capacidad del PepMV de diseminarse a través de la solución nutritiva en cultivos hidropónicos, aunque los análisis realizados no consiguieron detectar el virus en dicha solución (Fakhro *et al.,* 2005; Schwarz *et al.,* 2007).

3.4.3. Rango de hospedantes

Se han encontrado 32 especies hospedantes naturales de PepMV, pertenecientes a 14 familias botánicas diferentes (Tabla 1.4). Entre ellas, se encuentran las especies cultivadas *S. muricatum* y *S. lycopersicum*, otras especies de solanáceas silvestres descritas en el país de origen del virus (Soler *et al.,* 2002; 2005), especies de la flora arvense asociada al cultivo del tomate de distintas familias botánicas, como por ejemplo *Diplotaxis erucoides* (L.) DC, *Solanum nigrum* L. o *Sonchus oleraceus* L. recogidas en diferentes regiones de España (Jordá *et al.,* 2001b; Córdoba *et al.,* 2004a) y una planta aromática (*Ocimum basilicum* L.) procedente de invernaderos de Silicia (Davino *et al.,* 2009).

Tabla 1.4. Especies hospedantes naturales de PepMV.

Especie	**Familia**	**Referencia bibliográfica**
Amaranthus sp.	*Amaranthaceae*	Jordá *et al.,* 2001b
Bassia scoparia (L.) Voss.	*Chenopodiaceae*	Córdoba *et al.,* 2004a
Chenopodium murale L.	*Chenopodiaceae*	Córdoba *et al.,* 2004a
Calystegia sepium (L.)R.Br	*Convolvulaceae*	Córdoba *et al.,* 2004a
Convolvulus althaeoides L.	*Convolvulaceae*	Córdoba *et al.,* 2004a
C. arvensis L.	*Convolvulaceae*	Córdoba *et al.,* 2004a
Conyza albida Willd. Ex Spreng.	*Compositae*	Córdoba *et al.,* 2004a
Coronopus sp.	*Cruciferae*	Córdoba *et al.,* 2004a
Diplotaxis erucoides (L.) DC	*Brassicaceae*	Córdoba *et al.,* 2004a
Echium humile Desf.	*Boraginaceae*	Córdoba *et al.,* 2004a
E. creticum L.	*Boraginaceae*	Córdoba *et al.,* 2004a
Heliotropium europaeum L.	*Boraginaceae*	Córdoba *et al.,* 2004a
Malva parviflora L.	*Malvaceae*	Jordá *et al.,* 2001b
Moricandia arvensis (L.) DC	*Brassicaceae*	Córdoba *et al.,* 2004a
Nicotiana glauca Graham	*Solanaceae*	Jordá *et al.,* 2001b

Tabla 1.4. *(continuación)*

Especie	Familia	Referencia bibliográfica
Ocimum basilicum L.	*Lamiaceae*	Davino *et al.*, 2009
Onopordum sp.	*Asteracea*	Córdoba *et al.*, 2004a
Piptatherum multiflorum (Cav.)Beauv.	*Poaceae*	Córdoba *et al.*, 2004a
Plantago afra L.	*Plantaginaceae*	Córdoba *et al.*, 2004a
Rumex sp.	*Polygonaceae*	Córdoba *et al.*, 2004a
Solanum chilense (Dunal) Reiche	*Solanaceae*	Soler *et al.*, 2002
S. chmielewskii (C.M.Rick *et al.*) D.M.Spooner *et al.*	*Solanaceae*	Soler *et al.*, 2002
S. lycopersicum L.	*Solanaceae*	van der Vlugt *et al.*, 2000
S. muricatum Ait.	*Solanaceae*	Jones *et al.*, 1980
S. nigrum L.	*Solanaceae*	Jordá *et al.*, 2001b
S. parviflorum Cav.	*Solanaceae*	Soler *et al.*, 2002
S. peruvianum L.	*Solanaceae*	Soler *et al.*, 2002
S. pimpinellifolium L.	*Solanaceae*	Soler *et al.*, 2005
Sonchus oleraceus L.	*Compositae*	Jordá *et al.*, 2001b
S. tenerrimus L.	*Compositae*	Córdoba *et al.*, 2004a
Sysimbrium irio L.	*Brassicaceae*	Córdoba *et al.*, 2004a
Taraxacum vulgare (Lam.) Shrank.	*Compositae*	Córdoba *et al.*, 2004a

Asimismo, en diversos estudios efectuados se han determinado experimentalmente una serie de especies hospedantes artificiales del virus, todas pertenecientes a la familia *Solanaceae* (Tabla 1.5), así como una serie de plantas habitualmente empleadas como plantas indicadoras que han resultado siempre no hospedantes (Tabla 1.6). Cabe destacar que existen dos especies, *Chenopodium murale* L. (Jones *et al.*, 1980; Aguilar *et al.*, 2002) y *O. basilicum* (Salomone y Roggero, 2002; Davino *et al.*, 2009) que a pesar de resultar negativas al ser inoculadas artificialmente, han sido descritas como hospedantes naturales del virus (Tabla 1.4). Del mismo modo, se han encontrado plantas que

tras su inoculación presentaron resultados variables (infectadas o no) en función del aislado empleado, el cultivar o el experimento concreto realizado como se indica en la Tabla 1.7.

Tabla 1.5. Especies descritas como hospedantes tras su inoculación experimental con PepMV.

Especie	Familia	Referencia bibliográfica
Datura inoxia P.Mill	*Solanaceae*	Pospieszny *et al.,* 2008
D. estramonium L.	*Solanaceae*	Jones *et al.,* 1980; Jordá *et al.,* 2001a; Salomone y Rogero, 2002; van der Vlugt *et al.,* 2002; Verhoeven *et al.,* 2003
Nicotiana affinis T.Moore	*Solanaceae*	Pospieszny *et al.,* 2008
N. benthamiana Domin.	*Solanaceae*	Jordá *et al.,* 2001a; Aguilar *et al.,* 2002; Salomone y Roggero, 2002; van der Vlugt *et al.,* 2002; Davino *et al.,* 2008; Pospieszny *et al.,* 2008
N. bigelovii (Torr.) S. Wats	*Solanaceae*	Jones *et al.,* 1980; van der Vlugt *et al.,* 2002
N. clevelandii Gray	*Solanaceae*	Jordá *et al.,* 2001a; Aguilar *et al.,* 2002; Salomone y Roggero, 2002; Pospieszny *et al.,* 2008
N. debneyi Domin.	*Solanaceae*	Jones *et al.,* 1980; van der Vlugt *et al.,* 2002; Pospieszny *et al.,* 2008
N. hesperis (N.T. Burb.) P. Horton	*Solanaceae*	van der Vlugt *et al.,* 2002
N. magalopsidon (Huerck) Mueller	*Solanaceae*	Salomone y Roggero 2002
N. occidentalis H.-H. Wheeler	*Solanaceae*	Jones *et al.,* 1980; van der Vlugt *et al.,* 2002; Verhoeven *et al.,* 2003
N. tabacum L. cv. Xanthi	*Solanaceae*	Pospieszny *et al.,* 2008
Nicandra physaloides (L.) Gartn.	*Solanaceae*	Jones *et al.,* 1980; Jordá *et al.,* 2001a; Pospieszny *et al.,* 2008
Physalis peruviana L.	*Solanaceae*	Jones *et al.,* 1980
Solanum basendopogon Bitter	*Solanaceae*	Soler-Aleixandre *et al.,* 2007
S. brachycarpum Correll.	*Solanaceae*	Jones *et al.,* 1980
S. brevidens Phil.	*Solanaceae*	Jones *et al.,* 1980

Tabla 1.5. *(continuación)*

Especie	Familia	Referencia bibliográfica
S. canense Rydb.	*Solanaceae*	Soler-Aleixandre *et al.*, 2007
S. cardiophyllum Lindl.	*Solanaceae*	Jones *et al.*, 1980
S. caripense Humb. & Bonpl. ex Dunal	*Solanaceae*	Soler-Aleixandre *et al.*, 2007
S. chancayense Ochoa	*Solanaceae*	Jones *et al.*, 1980
S. cheesmaniae (L.Riley) Fosberg	*Solanaceae*	Soler-Aleixandre *et al.*, 2007
S. curtilobum Juz & Bukasor	*Solanaceae*	Jones *et al.*, 1980
S. demissum Lindl.	*Solanaceae*	Jones *et al.*, 1980
S. demissum x S.tuberosum	*Solanaceae*	Jones *et al.*, 1980
S. megistacrolobum Bitter	*Solanaceae*	Jones *et al.*, 1980
S. melongena L.	*Solanaceae*	Salomone y Roggero 2002; van der Vlugt *et al.*, 2002; Verhoeven *et al.*, 2003; Pospieszny *et al.*, 2008
S. microdontum Bitter	*Solanaceae*	Jones *et al.*, 1980
S. mochicense Ochoa	*Solanaceae*	Jones *et al.*, 1980
S. neorickii D.M.Spooner, G.J.Anderson & R.K. Jansen	*Solanaceae*	Soler-Aleixandre *et al.*, 2007
S. pennelli Correll	*Solanaceae*	Soler-Aleixandre *et al.*, 2007
S. raphanifolium Cardenas & Hawkes	*Solanaceae*	Jones *et al.*, 1980
S. stenotomum Juz & Bukasor	*Solanaceae*	Jones *et al.*, 1980
S. stoloniferum Schltdl.	*Solanaceae*	Jones *et al.*, 1980

Tabla 1.6. Especies descritas como no hospedantes tras su inoculación experimental con PepMV.

Especie	Familia	Referencia bibliográfica
Amarathus caudatus L.	*Amaranthaceae*	Jones *et al.*, 1980
A. edulis Speg.	*Amaranthaceae*	Jones *et al.*, 1980
A. retroflexus L.	*Amaranthaceae*	Salomone y Roggero, 2002
Ammi majus L.	*Apiaceae*	van der Vlugt *et al.*, 2002
Anthriscus cerefolium L.	*Umbelliferae*	Jones *et al.*, 1980
Beta vulgaris L.	*Chenopodiaceae*	Davino *et al.*, 2008
Brassica pekinensis (Lour.) Rupr.	*Cruciferae*	Jones *et al.*, 1980
Chenopodium amaranticolor Coste & A.Reyn	*Chenopodiaceae*	Jones *et al.*, 1980; Jordá *et al.*, 2001a; Aguilar *et al.*, 2002; Salomone y Roggero, 2002; van der Vlugt *et al.*, 2002
Capsicum sinense Jacq.	*Solanaceae*	Jones *et al.*, 1980
Coriandrum sativum L.	*Umbelliferae*	Jones *et al.*, 1980
Cucurbita pepo L.	*Cucurbitaceae*	Salomone y Roggero, 2002
Cyamopsis tetragonoloba (L.) Taub.	*Leguminoseae*	Jones *et al.*, 1980
Montia perfoliata (Donnex Willd.) Howell	*Portulacaceae*	Jones *et al.*, 1980
Petunia hybrida Hort. Ex E.Vilm	*Solanaceae*	Jordá *et al.*, 2001a; Aguilar *et al.*, 2002; Salomone y Rogero, 2002; van der Vlugt *et al.*, 2002; Pospieszny *et al.*, 2008
Phaseolus vulgaris L.	*Leguminoseae*	Jones *et al.*, 1980; Jordá *et al.*, 2002; van der Vlugt *et al.*, 2002; Davino *et al.*, 2008
Pisum sativum L.	*Leguminoseae*	Davino *et al.*, 2008
Solanum pseudocapsicum L.	*Solanaceae*	Soler-Aleixandre *et al.*, 2007
Tetragonia expansa Murria	*Aizoaceae*	Jones *et al.*, 1980
Vicia faba L.	*Leguminoseae*	Davino *et al.*, 2008; van der Vlugt *et al.*, 2002
Vigna unguiculata (L.) Walp.	*Leguminoseae*	Davino *et al.*, 2008

Tabla 1.7. Especies que han presentado una respuesta variable tras su inoculación mecánica artificial con PepMV en los diferentes ensayos realizados.

Especie	Familia	Referencia bibliográfica
Capsicum annuum L.	*Solanaceae*	Jorda *et al.,* 2001 (ni); Salomone y Rogero, 2002 (ni); van der Vlugt *et al.,* 2002 (ni); Verhoeven *et al.,* 2003 (v*); Davino *et al.,* 2008 (ni); Pospieszny *et al.,* 2008 (ni)
Chenopodium quinoa Willd.	*Chenopodiaceae*	Jones *et al.,* 1980 (ni); Jordá *et al.,* 2001a (ni); Salomone y Roggero, 2002 (ni); van der Vlugt *et al.,* 2002 (ni); Verhoeven *et al.,* 2003 (ni); Davino *et al.,* 2008 (ni); Pospieszny *et al.,* 2008 (l)
Cucumis sativus L.	*Cucurbitaceae*	Jones *et al.,* 1980 (l); Jordá *et al.,* 2001a (ni); Aguilar *et al.,* 2002 (ni); Salomone y Rogero, 2002 (ni); van der Vlugt *et al.,* 2002 (ni); Davino *et al.,* 2008 (l)
Datura metel L.	*Solanaceae*	Jones *et al.,* 1980 (s); van der Vlugt *et al.,* 2002 (s); Verhoeven *et al.,* 2003 (s); Davino *et al.,* 2008 (l)
Gomphrena globosa L.	*Amaranthaceae*	Jones *et al.,* 1980 (s); Jorda *et al.,* 2001 (s); Aguilar *et al.,* 2002 (s); Salomone y Roggero, 2002 (ni)
Nicotiana glutinosa L.	*Solanaceae*	Jones *et al.,* 1980 (s); Jordá *et al.,* 2001a (ni); Aguilar *et al.,* 2002 (ni); Salomone y Roggero, 2002 (ni); van der Vlugt *et al.,* 2002 (v*); Verhoeven *et al.,* 2003 (v); Pospieszny *et al.,* 2008 (s)
N. rustica L.	*Solanaceae*	Jones *et al.,* 1980; Jordá *et al.,* 2001a; (ni); Aguilar *et al.,* 2002 (ni);Salomone y Roggero, 2002 (local) van der Vlugt *et al.,* 2002
N. tabacum L. cv. Samsun	*Solanaceae*	Jones *et al.,* 1980 (s); Jordá *et al.,* 2001a; (ni); Aguilar *et al.,* 2002 (ni); Pospieszny *et al.,* 2008 (s)
N. tabacum cv. White Burley	*Solanaceae*	Jones *et al.,* 1980 (s); Jordá *et al.,* 2001a (ni); Aguilar *et al.,* 2002 (ni); Salomone y Roggero, 2002 (l); van der Vlugt *et al.,* 2002 (v*); Verhoeven *et al.,* 2003 (v); Pospieszny *et al.,* 2008 (s)
Physalis floridana Rydb.	*Solanaceae*	Jones *et al.,,* 1980 (s); Jorda *et al.,* 2001 (ni); van der Vlugt *et al.,* 2002 (v*); Verhoeven *et al.,* 2003 (v*); Pospieszny *et al.,* 2008 (v)
Solanum habrochaites S.Knapp & D.M. Spooner	*Solanaceae*	Soler-Aleixandre *et al.,* 2007 (da); Ling y Scott, 2007 (da)
S. ochranthum Dunal	*Solanaceae*	Soler-Aleixandre *et al.,* 2007 (da)
Solanum tuberosum L.	*Solanaceae*	Jones *et al.,* 1980 (v); Jordá *et al.,* 2001a (s); Salomone y Roggero, 2002 (ni); Davino *et al.,* 2008 (ni); van der Vlugt *et al.,* 2002 (v); Pospieszny *et al.,* 2008 (ni)
Vigna sinensis (L.) Hassk.	*Leguminoseae*	Jones *et al.,* 1980 (s); Jorda *et al.,* 2001 (s); Aguilar *et al.,* 2002 (ni)

da=dependiendo de la accesión de la especie inoculada; l= infección local; ni= no infectada; s= infección sistémica; v= variable en función del aislado del virus o el cultivar inoculado; v*= variable en función de si se trata del aislado original procedente de pepino dulce o de un aislado descritos en tomate.

3.5. Variabilidad genética

El PepMV es un virus con una gran variabilidad genética, del que se han caracterizado diferentes aislados a lo largo de los años, presentando una organización genómica similar, aunque se han encontrado polimorfismos significativos entre ellos (Ling *et al.,* 2007). Actualmente se diferencian cinco genotipos diferentes del virus: Peruano (PE), Europeo (EU), Chileno 2 (CH2), CH1/US1 y US2 (Hanssen *et al.,* 2008), que se agrupan en cuatro cepas (*strains*) diferentes PE, EU, CH2 (en la cual se engloba el genotipo US2) y CH1/US1 (Ling, 2007). Esta diferenciación entre genotipos y cepas radica en que la variación de las secuencias de los aislados CH2 y US2 se concentra únicamente en la parte central del genoma, concretamente en las regiones de TGB1 y los dominios de la helicasa y la RdRp (2307 al 4738 nt del CH2), donde únicamente presentan el 80% de identidad. En cambio, en el resto de la secuencia nucleotídica tienen una identidad del 98% (Ling *et al.,* 2008). Esta diferenciación se observa claramente en la Figura 1.9, donde en la región de la replicasa y el TGBp1 se diferencian claramente cinco grupos, en cambio en el resto de las regiones el US2 se agrupa con los aislados del tipo CH2, obteniéndose únicamente cuatro grupos. Actualmente, se afirma que el aislado US2 es un recombinante entre los genotipos CH1/US1 y CH2 (Hanssen *et al.,* 2009a)

En la Tabla 1.8 se recoge información de las secuencias completas del virus publicadas en la base de datos del GenBank (www.ncbi.nlm.nih.gov). Como puede apreciarse, la organización genómica es la misma para todos los aislados pero presentan diferencias en cuanto al número de nucleótidos en algunas zonas, fundamentalmente en los extremos 5´y 3´ UTR (López *et al.,* 2005) y a otras características como, por ejemplo, si el extremo 3´ de la señal de poliadenilación (5´AAUAAA 3´), se encuentra formando o no parte de la cola poli-A (Maroon-Lango *et al.,* 2005; Hasiów *et al.,* 2007). Sin embargo, las

diferencias más significativas se encuentran a nivel de identidad y similitud de secuencias, describiéndose como zona variable para la diferenciación entre genotipos el TGBp1 y TGBp3, frente a la replicasa y la CP que son más conservadas (Maroon-Lango *et al.,* 2005). Hasiów *et al.* (2007) apuntaron como zona de mayor variabilidad entre los aislados pertencientes a la cepa CH2 tanto el extremo 5´UTR como la zona comprendida entre la segunda mitad de la replicasa (ORF1) y la primera mitad del TGBp1 (ORF2).

Tal y como se ha señalado anteriormente, desde la primera detección del PepMV en pepino dulce hasta la detección del virus en tomate pasaron unos 25 años, si bien cabe destacar que ha sido en los últimos diez años cuando se ha incrementado la expansión del virus y ha variado en mayor medida su población genética. En un primer momento, la detección del virus en este nuevo hospedante (tomate) condujo al estudio comparativo de fragmentos del genoma del virus del nuevo aislado detectado con el original encontrado en pepino dulce. A raíz de dichos estudios, y debido a las diferencias biológicas y moleculares observadas, distintos autores consideraron que el aislado de tomate constituía una nueva cepa (*strain*) del virus que se propuso denominar como cepa tipo tomate frente a la original denominada tipo pepino o peruana (van der Vlugt *et al.,* 2002). Por tanto, inicialmente solo había dos cepas descritas: la cepa tipo tomate (posteriormente denominada europea) y la cepa tipo pepino o peruana.

Tabla 1.8. Características de las secuencias completas de PepMV[a].

Nº de accesión	Sec. (nt)	5´UTR (nt)	Replicase (nt)	TGB1 (nt)	TGB2 (nt)	TGB3 (nt)	CP (nt)	3´UTR (nt)	Referencias bibliográficas	Nombre del aislado	Genotipo
AM109896	6468	1-84	85-4404	4430-5134	5115-5486	5338-5598	5631-6344	6345-6408	Pagán *et al.*, 2006	SM74	PE
FJ940223	6420	1-86	87-4406	4432-5136	5117-5488	5340-5594	5633-6346	6347-6410	van der Vlugt *et al.*, 2002	EU-tomato	EU
AF484251	6450	1-86	87-4406	4432-5136	5117-5488	5340-5594	5633-6346	6347-6410	Aguilar *et al.*, 2002	Sp-13	EU
AJ438767	6410	1-86	87-4406	4432-5136	5117-5488	5340-5594	5633-6346	6347-6410	Cotillon *et al.*, 2002		EU
AM491606	6449	1-86	87-4406	4432-5136	5117-5488	5340-5594	5632-6345	6346-6412	Forray *et al*., 2004	PepMV-H	EU
AJ606361	6448	1-84	85-4404	4430-5134	5115-5486	5338-5598	5631-6344	6345-6408	López *et al.*, 2005	LP2001	PE
AJ606360	6449	1-86	87-4406	4432-5136	5117-5488	5340-5594	5633-6345	6346-6409	López *et al.*, 2005	LE-2002	EU
AJ606359	6450	1-86	87-4406	4432-5136	5117-5488	5340-5594	5633-6346	6347-6410	López *et al.*, 2005	LE-2000	EU
AY509927	6410	1-84	85-4401	4426-5130	5111-5482	5334-5582	5628-6341	6342-6410	Maroon-Lango *et al.*, 2005	US2	US2
AY509926	6413	1-85	86-4405	4430-5134	5115-5486	5338-5586	5633-6346	6347-6413	Maroon-Lango *et al.*, 2005	US1	US1
DQ000984	6414	1-85	85-4404	4429-5133	5114-5485	5337-5585	5632-6345	6346-6414	Ling., 2007	CH1	US1
DQ000985	6412	1-85	86-4402	4428-5132	5113-5484	5336-5584	5630-6343	6344-6412	Ling., 2007	CH2	CH2
EF408821	6412	1-85	86-4402	4428-5132	5113-5484	5336-5584	5630-6343	6344-6412	Hasiów *et al.*, 2007	PepMV-Pk	CH2
FJ612601	6412	1-85	86-4402	4428-5132	5113-5484	5336-5584	5630-6343	6344-6412	Hasiów-Jaroszewska *et al.*, 2009b	PepMV-Pa	CH2
FJ940225	6441	1-85	86-4405	4430-5134	5115-5486	5338-5586	5633-6346	6347-6415	van der Vlugt *et al.*, 2009 (DS)	US1	US1
FJ940224	6414	1-86	87-4406	4432-5136	5117-5488	5340-5594	5632-6345	6346-6409	van der Vlugt *et al.*, 2009 (DS)	DB1	CH2
FJ212288	6440	1-84	85-4401	4427-5131	5112-5483	5335-5583	5629-6342	6343-6440	Adams *et al*., 2009		CH2

DS= Enviado directamente (direct submission).

[a]Fuente: Datos obtenidos tras la consulta de la base de datos del GenBank (www.ncbi.nlm.nih.gov) en Agosto de 2009.

Todos los estudios comparativos de los aislados del tipo tomate detectados en diferentes países europeos, coinciden en la alta identidad a nivel de secuencia nucleotídica (<99%) entre ellos, reduciéndose al ser comparados con secuencias del aislado original (aproximadamente 95%). Las diferencias encontradas a nivel de aminoácidos fueron menores (Cotillon *et al.,* 2002; Aguilar *et al.,* 2002; Verhoeven *et al.,* 2003). Sin embargo, Martínez-Culebras *et al.* (2002) detectaron en España cinco aislados que presentaban una secuencia nucleotídica (correspondiente a un fragmento de la RdRp) diferente a la de las otras dos cepas (tipos tomate y pepino) conocidas hasta ese momento (79-80% identidad). Estos aislados presentaban este "nuevo tipo" de PepMV en infección simple e incluso en infección mixta con la cepa tipo tomate. En 2005, se describieron dos aislados en Estados Unidos denominados US1 y US2, que únicamente mostraban un 78,6-82,2% de homología de secuencia de nucleótidos con los aislados de las cepas tipo tomate y peruana. Se sugiere la posibilidad de que los aislados encontrados pertenecientes al "nuevo tipo" de PepMV en España descritos por Martínez-Culebras *et al.* (2002) podrían pertenecer al grupo US2 (Maroon-Lango *et al.,* 2005). A partir de ese momento, pareció generalizarse la denominación de cepa europea (EU) para referirse a la descrita como tipo tomate, así como peruana (PE) para el tipo pepino. Posteriormente, aparecieron dos aislados identificados como aislados Chilenos (CH1 y CH2), ya que procedían de semillas importadas a Estados Unidos desde ese país. Estos aislados presentaban 99,3% y 90,9% de identidad a nivel de secuencia nucleotídica con los aislados US1 y US2, respectivamente y, entre un 78,4% a un 82,2% con los aislados EU y PE. Con estos datos, se sugirió la existencia de cuatro cepas claramente diferenciadas: EU, PE, CH1/US1 y CH2 (Ling, 2007). La elevada homología encontrada entre los aislados CH1 y US1, hace que constituyan una cepa denominada CH1/US1. Desde entonces, se describe la presencia de nuevos aislados en diferentes países europeos, y además comienzan

a realizarse estudios para determinar la población genética del virus. Los aislados de PepMV detectados en plantas de tomate de diversos invernaderos sicilianos en 2005, así como las plantas afectadas de albahaca recogidas de estos mismos invernaderos 3 años después, resultaron pertenecientes a la cepa CH2 (Davino *et al.,* 2008). En Polonia, se describió un nuevo aislado (PepMV-Pk) que pertenece a la cepa CH2 debido a la gran identidad nucleotídica de su secuencia (98,3%), con los otros aislados de esta cepa, aunque contenía algunas mutaciones no sinónimas que podrían indicar la selección de las mejores variantes genéticas del virus para sobrevivir en un determinado hospedante (Hasiów *et al.,* 2007). Sorprendentemente, este aislado polaco no produjo síntomas en las hojas de tomate o éstos fueron extremadamente suaves, aunque causó jaspeados suaves en fruto (Pospieszny *et al.,* 2008). Por otro lado, se detectó en las Islas Canarias un aislado perteneciente al genotipo CH1/US1, descrita por primera vez en un emplazamiento diferente al original (Alfaro-Fernández *et al.,* 2008a).

Diversos estudios de distribución genética de poblaciones de PepMV han determinado la presencia de diversos genotipos, encontrándose mayoritariamente distribuidos el EU y el CH2. En España, el genotipo presente en mayor proporción en las muestras analizadas, recogidas en distintas zonas geográficas del país entre 2001 y 2004 fue el EU, encontrándose en el 80% de ellas. Con menor frecuencia relativa, se encontraron el PE y el US2 (actualmente denominado CH2), presentándose habitualmente en infecciones mixtas con aislados EU, aunque los aislados estudiados solo se agrupaban en estos dos genotipos en dos zonas del genoma, el TGB en el caso de los aislados del tipo PE y la CP en los aislados del tipo US2. Asimismo, se detectó la presencia de dos aislados recombinantes entre EU y US2, intercambiando fragmentos del TGB y CP (Pagán *et al.,* 2006). En estudios similares realizados en Bélgica, se caracterizó la convivencia de aislados del genotipo EU y CH2, y su coinfección

en muchas de las plantas. En los invernaderos estudiados se determinó que los aislados CH2, genotipo predominante, se dispersaban más rápidamente que los EU, presentando, por tanto, una ventaja biológica. En este caso, también se detectó la presencia de aislados recombinantes unidireccionales, presentando el extremo 5´ una secuencia similar al genotipo CH2 y el extremo 3´ al EU. Estos recombinantes fueron lo suficientemente estables para transmitirse de una planta a otra (Hanssen *et al.,* 2008).

En América del Norte, contrariamente a lo que podría esperarse tras la caracterización por Maroon-Lango *et al.* (2005) de los aislados US1 y US2 en Estados Unidos, los estudios de población genética del virus revelaron la presencia mayoritaria de aislados del genotipo EU, aunque también se detectaron US1 y US2 en Arizona y Colorado, y CH2 en Texas, siempre en infección mixta con el EU. Las muestras analizadas procedentes de Canadá contenían únicamente aislados del genotipo EU de PepMV (Ling *et al.,* 2008). En resumen, los genotipos de PepMV más ampliamente distribuidos en Europa son el EU y el CH2 (Pagán *et al.,* 2006; Hanssen *et al.,* 2008; Hasiów *et al.,* 2007). Sin embargo en América del Norte, a pesar de haberse descrito los genotipos US1, US2 y CH2, el más extendido es el genotipo EU (Ling *et al.,* 2008).

Con respecto a la asociación entre la variabilidad genética y sintomatológica, no se conoce en la actualidad qué zona del genoma la induce o cuáles son los nucleótidos y aminoácidos determinantes responsables de las características biológicas de los aislados (Maroon-Lango *et al.,* 2005; Pagán *et al.,* 2006; Hanssen *et al.,* 2008; Hasiów-Jarosweska *et al.,* 2009a). Sin embargo, se ha demostrado que la coinfección en algunas plantas de los genotipos EU y CH2 genera una mayor agresividad en los síntomas de PepMV observados (Hanssen *et al.,* 2008), lo que se asocia a una mayor acumulación viral frente a las infecciones simples (Hanssen *et al.,* 2009b).

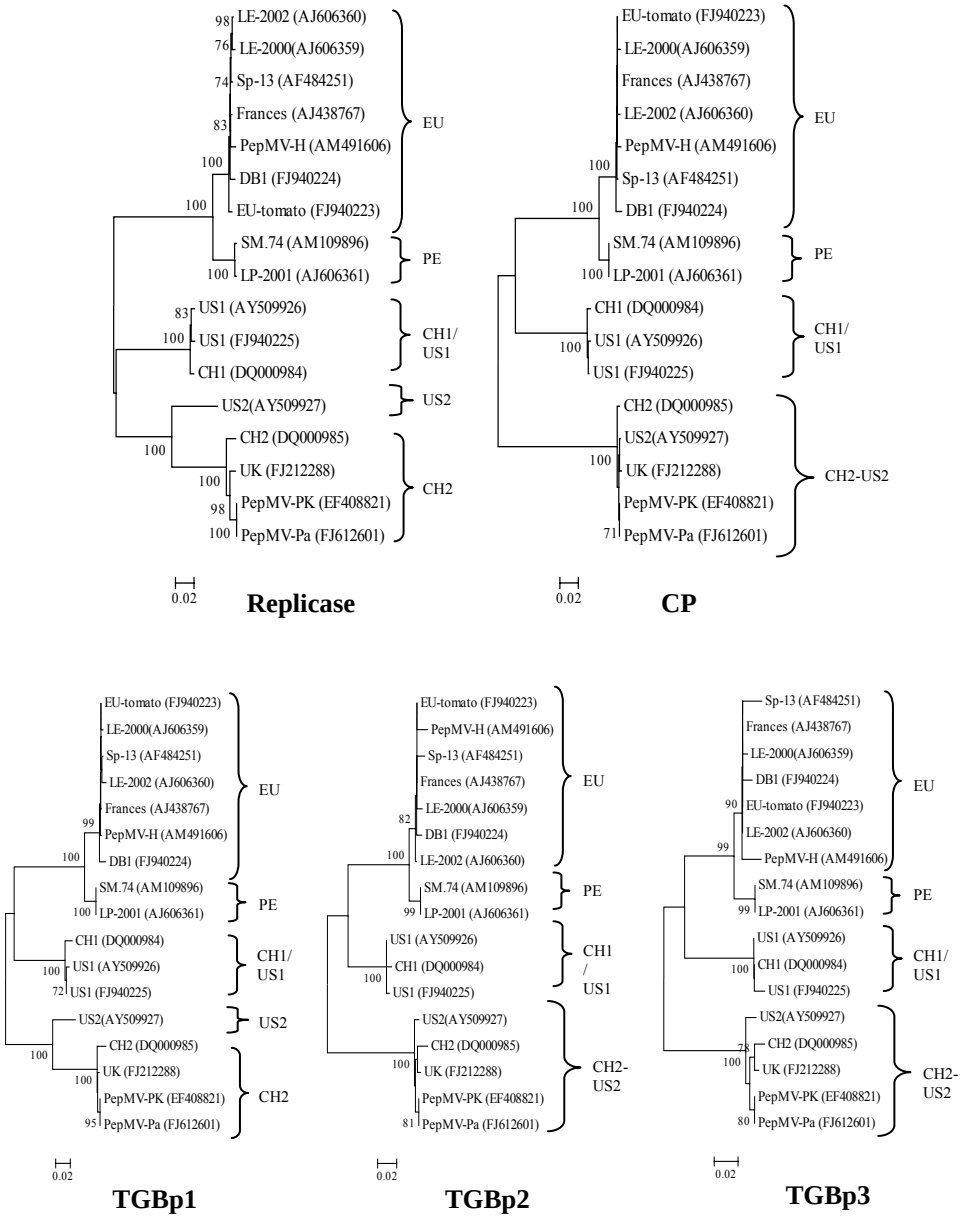


Figura 1.9. Árboles filogenéticos basados en los alineamientos de las secuencias de aminoácidos de las cinco regiones codificantes del PepMV calculados según el método de unión de vecinos (*neighbour joinng*, NJ) implementados con 1000 pseudo-réplicas a través del programa informático Mega 3.1. Las secuencias proceden de la base de datos de GenBank (www.ncbi.nlm.nih.gov).

Actualmente, se acepta la hipótesis de que únicamente pequeñas diferencias a nivel nucleotídico pueden producir grandes diferencias biológicas entre aislados (Hanssen *et al.,* 2009b), pudiendo presentar incluso efectos dramáticos en la virulencia de los aislados que causan severos cambios fenotípicos como es el caso de los aislados necróticos, que inducen necrosis en tomate y que han sido caracterizados como pertenecientes al genotipo CH2 (Hasiów-Jarosweska *et al.,* 2009b) o EU (Verhoeven *et al.,* 2003). Se han determinado por el momento diversos polimorfismos entre aislados, a pesar que aún no se sabe si tienen algún papel en la expresión de los síntomas (Hanssen *et al.,* 2009b; Hasiów-Jarosweska *et al.,* 2009b). De esta manera, la sintomatología sería atribuida, al menos parcialmente, al aislado presente en la planta (Pagán *et al.,* 2006; Hanssen *et al.,* 2009b). Para elucidar cuáles son los factores responsables a nivel de secuencia de los aislados de dicha variabilidad biológica, algunos autores propusieron la creación de clones infecciosos con la secuencia completa del virus (Maroon-Lango *et al.,* 2005). Se debe señalar que se ha conseguido recientemente la construcción de un clon de cDNA de un aislado necrótico de PepMV perteneciente al genotipo CH2, cuyo RNA trascrito resultó infeccioso, y produjo la misma sintomatología y presentabó la misma secuencia de nt que el aislado original (Hasiów-Jarosweska *et al.,* 2009a).

3.6. Métodos de diagnóstico

3.6.1. Observación de síntomas en campo e inoculación de plantas indicadoras

Como se ha descrito anteriormente el PepMV es un virus que presenta una gran variabilidad sintomatológica y algunos de sus síntomas pueden ser confundidos con los causados por otros factores. A pesar de ello, la observación de síntomas proporciona una idea preliminar de la posible presencia del virus en las plantas afectadas, aunque no resulte fiable, ya que no todas las plantas

infectadas manifiestan síntomas de la enfermedad. Tradicionalmente se ha empleado la inoculación y observación de síntomas en plantas indicadoras para el diagnóstico de virus (Hull, 2002). En el caso del PepMV, se describieron en un primer momento como buenas plantas indicadoras *Nicotiana glutinosa* L., *Nicotiana debneyi* Domin., *Datura metel* L. y *Datura stramonium* L. (Jones *et al.,* 1980). No obstante, hay que tener en cuenta que, estas especies fueron inoculadas con el aislado original de pepino dulce (PE), y como se demostró posteriormente, aislados pertenecientes a otras cepas del virus o incluso dentro de la misma cepa pueden producir síntomas diferentes en algunas de las plantas indicadoras (van der Vlugt *et al.,* 2002; Verhoeven *et al.,* 2003). Asimismo se ha descrito *Datura inoxia* P. Mill. como especie indicadora para diferenciar entre aislados necróticos de no necróticos dentro del genotipo CH2 (Hasiów-Jarosweska *et al.,* 2009b). En este punto, se debe remarcar la idea anteriormente comentada de la ausencia de información en cuanto a la asociación entre la variabilidad sintomatológica y genética de los diferentes aislados del virus.

3.6.2. Observación al microcopio electrónico de transmisión

Para un primer diagnóstico, se ha empleado la microscopia electrónica para la observación de las partículas virales, determinando por su morfología y tamaño medio aproximado su pertenencia al grupo de los *Potexvirus.* Además, se puede realizar el estudio histopatológico del virus en las hojas procedentes de plantas infectadas tras su inclusión en resinas, corte con microtomo y observación al microscopio electrónico de transmisión, determinándose con facilidad los cuerpos de inclusión formados por masas de partículas virales en el citoplasma de células parenquimáticas del mesófilo (Jordá *et al.,* 2000b).

3.6.3. Serología

La técnica serológica ELISA (enzyme-linked inmunosorbent assay) es el método más comúnmente empleado para el diagnóstico rutinario de virus vegetales, ya que permite el análisis de gran número de muestras con gran fiabilidad a un coste económico reducido (Uga y Tsuda, 2005). En un primer momento, y dada la relación serológica del PepMV con el NMV (Jones *et al.,* 1980), los primeros diagnósticos se realizaban con el antisuero específico de este último, obteniéndose gran cantidad de falsos negativos. Posteriormente, con la aparición en el mercado de los antisueros específicos de PepMV, se observó la gran resolución de éstos frente al heterólogo NMV, existiendo aún así diferencias en la sensibilidad entre los específicos de distintas casas comerciales (Jordá *et al.,* 2000b). Asimismo, en los primeros estudios se empleó la técnica de la inmunoimpresión descrita por Lin *et al.* (1990) para diversos virus, que consiste en la impresión del tejido sobre membrana de nitrocelulosa, bloqueo con proteína, y reconocimiento con la γ-globulina del antisuero del virus (Jordá *et al.,* 2000b). Actualmente existen en el mercado gran número de antisueros específicos del PepMV de diversas casas comerciales, que son los comúnmente empleados para el diagnóstico del virus.

Por otro lado, se ha utilizado también la técnica de inmunomicroscopía electrónica (inmunoelectron microscopy, ISEM) con antisuero específico del aislado original de PepMV. Esta técnica permite la observación clara de la partícula viral, confirmación de la identidad serológica del virus e incluso cuantificación del nivel de virus marcado (Hyatt y Eaton, 1993; Hull, 2002). Esta técnica fue empleada en los primeros diagnósticos del virus, determinándose la presencia de aislados de la cepa EU de PepMV, aunque la concentración de virus detectado fue menor que la obtenida con el aislado original del virus (French *et al.,* 2001; Roggero *et al.,* 2001). Sin embargo, el estudio comparativo de los antisueros realizados a partir del aislado original de

pepino y el de tomate, determinó que los aislados analizados (pertenecientes a las cepas EU y PE) eran serológicamente indistinguibles (van der Vlugt *et al.*, 2002).

3.6.4. Técnicas moleculares

3.6.4.1. Técnica RT-PCR y secuenciación

Otra de las técnicas frecuentemente empleadas para el diagnóstico de PepMV es la transcripción reversa de la reacción en cadena de la polimerasa (RT-PCR, *reverse-transcription polymerase chain reaction*). Esta metodología presenta como gran ventaja su elevada sensibilidad unida a la gran especificidad que se consigue con el diseño de cebadores (oligonucleótidos o *primers*) complementarios a un fragmento del DNA molde y del que se amplificarán múltiples copias en la reacción empleando una DNA polimerasa termo estable (Hull, 2002). A lo largo de los años, se han publicado diversas secuencias de cebadores degenerados diseñados en zonas conservadas comunes a diferentes virus dentro del género *Potexvirus* (Gibbs *et al.,* 1998; van der Vlugt y Berendsen, 2002), así como diversas secuencias de cebadores específicos de PepMV, que hibridan con fragmentos del genoma de algún aislado o cepa concreta de PepMV, o son generales de la especie (Mumford y Metcalfe, 2001; French *et al.,* 2001; Martínez-Culebras *et al.,* 2002; Aguilar *et al.,* 2002; Cotillon *et al.,* 2002; López *et al.,* 2005; Pagán *et al.,* 2006; Ling, 2007; Alfaro-Fernández *et al.,* 2008a; Ling *et al.,* 2008; Hanssen *et al.,* 2009b).

Previamente a la publicación de secuencias de PepMV y a la disponibilidad de un antisuero comercial, se publicó un método de inmunocaptura con RT-PCR para la detección del virus, que combinaba el uso de un antisuero general para las dobles cadenas de RNA (dsRNA), seguido de una RT-PCR con cebadores degenerados para *Potexvirus* (Mansilla *et al.,* 2003).

La aparición de mayor información acerca de la secuencia del virus, así como la comercialización de antisueros, hizo que otras técnicas más rutinarias desbancaran dicha metodología.

Para conocer la secuencia concreta del virus contenido en la muestra analizada, el producto amplificado durante la RT-PCR debe ser secuenciado, consiguiendo así, mediante comparación con las secuencias contenidas en las bases de datos, conocer la identidad y similitud del fragmento amplificado de dicho aislado. En el caso del PepMV existen numerosas secuencias publicadas en la base de datos del NCBI, aunque de ellas únicamente 17 presentan la secuencia del genoma completo (anteriormente descritos en la Tabla 1.7).

3.6.4.2. Técnica RT-PCR y diferenciación mediante RFLP

La técnica del análisis de polimorfismos en la longitud de los fragmentos de restricción o RFLP (*Restriction Fragment Lenght Polimorsphism*), se basa en la capacidad de los enzimas o endonucleasas de restricción de reconocer y cortar secuencias específicas en el DNA, que pueden variar entre individuos (polimorfismos). Primeramente se debe amplificar el fragmento del genoma a estudiar mediante RT-PCR con cebadores específicos, y posteriormente se realiza la digestión del fragmento con los enzimas de restricción necesarios para su diferenciación. Esta técnica es muy útil, entre otras aplicaciones, para la diferenciación de aislados o cepas dentro de una especie, como se determinó para el PepMV (Martínez-Culebras *et al.,* 2002; Hanssen *et al.,* 2008). La primera vez que se diseñó una técnica de RT-PCR con cebadores específicos seguida del análisis por RFLP, se determinó la existencia de tres tipos de aislados de PepMV diferentes (P1, P2 y P3), que presentaban un patrón diferencial tras ser digerido el producto amplificado correspondiente a un fragmento de 375 pb de la replicasa con el enzima *Sau*3A. Estos tipos parecían corresponder a la cepa EU, PE y otra no descrita hasta el momento que

presentaba grandes diferencias respecto a las otras dos (Martínez-Culebras *et al.,* 2002). Al describirse posteriormente nuevos aislados, se indicó que probablemente este tipo P3 correspondiera al grupo de aislados agrupados con el genotipo US2 (Maroon-Lango *et al.,* 2005). Recientemente, y debido a la aparición de estos nuevos aislados y la clasificación de éstos en cinco genotipos, esta técnica no fue capaz de detectar alguno de ellos, lo que propició el diseño de una nueva técnica RT-PCR-RFLP. Este método consistía en la diferenciación del genotipo de PepMV presente en la muestra de entre los cinco descritos (EU, PE, CH2, CH1/US1 y US2), gracias a la digestión con diferentes enzimas de restricción de dos fragmentos de diferentes zonas del genoma del virus, previamente amplificados. Uno de ellos correspondía a 625 bp de la RdRp, que se digería posteriormente con *Eco*RI y *Bgl*II, diferenciándose así únicamente el genotipo CH2, ya que no diferenciaba entre los genotipos EU y PE, ni entre US2 y CH1/US1 que generaban patrones de bandas similares. El otro fragmento amplificado correspondía a la CP (845 bp) y se digería con *Hind*III, *Nde*I, *Pvu*II y *Sac*I, diferenciándose una vez comparados los resultados de todos los cortes los cinco genotipos existentes (Hanssen *et al.,* 2008).

3.6.4.3. Técnica RT-PCR en tiempo real

La PCR en tiempo real o real-time PCR es una técnica basada en la PCR convencional que permite la cuantificación del DNA amplificado, ya que este es proporcional a la cantidad de fluorescencia emitida en cada ciclo de PCR. La mayor sensibilidad, la capacidad de cuantificación de la cantidad de DNA (o RNA) y la eliminación de la manipulación de los productos post-PCR suponen grandes ventajas frente a la PCR convencional. Para la detección de PepMV se han desarrollado dos variantes de la RT-PCR en tiempo real: sistema Taqman™ que emplea una sonda molecular específica de PepMV (Ling *et al.,* 2007) y el sistema SYBR Green que emplea fluorocromos no específicos (Hasiów *et al.,*

2008). En el primer caso, previamente a la reacción se realizó la inmunocaptura de los viriones del virus con un antisuero específico, quedando éstos absorbidos en los tubos o en la placa, permitiendo así realizar el ensayo completo en un único tubo. Posteriormente, se empleó una sonda específica que hibridaba con todos los genotipos de PepMV en la zona del TGB2 marcada con un fluorocromo en su extremo 5´, y con otro fluorocromo "quencher" (inhibidor de la fluorescencia) en su extremo 3´; incluía dos parejas de cebadores específicos de esa misma zona del genoma para la detección de los diferentes genotipos de PepMV, (una para los genotipos CH2 y US2, y otra para el resto). La técnica presentó una gran sensibilidad, pudiendo cubrir a priori el diagnóstico de las diferentes cepas descritas, si bien la sonda presentaba un nucleótido de divergencia con el genotipo PE, y debido a que los autores no disponían de ninguna muestra infectada con ese genotipo concreto no pudieron evaluar la eficiencia de la técnica frente a éste (Ling *et al.,* 2007). Este sistema Taqman™ de RT-PCR en tiempo real con una sonda y cebadores específicos de PepMV (de la zona de la polimerasa en este caso), fue empleado con éxito para la detección del virus en abejorros polinizadores (Shipp *et al.,* 2008). En el otro sistema descrito, únicamente se requiere de los cebadores específicos del virus, diseñados en este estudio concreto para amplificar una zona de la CP conservada para todos los aislados, ya que la fluorescencia se emite debido a la adición del fluorocromo SYBR Green que se acopla en la secuencia de DNA. Esta técnica se empleó para detectar con éxito los aislados polacos pertenecientes a los genotipos CH2 y EU (Hasiów *et al.,* 2008).

3.7. Control

Debido a la facilidad de diseminación del virus de manera mecánica, es importante adoptar una serie de medidas para evitar la introducción y propagación del virus en el cultivo. Es imprescindible el cumplimiento de una

serie de medidas higiénicas para evitar su diseminación, tales como cambios periódicos de ropa por parte de los operarios que manipulan las plantas. Algunos autores recomiendan la desinfección de herramientas con diferentes productos como leche descremada con un contenido en proteínas de 3,5% (Fletcher, 2000) o desinfectantes que contengan amonios cuaternarios y aldehídos glutáricos (Lacasa *et al.,* 2001). Una vez detectada la infección y para evitar que el virus se propague, se debe eliminar cuidadosamente tanto la planta afectada como aquellas que se encuentren en contacto con ella, y destruir los restos infectados. Las estructuras de los invernaderos, material empleado durante el cultivo y superficies deben desinfectarse bien tras su utilización, así como el suelo (Fletcher, 2000; Lacasa *et al.,* 2000; Jones y Lammers, 2005).

El PepMV tiene el estatus de patógeno de cuarentena en semilla dentro de la UE desde 2001 y existen diversas regulaciones para prevenir su introducción y posterior dispersión en los países miembros. La Comisión Europea decretó medidas (normativa 2000/325/EC, 2001/536/EC y 2004/200/EC) para el control y entrada en Europa de semillas de tomate infectadas con PepMV, exigiendo la inspección y análisis de las semillas procedentes de terceros países, así como la extracción de semillas mediante un tratamiento ácido. Además, estas medidas exigen a los países miembros la inspección y análisis periódicos de plántulas en los semilleros, así como en instalaciones productoras de semillas. Como ya se ha comentado, se ha demostrado recientemente la transmisión por semilla del virus, estableciéndose asimismo como el tratamiento más eficaz para la desinfección de ésta la inmersión de las semillas durante tres horas en una solución de fosfato trisódico al 10% (Córdoba-Sellés *et al.,* 2007b).

En relación con la mejora genética tradicional, aún no existen variedades resistentes al virus, aunque se han detectado posibles fuentes de resistencia en algunas accesiones de especies del género *Solanum*, como son *Solanum chilense*

(Dunal) Reiche o *Solanum peruvianum* L. (Soler-Aleixandre *et al.,* 2007) y *Solanum habrochaites* S. Knapp & D.M. Spooner (Ling y Scott, 2007).

En ciertos lugares como los Países Bajos, algunos agricultores inoculan deliberadamente con PepMV las plantas de tomate al principio del cultivo, para inmunizar la planta frente a futuras infecciones del virus más tardías (Spence *et al.,* 2006). Esta inmunización está basada en el principio de la protección cruzada o pre-inmunización, que consiste en la inoculación de aislados del virus poco agresivos encontrados en la naturaleza, u obtenidos por mutagénesis para proteger a la planta de infecciones posteriores con aislados más agresivos del virus (Gosálvez y Garnsey, 1989). La protección cruzada ha sido empleada con éxito variable contra el *Tobacco mosaic virus* (TMV), *Citrus tristeza virus* (CTV), *Papaya ring spot virus* (PRSV) (Yeh y Gosálvez, 1984; Gosálvez y Garnsey, 1989) y *Zucchini yellow mosaic virus* (ZYMV) (Lecoq *et al.,* 1991; Perring *et al.,* 1995). En PepMV, a pesar de que se produce un efecto negativo de mayor entidad en la calidad de los frutos si las plantas se infectan tardíamente (Spence *et al.,* 2006), resultados obtenidos posteriormente indican que los aislados del genotipo EU no protegen frente a los aislados del genotipo CH2 y viceversa, lo cual sugiere que la inmunización llevada a cabo por algunos agricultores no es efectiva y podría, incluso incrementar el daño provocado por el virus (Hanssen *et al.,* 2008). Sin embargo, Ling (2007) apunta la existencia de propiedades de protección cruzada entre los aislados CH1 y CH2, aunque se trata únicamente de una hipótesis basada en la expresión de síntomas más suave que detectó en las plantas infectadas por la mezcla de dichos aislados, frente a la mayor agresividad descrita por otros en infección simple.

4. ENFERMEDADES EMERGENTES ASOCIADAS A LA PRESENCIA DE PepMV

Desde su detección afectando a tomate en 1999, se han encontrado algunas enfermedades que se han asociado a la presencia de este virus, siendo un claro ejemplo, el "colapso" del tomate. Esta enfermedad aparece en España en 1998, paralelamente a la observación de síntomas típicos de PepMV. En la campaña 1999/2000 la incidencia del "colapso" osciló entre un 15 y 90% en invernaderos de Murcia, resultando muy variable entre invernaderos. Los síntomas desarrollados por las plantas afectadas han sido descritos en apartados anteriores. En un primer momento, las plantas afectadas se analizaron frente a diversos agentes patógenos (hongos, virus y bacterias) para tratar de averiguar la etiología de esta enfermedad. Posteriormente, se observó una reiterada asociación de dicha enfermedad con la infección de la planta con PepMV. En diversos ensayos se demostró la relación directa entre la aparición de síntomas de "colapso" con la infección de PepMV en la parte aérea, en combinación con la presencia de *O. brassicae* sl en las raíces, unido a una bajada brusca y posterior recuperación de las temperaturas en el invernadero (Córdoba *et al.* 2004b). Otros autores observaron una fuerte acumulación de PepMV en los tallos de plantas afectadas, acompañada de una necrosis del sistema vascular de las mismas. De todos modos, al no poder reproducir los síntomas de la enfermedad concluyeron que esta acumulación del virus no era suficiente para el desarrollo del "colapso" (Soler-Aleixander *et al.,* 2005).

Por otro lado, en 2001 se detectó en invernaderos de Murcia una nueva sintomatología que se caracterizaba por producir necrosis en los foliolos, tallos y frutos de las plantas afectadas confiriéndoles un aspecto general como de "quemado" o "torrao", el cuál da nombre a la enfermedad: "torrao" del tomate (Figura 1.10a). En el intento de determinar el agente causal, se detectó la presencia de PepMV en una gran proporción de las plantas afectadas lo que llevó

a sugerir que este virus podría tratarse de uno de los agentes implicados en el desarrollo del síndrome (Alfaro-Fenández *et al.,* 2006). Sin embargo, estudios posteriores determinaron que el agente causal de dicha afección era un nuevo virus denominado Tomato torrado virus (ToTV) (Verbeek *et al.,* 2007a). En el siguiente apartado se describirán con detalle los aspectos más relevantes de esta nueva enfermedad.

5. "TORRAO" O "CRIBADO" DEL TOMATE

5.1. Antecedentes históricos y sintomatología

A principios de la primavera de 2001 en un invernadero de tomate de la variedad Thomas situado en Murcia, se observaron un 3% de plantas afectadas que presentaban los típicos síntomas de "torrao". Tres semanas más tarde, la proporción de plantas infectadas se había incrementado exponencialmente, teniendo lugar poco después el levantamiento del cultivo a principios de mayo. En septiembre de ese mismo año, se realizó en dicho invernadero una plantación de tomate cv. Boludo, reapareciendo de nuevo los mismos síntomas en octubre. A partir de ahí, los sucesivos cultivos presentaron síntomas de la enfermedad, encontrándose en 2002 extendida por toda la zona, con incidencias irregulares. En la primera campaña 2001/02 la intensidad y progresión de la enfermedad se mostraron temporales o estacionales, incrementándose los síntomas a mitad de la primavera y el otoño. Sin embargo, en la campaña 2002/03, los accesos infecciosos fueron continuos aunque la intensidad también fue mayor en primavera y otoño (Jordá *et al.,* 2003). Sin embargo, en las Islas Canarias existen informes que indican la presencia de plantas afectadas por primera vez en el sur de Gran Canaria en 1996 y en el suroeste de Tenerife en 1997, apareciendo sucesivamente en las campañas posteriores. La mayoría de los casos se presentaron en otoño, aunque la planta se recuperaba tras el primer ataque. Años

después se constató una grave incidencia de la enfermedad en las campañas 2004-05 y 2005-06 en Gran Canaria (Espino *et al.,* 2007). Finalmente, la enfermedad se observó afectado a cultivos de tomate de distintas variedades en diversas regiones de España: Murcia, Alicante, Mallorca, Canarias, Almería (Alfaro-Fernández *et al.,* 2006).

Los primeros síntomas se presentaron en nuevas brotaciones. Se observó en las hojas ligeros amarilleos en la parte basal del foliolo que posteriormente se necrosaban, evolucionando en algunas ocasiones a "cribado" (Figura 1.10b). En otras ocasiones las lesiones necróticas evolucionaron a modo de manchas internerviales avanzando por los foliolos desde la base hasta el ápice (Figura 1.10c). En los tallos y peciolos aparieron manchas necróticas longitudinales que en algunos casos se asemejaban a costuras, ejerciendo una tensión tal en los tejidos que produjeron un retorcimiento de la hoja en espiral. Algunas flores manifestaron necrosis, produciendo su abscisión. En los frutos se observaron lesiones o manchas necróticas, pudiendo ser éstas lineales o circulares, a veces a modo de costura (Figura 1.10d). Algunas plantas que presentaron infecciones en los brotes llegaron a recuperarse, emitiendo nuevas hojas y manteniendo como único síntoma patente el cribado y la necrosis en las hojas más viejas (Jordá *et al.,* 2003; Alfaro-Fernández *et al.,* 2006).

Se realizaron numerosos intentos para identificar el agente causal de la enfermedad, analizándose las plantas afectadas para multitud de virus que afectan habitualmente al cultivo del tomate, así como otros que inducen necrosis en este hospedante. Los resultados obtenidos revelaron que el único virus detectado en la mayoría de las muestras analizadas fue el PepMV. Asimismo se intentó la transmisión de la enfermedad mediante inoculación mecánica e injerto, aunque no dio resultados satisfactorios ya que, a excepción de algún caso aislado no se consiguió reproducir los síntomas de la enfermedad (Alfaro-Fernández *et al.,* 2006).

Figura 1.10. Síntomas de "torrao" observados en plantas afectadas. (**a**) Aspecto general de "torrao" o quemado de la planta. (**b**) Necrosis en la base del foliolo que evoluciona a "cribado". (**c**) Manchado necrótico internervial que avanza hacia la parte apical del foliolo. (**d**) Lesiones necróticas lineales en fruto a modo de costura.

En 2005, se señaló por primera vez como posible agente causal de la enfermedad a un nuevo virus denominado Tomato torrado virus, ToTV (Verbeek *et al.*, 2005). Posteriormente, se publicó una patente del mismo (van den Heuvel *et al.*, 2006), donde se describía tanto la secuencia, como diferentes características biológicas de este virus, además de cebadores específicos para su diagnóstico. Un año más tarde, parte del equipo holandés que desarrolló la patente, publicó la identificación y caracterización del virus. Se trata de un nuevo virus perteneciente al grupo de virus del tipo "picorna", y se propuso la creación de un nuevo género Torradovirus, ya que este virus poseía

características específicas que no permitían englobarlo en ninguno de los géneros determinados hasta ese momento, aunque presentaba cierta relación con los géneros *Sequivirus*, *Sadwavirus* y *Cheravirus* (Verbeek *et al.,* 2007a). Gracias a la publicación de la secuencia, se pudo analizar mediante RT-PCR con cebadores específicos de ToTV muestras recogidas en Murcia durante 2003-2006, encontrándose el virus en el 93% de éstas, de las cuales solo un 10% resultaron negativas a PepMV. En este estudió se determinó a su vez, que esta coinfección de ToTV y PepMV se producía fundamentalmente con el genotipo CH2 de este último virus, por lo que se planteó verificar tanto si existía un efecto sinérgico en la coinfección por ambos virus, como si la reiterada conjunción del tipo de aislado era relevante o fruto de una mayor distribución del genotipo CH2 en detrimento de otros (Alfaro-Fernández *et al.,* 2007b). Posteriormente, se determina la presencia de ToTV en muestras recogidas durante 2007 en las Islas Canarias, concretamente en Gran Canaria (Alfaro-Fernández *et al.,* 2007a), aunque los grupos de sanidad vegetal de dicha Comunidad Autónoma venían observando síntomas similares desde las campañas de 1996 y 1997, como ya se ha comentado (Espino *et al.,* 2007). Simultáneamente a los estudios realizados en Murcia, dichos grupos de sanidad vegetal detectaron el ToTV en todas las zonas productoras de tomate de Tenerife y Gran Canaria, encontrándose habitualmente en infección mixta con el genotipo EU de PepMV (Espino *et al.,* 2007).

Paralelamente a la identificación del ToTV, en ese mismo año, se caracterizaron dos nuevos virus denominados Tomato apex necrosis virus (ToANV) y Tomato marchitez virus (ToMarV), encontrados afectando a cultivos de tomate en la zona de Sinaloa (México), donde los productores se refieren a la enfermedad provocada por estos agentes como "marchitez" o "marchitez manchada", debido al aspecto que presentan las plantas afectadas. Estos virus producen síntomas de necrosis en hojas y en la parte apical de la

planta que presenta asimismo malformaciones. Además se observan anillos necróticos en frutos, que los deprecian comercialmente (Turina *et al.,* 2007; Verbeek *et al.,* 2007b). Algunos autores han sugerido que ambos virus son aislados o cepas de una misma especie, por la gran similaridad que presentan tanto molecularmente como en sus características biológicas (Verbeek *et al.,* 2007b). De esta forma, estos dos virus junto con el ToTV formarían parte del nuevo género Torradovirus (Sanfaçon *et al.,* 2009). Recientemente, se ha determinado la infección de cultivos de tomate por un nuevo virus en Guatemala que produce manchas necróticas y lesiones en hojas y peciolos, denominándose Tomato chocolate spot virus (ToCsV). Las propiedades morfológicas y genéticas de este nuevo patógeno han llevado a los autores que lo han identificado a proponer su demarcación dentro del nuevo género Torradovirus, agrupándose así con ToTV, ToMarV y ToANV (Kuo *et al.,* 2009).

5.2. Agente causal y organización genómica

Las partículas virales de ToTV son isométricas, de unos 28 nm de diámetro (Verbeek *et al.,* 2007a) y aparecen formando agregados cristalinos en el interior de los tejidos de plantas de tomate infectadas (Pospieszny *et al.,* 2009; Figura 1.11). La purificación de la partícula viral se consigue con rendimientos muy bajos del orden de 15-20 o 75-150 μg/100 g de hoja (Verbeek *et al.,* 2007a; Pospieszny *et al.,* 2009), atribuyéndose en parte a la formación de agregados cristalinos por las partículas virales (Pospieszny *et al.,* 2009).

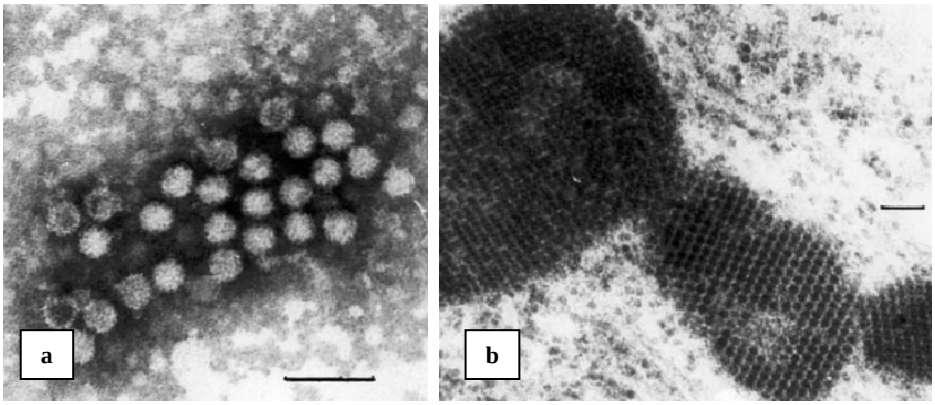


Figura 1.11. Observaciones de micoscopía electrónica de las partículas isométricas de ToTV en extracto de savia de plantas de *N. tabacum* infectadas **(a)** y los agregados cristalinos en células de hoja de tomate infectadas **(b)** (Fuente: Pospieszny *et al.*, 2009).

El genoma del virus es bipartito y está formado por dos RNAs poliadenilados de cadena simple con polaridad positiva (Verbeek *et al.*, 2007a). La organización genómica del ToTV puede observarse gráficamente en la Figura 1.12.

El RNA1 está compuesto por 7793 nt, sin incluir la cola poli-A del extremo 3´, conteniendo una pauta de lectura abierta (ORF1) que codifica para una poliproteína de 2158 aa, con una masa molecular de 241 kDa. Los codones de inicio (AUG) y parada (UGA) de la ORF1-RNA1 se sitúan en las posiciones 107-109 nt y 6581-6583 nt, respectivamente. Esta poliproteína presenta los motivos conservados típicos para el cofactor proteasa (PRO-CO) en las posiciones de aminoácidos (aa) 106-338, la helicasa (H) en 398-495 aa, la proteasa (P) en 1000-1100 aa y la RNA polimerasa RNA dependiente (RdRp) en 1303-1554 aa (Verbeek *et al.*, 2007a).

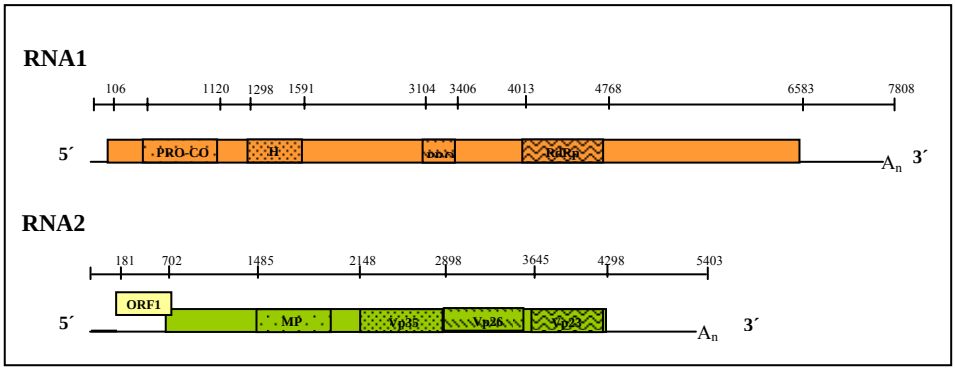


Figura 1.12. Representación esquemática de la organización genómica del ToTV. (Fuente: Adaptada de la información recogida en Verbeek *et al.* (2007a) y las secuencias completas del RNA1 y RNA2 del virus publicadas en la base de datos del GenBank con números de accesión DQ388879 y DQ388880).

El RNA2 con 5389 nt sin incluir la cola poli-A, contiene dos ORF. La primera ORF1 codifica una proteína de 187 aa con una masa molecular de 20 kDa, presentando el codón de inicio en el nucleótido 182 y el de parada en las posiciones 743-745 nt. Por el momento no se ha encontrado homología de esta proteína con otras contenidas en las bases de datos. La segunda ORF (ORF2-RNA2) codifica una poliproteína de 1198 aa con una masa molecular de 134 kDa. El codón de inicio de esta ORF2 se encuentra en las posiciones 702-704 nt y solapa parcialmente con la ORF1. El codón de parada UGA de la poliproteína se sitúa en las posiciones 4296-4298 nt. Esta poliproteína ORF2 contiene motivos típicos conservados (LxxPxL) de proteínas de movimiento (MP) de virus pertenecientes al género *Nepovirus*, encontrados en la posición 262-267 aa. Se encuentran además las tres subunidades para la proteína de la cápside; Vp35 (35kDa) en un área comprendida entre las posiciones 487-729 aa, Vp26 (26kDa) entre 730-983 aa y Vp23 (23kDa) entre 983-1195 aa. En los extremos 5´y 3´ de ambos RNAs presenta dos regiones no codificantes (UTR). La 5´UTR esta constituida por 106 nt en el RNA1 y 181 nt en el RNA2. En cambio las 3´UTRs

del RNA1 y RNA2 están conformadas por 1210 y 1092 nt, respectivamente (Verbeek *et al.,* 2007a).

5.3. Taxonomía

Las características del ToTV hacen que se encuadre taxonómicamente dentro del orden *Picornavirales*, denominados habitualmente virus del tipo "picorna" o miembros de la superfamilia o supergrupo "picorna". Las propiedades que comparten los miembros de este orden son (Sanfaçon *et al.,* 2009):

- Presentan partículas icosaédricas de 25-30 nm de diámetro con una simetría pseudo-T=3.
- Muestran una gran CP o una CP dividida en 2 o 3 subunidades más pequeñas.
- Tienen un genoma compuesto por RNA de polaridad positiva mono- o bipartito, con cada segmento codificando para una gran poliproteína, caracterizada por presentar proteinasas de cisteína.
- Contienen un bloque de replicación típico (Hel-Pro-Pol) que incluye una helicasa tipo III, una proteinasa tipo 3C y una RdRp tipo I.

Hasta el momento, en este gran grupo se incluían las familias *Comoviridae*, compuesta por los géneros *Nepovirus*, *Comovirus* y *Fabavirus*, y *Sequiviridae* compuesta por los géneros *Sequivirus* y *Waikavirus*. Asimismo, se englobaban en dicho grupo dos géneros no asignados a ninguna familia: *Cheravirus* y *Sadwavirus*. Además, existían otros virus que poseen las propiedades generales del orden, pero no pueden ser asignados a ninguno de los géneros existentes como el *Strawberry latent ringspot virus* (SLRSV), o los

nuevos virus Tomato torrado virus (ToTV) y Tomato marchitez virus (ToMarV). Recientemente se ha propuesto la creación de una nueva familia que englobe a todos estos virus denominada Secoviridae, que incluiría los géneros *Comovirus* (especie tipo *Cowpea mosaic virus*, CPMV), *Fabavirus* (especie tipo *Broad bean wilt virus 2*, BBWV2), *Nepovirus* (especie tipo *Tobacco ring spot virus*, TRSV), *Sequivirus* (especie tipo *Parsnip yellow fleck virus*, PYFV), *Waikavirus* (especie tipo *Rice tungro spherical virus*, RTSV), *Cheravirus* (especies tipo *Cherry rasp leaf virus*, CRLV), *Sadwavirus* (especie tipo *Satsuma dwarf virus*, SDV), así como un nuevo género denominado Torradovirus que englobara a los virus ToTV (especie tipo) y ToMarV. La propuesta para la creación de esta nueva familia y género fue aceptada en 2008 por la ICTV (*Internacional Committe on Taxonomy of Viruses*) y en este momento, se encuentra pendiente de ratificación por los miembros de dicho comité. Las propiedades comunes que compartirían los miembros de esta nueva familia Secoviridae, además de las anteriormente indicadas, serían (Sanfaçon *et al.,* 2009):

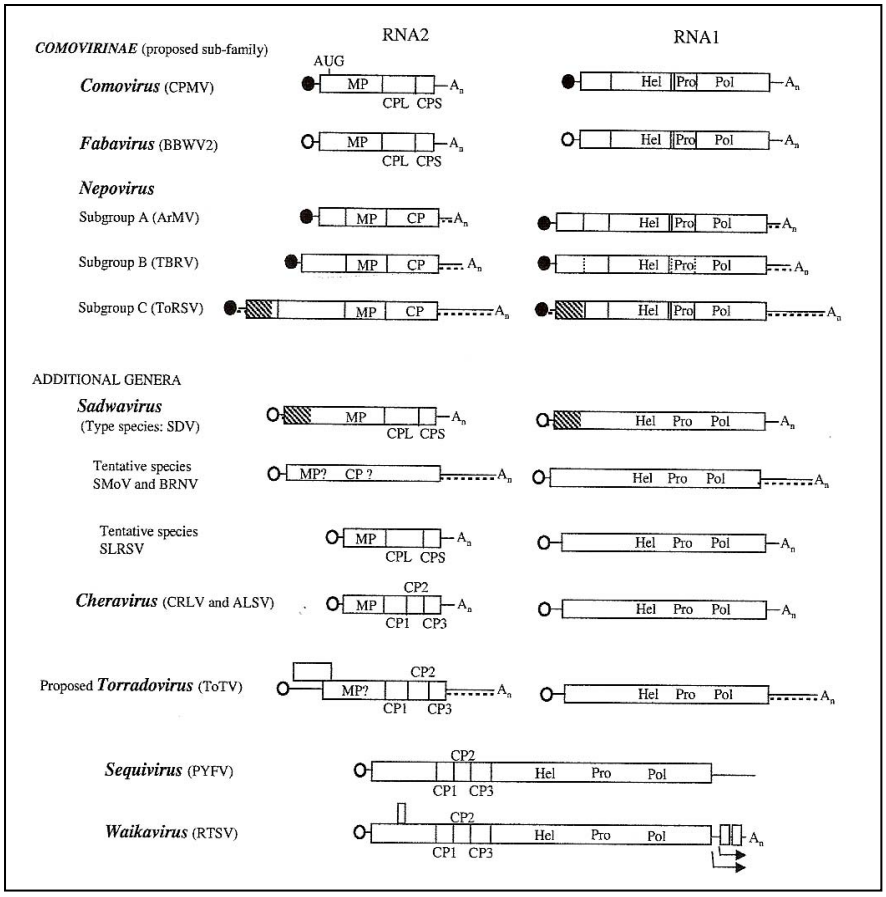
- Infectan plantas empleando proteínas especializadas llamadas proteínas de movimiento y/o proteínas de la cápside adaptadas para dicha función, que le permiten el movimiento tanto célula a célula como a larga distancia.
- Se agrupan en una única rama del dendrograma Pro-Pol (dominios conservados entre el motivo CG de la proteinasa 3C y el motivo GDD de la polimerasa) frente a miembros del orden picornavirales que infectan a otros reinos (Figura 1.14).

Como criterios para la demarcación de géneros dentro de esta familia se incluirían (Sanfaçon *et al.,* 2009):

- Número de RNA genómicos.
- Número de dominios de proteínas.
- Número de CPs.

- Presencia de ORF adicionales y/o RNA sugenómicos.
- Agruparse en una única rama en el dendrograma Pro-Pol al compararse con otros géneros de la misma familia.

La organización genómica de los distintos géneros recogidos en la nueva familia puede observarse en la Figura 1.13. El modo de transmisión no se incluye como criterio de demarcación ya que no todos los virus presentan un vector definido, además de encontrarse variación entre las especies dentro del mismo género, por ejemplo, en el género *Nepovirus* la mayor parte de las especies se transmiten por nemátodos, sin embargo *Blackcurrant reversion virus*, BRV se transmite por ácaros (Sanfaçon *et al.*, 2009).

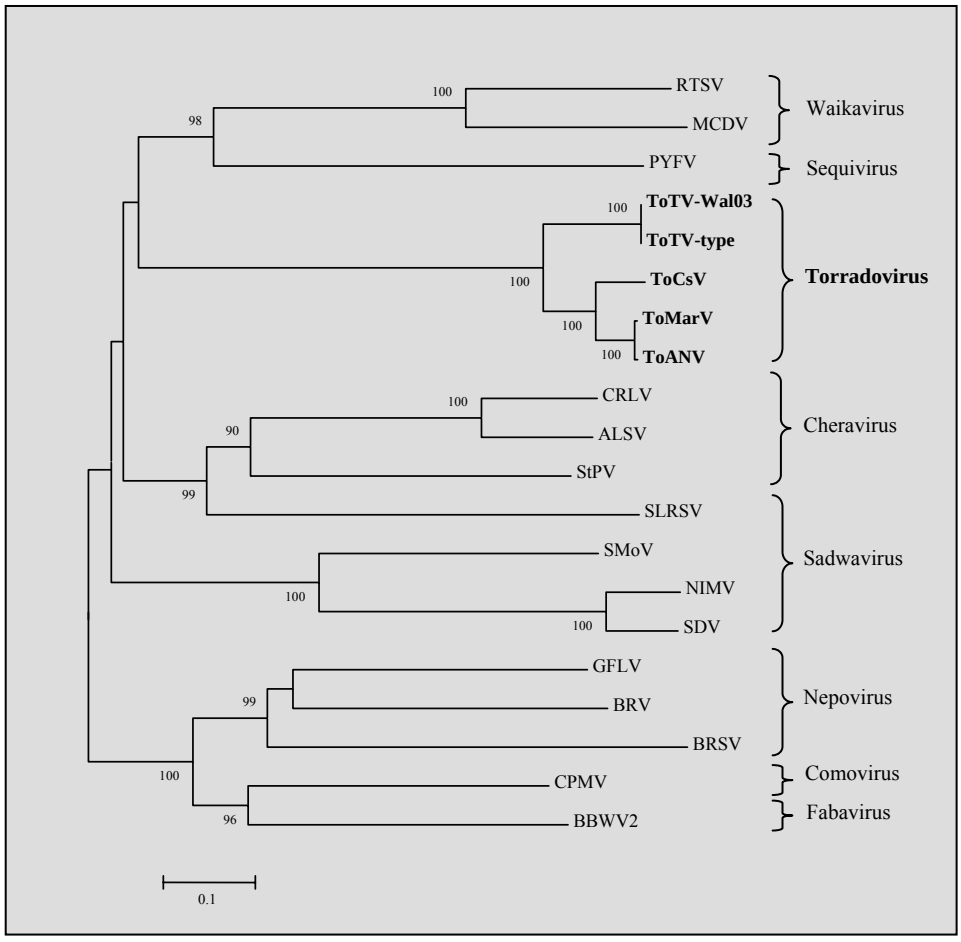


El nuevo género propuesto denominado Torradovirus, englobaría a ToTV, ToCSV, ToANV y ToMarV, ya que se agrupan en una única rama diferenciada en el dendrograma Pro-Pol (Figura 1.14), presentando además una ORF adicional en el RNA2 respecto a otros géneros propuestos. Como se ha indicado anteriormente, estos virus presentan características similares a los miembros de los géneros *Sequivirus*, *Waikavirus*, *Cheravirus*, *Sadwavirus*, sin embargo, comparten más características estructurales con los miembros del género *Cheravirus*. El recientemente descrito Tomato apex necrosis virus (ToANV) se consideraría un aislado dentro de la especie de ToMarV debido a su elevada similitud. Se establecen así los criterios que definen especies dentro de este nuevo género (Sanfaçon *et al.,* 2009):

- Presentar menos de un 75% de identidad en la secuencia de aa de las CPs.
- Presentar menos de un 80% de identidad en la secuencia de aa de la region Pro-Pol.
- El tipo de vector.
- El rango de hospedantes.
- La ausencia de relación serológica cruzada.
- La ausencia de protección cruzada.

◄ **Figura 1.13.** Organización genómica de los géneros incluidos en la nueva familia Secoviridae. Hel=helicasa, Pro=proteinasa tipo 3C, Pol=RNA polimerasa RNA dependiente, CP=proteína de la capside (L= large y S= small), MP=proteína de movimento, A_n=cola poliA. Los interrogantes indican que la posición y/o función de la proteína es tentativa. Las flechas indican posibles RNA subgenómicos.

ToANV y ToMarV presentan no sólo elevada identidad en sus secuencias, sino también características biológicas similares, por lo que se propone que sean incluidos como dos aislados o cepas dentro de la misma especie perteneciente al género Torradovirus (Verbeek *et al.,* 2007b). El recientemente descrito ToCsV se agrupa filogenéticamente con ToANV, ToMarV y ToTV en el dendrograma Pro-Pol, proponiendose asimimo su inclusión en dicho nuevo género (Kuo *et al.,* 2009).



Pospieszny *et al.* (2007) apuntaron la posible relación del ToTV con el virus Tomato necrosis dwarf virus (TNDV) descrito por Larsen *et al.* (1984) que inducía necrosis en tomate y era transmitido por la mosca blanca *B. tabaci*. A pesar de mostrar propiedades similares en sintomatología, rango de hospedante, estructura de la partícula viral, e incluso de reaccionar serológicamente con un antisuero del TNDV, no se continuaron y ampliaron los trabajos correspondientes debido a la pérdida del aislado existente de TNDV (Pospieszny *et al.,* 2009).

5.4. Aspectos epidemiológicos

ToTV es un virus que se encuentra en expansión, no solo en España, sino también en Europa, Centro-América y Australia. Ha sido detectado en Polonia en muestras recogidas durante 2003 y 2004 en la región de Wielkopolska (Pospieszny *et al.,* 2007) y en la región de Mazowsze durante 2007 (Pospieszny *et al.,* 2009). También se ha detectado en Hungría, en cultivos de tomate de tres regiones (Szeged, Öcsöd y Csongrád) durante 2007 y 2008 (Alfaro-Fernández *et al.,* 2009a), y en Panamá a partir de muestras procedentes de cuatro regiones del país (Coclé, Herrera, Los Santos y Veraguas) recogidas durante 2008 (Herrera-Vásquez *et al.,* 2009a).

◄ **Figura 1.14.** Análisis filogenético de virus relacionados con el ToTV. (Fuente: elaborada en base al alineamiento de la región entre el motivo CG de la proteasa y el GDD de la RdRp de virus pertenecientes a diferentes familias y géneros. ALSV, *Apple latent spherical virus*; BRSV, *Beet ringspot virus*; BBWV2, *Broad bean wilt virus 2*; CRLV, *Cherry rasp leaf virus*; CPMV, *Cowpea mosaic virus*; GFLV, *Grapevine fanleaf virus*; MCDV, *Maize chlorotic dwarf virus*; NIMV, *Navel orange infectious mottling virus*; PYFV, *Parnship yellow fleck virus*; RTSV, *Rice tungro spherical virus*; SDV, *Satsuma dwarf virus*; SMoV, *Strawberry mottle virus*; SLRSV, *Strawberry latent ring spot virus*; StPV, *Stocky prune virus*; ToANV: Tomato apex necrosis virus; ToCsV, Tomato chocolate spot virus; ToMarV, Tomato marchitez virus, ToTV, Tomato torrado virus. Fuente: Elaborado a partir de Verbeek *et al.* (2007b).

En octubre de 2008, fue detectado en el Sur de Australia, en cultivos protegidos de la zona norte de Adelaida, aunque existe la sospecha de su presencia desde 2005 en el continente austral, ya que algunas muestras preservadas desde entonces han resultado positivas al virus (IPPC, 2008).

En cuanto al modo de transmisión del virus, desde la observación de las primeras infecciones, se relacionó el virus con la presencia de la mosca blanca *T. vaporariorum* (Jordá *et al.,* 2003; Verbeek *et al.,* 2007a). Posteriormente se demostró la transmisión de ToTV por dicho vector con una elevada eficiencia (hasta del 100%), comparado con la transmisión mecánica artificial que resultó mucho menos efectiva, oscilando entre 50-70% o 70-90% (Pospieszny *et al.,* 2007; 2009), si bien depende en gran medida de la especie de hospedante empleada para multiplicar el virus. El inóculo del virus procedente de *Physalis floridana* Rydb. o *N. benthamiana* fue más efectivo para la inoculación mecánica que el procedente de tomate infectado (Pospieszny *et al.,* 2009). Otros ensayos de transmisión de ToTV con *T. vaporariorum* determinaron el aumento de plantas afectadas, así como de la expresión de síntomas, con el incremento de la duración del fotoperiodo diurno a lo largo del ensayo (Juárez y Cámara, 2008). Asimismo, se consiguió detectar el virus en el vector mediante RT-PCR empleando cebadores específicos que amplifican un fragmento del RNA2 situado en la subunidad Vp23 de la CP (Pospieszny *et al.,* 2009). Otros autores han afirmado que la transmisión del virus no solo por *T. vaporariorum*, sino también por la mosca blanca *Bemisia tabaci* (Gennadius) (Amari *et al.,* 2008). Sin embargo, no se produjo transmisión del ToTV en los ensayos realizados con el áfido *M. persicae*, tras un periodo de adquisición de 24 horas y su liberación posterior en plantas de tomate sanas (Pospieszny *et al.,* 2009).

Por lo que se refiere al rango de hospedantes, únicamente el tomate se ha descrito como hospedante natural del virus. Sin embargo, se han realizado diversos estudios experimentales para determinar el rango de hospedantes del

ToTV, concluyendo que éste se restringe a especies de la familia *Solanaceae* (Tablas 1.9 y 1.10). El tomate (*S. lycopersicum*) presentó una reacción variable a la inoculación con ToTV en función del cultivar, apareciendo cultivares con severos síntomas de necrosis y deformaciones, otros con síntomas suaves o asintomáticos e incluso, algunos de los cultivares ensayados (Emotion, Raisa, Robin y Jaga) con una posible resistencia natural al virus, puesto que no resultaron infectados (Budziszewska *et al.,* 2008; Pospieszny *et al.,* 2009). Recientemente, se han patentado plantas de tomate resistentes al virus, así como los métodos para producirlas, dado que el tomate presenta un gen recesivo que le confiere resistencia natural al virus. De este modo se expresa resistencia a ToTV cuando este gen está presente en la planta en homocigosis, mientras que las plantas heterocigóticas son susceptibles (Maris *et al.,* 2007). Todas las especies recogidas en la Tabla 1.9 presentaron infección sistémica del virus, la mayoría de ellas con síntomas evidentes, a excepción de *N. rustica* que resultó asintomática en los estudios realizados (Verbeek *et al.,* 2007a; Amari *et al.,* 2008). *S. melongena* y *N. tabacum* cv. White Burley presentaron una respuesta diferencial en función del estudio realizado, probablemente atribuible al aislado empleado. Las plantas de berenjena inoculadas en el estudio de Amari *et al.* (2008) resultaron asintomáticas seis semanas tras la inoculación, sin embargo Pospieszny *et al.* (2009) apuntaron la existencia de clorosis en las plantas infectadas. En el caso de *N. tabacum* cv. White Burley, el aislado inoculado por Verbeek *et al.* (2007a) no produjo síntomas, contrariamente al aislado polaco Wal03 que indujo clareado de venas y clorosis, además de anillos en las hojas inoculadas (Pospieszny *et al.,* 2009).

Tabla 1.9. Especies hospedantes de ToTV tras su inoculación mecánica o su transmisión mediante *T. vaporariorum*.

Especie	Familia	Referencia bibliográfica
Capsicum annuum L.	*Solanaceae*	Amari *et al.*, 2008; Pospieszny *et al.*, 2009
Datura inoxia P.Mill	*Solanaceae*	Pospieszny *et al.*, 2009
Nicotiana affinis T. Moore	*Solanaceae*	Pospieszny *et al.*, 2009
N. benthamiana Domin.	*Solanaceae*	van den Heuvel *et al.*, 2006; Verbeek *et al.*, 2007a; Amari *et al.*, 2008; Pospieszny *et al.*, 2009
N. clevelandii Grey	*Solanaceae*	Verbeek *et al.*, 2007a; Pospieszny *et al.*, 2009
N. debneyi Domin.	*Solanaceae*	Pospieszny *et al.*, 2009
N. glutinosa L.	*Solanaceae*	van den Heuvel *et al.*, 2006; Verbeek *et al.*, 2007a; Amari *et al.*, 2008; Pospieszny *et al.*, 2009
N. hesperis (N.T.Burb.) P. Horton	*Solanaceae*	van den Heuvel *et al.*, 2006; Verbeek *et al.*, 2007a
N. occidentalis H.-H. Wheeler	*Solanaceae*	van den Heuvel *et al.*, 2006; Verbeek *et al.*, 2007a
N. tabacum L. cv. White Burley	*Solanaceae*	Verbeek *et al.*, 2007a; Pospieszny *et al.*, 2009
N. tabacum L. cv. Xanthi	*Solanaceae*	Amari *et al.*, 2008; Pospieszny *et al.*, 2009
N. tabacum cv. Samsum	*Solanaceae*	Pospieszny *et al.*, 2007; Amari *et al.*, 2008
N. rustica L.	*Solanaceae*	Verbeek *et al.*, 2007a; Amari *et al.*, 2008
Nicandra physaloides (L.) Gaerth.	*Solanaceae*	Pospieszny *et al.*, 2009
Petunia hybrida Hort. *ex* E.Vilm.	*Solanaceae*	Pospieszny *et al.*, 2009
Physalis floridana Rydb.	*Solanaceae*	Verbeek *et al.*, 2007a; Amari *et al.*, 2008; Pospieszny *et al.*, 2009
S. lycopersicum L.	*Solanaceae*	Amari *et al.*, 2008; Pospieszny *et al.*, 2009
S. melongena L.	*Solanaceae*	Amari *et al.*, 2008; Pospieszny *et al.*, 2009
S. tuberosum L.	*Solanaceae*	Pospieszny *et al.*, 2009

En cambio, especies de otras familias botánicas no resultaron infectadas con el virus tras su inoculación artificial (Tabla 1.10). A pesar de presentar el mismo rango de hospedantes, se observaron diferencias de virulencia entre tres aislados polacos de ToTV al comparar los síntomas inducidos por éstos en las plantas indicadoras. Esta variabilidad sintomatológica podría estar causada por algunos cambios en el genoma, ya que resultaban más agresivos los aislados recogidos más recientemente (2007), frente al aislado de 2003 (Pospieszny *et al.,* 2009). *P. floridana* resultó ser un buen indicador de la infección por ToTV, ya que produjo síntomas severos de moteado, necrosis de hojas y muerte de plantas, frente a otras solanáceas testadas que únicamente manifiestaron mosaicos suaves o clareado de venas (Pospieszny *et al.,* 2009). Además, esta especie puede emplearse como hospedante diferencial para distinguir entre ToTV y ToMarV, ya que este último le induce a las plantas de *P. floridana* síntomas más suaves de moteado y necrosis local. Otro hospedante diferencial entre ambos virus sería *Chenopodium quinoa* Willd., ya que el ToMarV induce lesiones necróticas locales, mientras que el ToTV no (Verbeek *et al.,* 2007b).

Tabla 1.10. Especies no hospedantes de ToTV tras su inoculación experimental mecánica o su transmisión mediante *T. vaporariorum*.

Especie	**Familia**	**Referencia bibliográfica**
Chenopodium quinoa Willd.	*Chenopodiaceae*	Verbeek *et al.,* 2007a; Pospieszny *et al.,* 2007; Amari *et al.,* 2008
Gomphrena globosa L.	*Amaranthaceae*	Verbeek *et al.,* 2007a
Phaseolus vulgaris L.	*Leguminoseae*	Pospieszny *et al.,* 2007
Pisum sativum L.	*Leguminoseae*	Pospieszny *et al.,* 2007
Cucumis sativus L.	*Cucurbitaceae*	Pospieszny *et al.,* 2007; Amari *et al.,* 2008
Beta vulgaris L.	*Chenopodiaceae*	Pospieszny *et al.,* 2007
Cucumis melo L. var. cantaloupe	*Cucurbitaceae*	Amari *et al.,* 2008
Cucurbita pepo L. var. negro belleza	*Cucurbitaceae*	Amari *et al.,* 2008
Chenopodium album ssp. *amaranticolor* Coste & A. Reinier	*Chenopodiaceae*	Amari *et al.,* 2008

5.5. Variabilidad genética

Hasta la fecha, se han secuenciado completamente dos aislados de ToTV: el aislado tipo PRI-ToTV0301 (Nº Accesión DQ388879 y DQ388880), procedente de Murcia y recogido durante 2003 (Verbeek *et al.,* 2007a) y el aislado polaco Wal03 (No. Accesión EU563947 y EU563948) recogido en 2003 (Budziszewska *et al.,* 2008). Sin embargo existen en el GenBank diversas secuencias parciales de aislados, la mayor parte españoles, aunque también están publicados tres aislados húngaros y uno de Panamá. Las diferencias más notables encontradas entre el aislado tipo y el aislado polaco Wal03 es su longitud, debida a una inserción en el extremo 3´ y el tamaño de sus CP (Budziszewska *et al.,* 2009). Las diferencias en el tamaño de las subunidades CP también ha sido observada en otros aislados polacos, y se atribuye a un mayor contenido de cadenas hidrofílicas que pueden unirse transitoriamente con agua a través de puentes de hidrógeno dando como resultado una mayor masa molecular de estas subunidades (Pospieszny *et al.,* 2009).

Por otro lado, las diferencias observadas a nivel de secuencia entre el aislado Wal03 y el aislado tipo de ToTV determinaron la presencia de 3 cambios en aminoácidos en la región Vp35 y Vp23, y uno en la región de la RdRp, siendo el resto de sustituciones de nucleótidos sinónimas (Budziszewska *et al.,* 2008). Los análisis de las secuencias de aminoácidos de los aislados polacos determinaron que existía un elevado porcentaje de identidad entre ellos en diferentes zonas del genoma y con el aislado tipo PRI-ToTV0301. En el RNA1, únicamente se analizó el primer aislado polaco encontrado, presentando éste respecto al aislado tipo un 99-100% de identidad en su secuencia de aa en las zonas de la proteasa, helicasa y polimerasa. En cuanto a las subunidades CP, estudios comparativos entre los tres aislados polacos, la identidad entre sus secuencias de aminoácidos fue de un 98-100% (Budziszewska *et al.,* 2008; Pospieszny *et al.,* 2009).

En el resto de aislados descritos de ToTV, únicamente se analizaron zonas concretas del genoma donde su identidad a nivel de nt con el aislado tipo fue siempre de más de un 98% (Alfaro-Fernández *et al.,* 2007a; Amari *et al.,* 2008; Herrera-Vásquez *et al.,* 2009a; Alfaro-Fernández *et al.,* 2009a). En cambio, el ToMarV y el ToANV mostraron identidades en sus secuencias mucho menores con el ToTV, aunque entre ambos virus presentaron una relación más estrecha (Tabla 1.11), por lo que algunos autores plantearon que se tratasen como dos aislados o cepas del mismo virus como se ha indicado anteriormente (Verbeek *et al.,* 2007b). Hay que destacar que el ToANV aún no está completamente secuenciado, por lo que no se ha podido comparar el total de su secuencia (Verneek *et al.,* 2007b), además de considerarse actualmente como una cepa de ToMarV (Sanfaçon *et al.,* 2009).

Tabla 1.11. Porcentaje de identidad de las secuencias de aminoácidos de las diferentes zonas del genoma de ToMarV comparadas con el aislado tipo de ToTV (ToTV-PRI0301), el aislado polaco Wal03 de ToTV, y el aislado parcialmente secuenciado de ToANV VE434.

	RNA1			**RNA2**					
	ORF	**Helicase**	**RdRp**	**ORF1**	**ORF2**	**MP**	**Vp35**	**Vp26**	**Vp23**
ToTV-tipo	65%	95.6%	85%	63%	69.9%	62.1%	72%	86%	71%
ToTV-Wal03	69.7%	89%	86%	60%	73.1%	-	70%	86%	71%
ToANV	99%	100%	100%	-	90%	-	89%	98%	94%
ToCsV	83%	-	-	70%	70%	-	-	-	-

El aislado polaco Wal03 presentó el mismo orden de homología con ToMarV y ToANV que el aislado tipo de ToTV (Budziszewska *et al.,* 2008). El análisis molecular de las secuencias de aa de las tres subunidades CP de los aislados polacos Ros y Kas determinaron que estos presentaban 98-100% con las del aislado tipo ToTV, en cambio con ToMarV y ToANV mostraron 69-86% y

71-85%, respectivamente. El recientemente detectado ToCsV presenta un 74% de identidad de su RNA1 con el de ToMarV y ToANV, aunque esta identidad es menor comparándolo con el ToTV. En cambio, la identidad de su RNA2 es del 70% con el de ToTV y ToMarV (Kuo *et al.,* 2009).

Los estudios realizados han revelado una gran homología entre los aislados de ToTV estudiados hasta el momento, procedentes de España y Polonia. Los aislados polacos presentan una distancia evolutiva muy pequeña lo que hace pensar que los aislados Ros y Kra encontrados en 2007, se hayan originado a partir de Wal03 encontrado en 2003 y hayan evolucionado acumulando pequeños cambios en su genoma (Pospieszny *et al.,* 2009).

Capítulo 2

Justificación y objetivos

JUSTIFICACIÓN Y OBJETIVOS

El tomate es un cultivo de gran importancia económica a nivel mundial, que se ve afectado por enfermedades de etiología diversa, entre las cuales destacan las causadas por virus, debido a que ocasionan cuantiosas pérdidas económicas y son de difícil control. Uno de los virus que afectan a este cultivo es el virus del mosaico del pepino dulce (PepMV), que se encuentra ampliamente extendido por las principales áreas productoras de tomate de Europa, gracias a su eficaz transmisión mecánica y probablemente a su recientemente demostrada transmisión por semilla (Córdoba-Sellés *et al.,* 2007b). A pesar de ser un virus muy estudiado desde su primera detección en tomate en 1999 (van der Vlugt *et al.,* 2000), todavía existen algunos aspectos de su epidemiología y variabilidad molecular que se desconocen, entre otros, la relación entre la variabilidad molecular y sintomatológica, su distribución en la planta, o los efectos sinérgicos entre los genotipos descritos. Asimismo, la caracterización de diferentes genotipos del virus y su distribución en campo, dificultan el diagnóstico por las técnicas convencionales. Por todas estas razones, en este trabajo se planteó el diseño de una técnica de diagnóstico eficaz para la detección de los diversos genotipos del PepMV. Por otro lado, la construcción de clones infecciosos de virus así como, la incorporación del uso de proteínas fluorescentes en el campo de la Virología Vegetal ha permitido avanzar en el estudio de la biología molecular de los virus de RNA. Estas metodologías permiten conocer las diferentes etapas del ciclo de infección viral o reconocer regiones del genoma responsables de diversas propiedades biológicas (Hasiów-Jarowseska *et al.,* 2009; Sánchez-Navarro y Pallás, 2008). Por ello, en el desarrollo de este trabajo, se consideró necesario intentar construir

un clon infeccioso de PepMV, y marcarlo posteriormente con la proteína fluorescente verde (GFP, *green fluorescent protein*) para futuros estudios de la distribución y acumulación del virus en la planta o incluso para determinar las interacciones a nivel celular de los diversos genotipos mediante el marcaje diferencial con diversas proteínas fluorescentes.

En estudios anteriores a este trabajo, se relacionó la presencia del PepMV y el hongo *O. brassicae* sl con la manifestación del síndrome conocido como "colapso" del tomate (Córdoba *et al.,* 2004b). Sin embargo, y a pesar de la asociación de estos dos agentes, no se estudió la posible transmisión de este virus mediante dicho hongo vector.

En otro orden de cosas, el cultivo del tomate presenta una complejidad fitosanitaria, causada en cierto modo por la gran intensificación de su cultivo, y unida a la presencia de virosis ampliamente extendidas como la causada por el PepMV, que conduce a la aparición de nuevas enfermedades de etiología diversa. El "torrao" o cribado del tomate, cuyo agente causal ha sido identificado como Tomato torrado virus (ToTV) (Verbeek *et al.,* 2007a), es una enfermedad presente en España desde hace más de ocho años (Jordá *et al.,* 2003) que produce infecciones muy graves tanto por sí solo, como en infecciones mixtas, encontrándose en la mayoría de los casos junto al PepMV, lo que agrava sus síntomas e incluso llega a confundirlos. A pesar de los nuevos trabajos de caracterización del ToTV, existe poca información en cuanto a su forma de transmisión, sintomatología concreta y efecto sinérgico con otros virus que comúnmente afectan al tomate. Se trata, por tanto de un problema en expansión por todas las zonas productoras de tomate que, además puede verse agravado con la presencia en las plantaciones de otras entidades virales. En el presente trabajo se pretende profundizar en la epidemiología, distribución y variabilidad de este nuevo virus.

Por todo ello, se plantearon los siguientes objetivos concretos en la siguiente tesis doctoral:

1. Desarrollar una técnica de multiplex RT-PCR para la detección e identificación simultánea de los diferentes genotipos de PepMV descritos hasta el momento.

2. Construir un clon infeccioso de PepMV e introducirle posteriormente el gen de la proteína fluorescente verde GFP (*green fluorescent protein*), creando un nuevo clon infeccioso mutado que expresara dicha proteína, estableciendo un precedente para futuros estudios de distribución y acumulación del virus en la planta y comparación entre sus genotipos.

3. Comprobar la capacidad del hongo vector *O. brassicae* sl (concretamente *O. virulentus*) de transmitir PepMV.

4. Evaluar la incidencia y distribución de la enfermedad conocida como "torrao" en España, determinando la presencia de su agente causal ToTV, y otros virus que afectan habitualmente al tomate entre ellos el PepMV, así como el diagnóstico del ToTV mediante hibridación molecular.

5. Analizar los posibles reservorios del ToTV entre la flora arvense asociada al cultivo del tomate en España.

6. Estudiar la citopatología del ToTV al infectar hojas de tomate y el posible efecto sinérgico de la coinfección de éste con otros virus que afectan al tomate.

7. Estudiar la variabilidad molecular de una población de aislados españoles de ToTV procedentes de diversas regiones del país y recogidos desde el año 2001 hasta 2009.

Los distintos objetivos planteados en esta tesis doctoral se abordan en los siguientes capítulos como trabajos independientes con formato artículo, ya que la mayor parte de ellos han sido aceptados para su publicación en diferentes revistas internacionales.

Capítulo 3

Simultaneous detection and identification of *Pepino mosaic virus* (PepMV) isolates by multiplex one-step RT-PCR

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ABSTRACT

A one-step reverse transcription-polymerase chain reaction (RT-PCR) was developed for the simultaneous detection and identification of three groups of *Pepino mosaic virus* (PepMV): European/Peruvian, Chilean 1/US1 and Chilean 2/US2 groups, followed by a restriction analysis that allowed the separation of the European, Peruvian, Chilean 2 and US2 isolates (patent pending). The multiplex RT-PCR reaction was performed by a mix of six primers that amplified a part of the RNA-dependent RNA polymerase gene of PepMV plus an internal control. Amplifications resulted in a 980, 703 or 549 bp PCR products for European/Peruvian, Chilean 1/US1 or Chilean 2/US2 groups, respectively. For the identification of the isolates present within the European/Peruvian and Chilean 2/US2 groups, the amplified PCR fragments were directly digested with *Sac*I enzyme. The multiplex RT-PCR method presented higher sensitivity to detect CH1/US1 isolates in field samples than RFLP-PCR method described by Hanssen *et al.* (2008). The detection limit observed with the multiplex RT-PCR was equal or 3125 times higher when compared to single RT-PCR or ELISA-DAS and molecular hybridization methods, respectively. The use of multiplex RT-PCR method in routine analysis of field tomato samples allowed the detection of 36.2 and 33.4% more positives when compared to the serological and molecular hybridization methods, respectively, and the identification of plants infected with one, two or three isolates of PepMV.

INTRODUCTION

Pepino mosaic virus (PepMV) is a *Potexvirus* which was first described on Pepino (*Solanum muricatum* Ait.) in Peru (Jones *et al.,* 1980). In 1999, PepMV was reported infecting tomato crops (*Solanum lycopersicon* L.) in the Netherlands (van der Vlugt *et al.,* 2000). Since then, PepMV has spread rapidly through the main tomato production areas worldwide, where it produces significant economic losses.

PepMV is a positive, single stranded RNA (ssRNA) virus. The total genome of PepMV is approximately 6500 bp which includes five open reading frames (ORFs). Different strains of PepMV have been identified. The biological and molecular differences first observed between the tomato and pepino isolates of PepMV suggested that the tomato isolate of PepMV as a different strain, and was named 'tomato strain' (van der Vlugt *et al.,* 2002). Further studies demonstrated that PepMV tomato strain isolates from different locations (Europe, North America and Canada) showed clear differences in symptom aggressiveness and nucleotide sequences when compared to the original pepino isolate (hereafter referred to as Peruvian isolate, PE) but minor differences among them (Verhoeven *et al.,* 2003). Furthermore, the North American strains US1 and US2, which showed only a 79-83% overall sequence homology to both European (EU) and Peruvian strains (PE), have been described (Table 3.1; Maroon-Lango *et al.,* 2005). Recently, new isolates have been identified as Chilean isolates, CH1 (GenBank accession number DQ000984) and CH2 (GenBank accession number DQ000985), from a commercial tomato seed lot produced in Chile (Ling, 2007), the highest sequence homology of which was to isolates US1 (98%) and US2 (91%), respectively (Table 3.1; Ling, 2007). Moreover, a new Polish isolate highly distinct from the currently identified

isolates has been described and referred to as the PK isolate (GenBank accession number EF408821). The Polish isolate PK shows 81% or less nucleotide sequence identity with EU, PE or US strains (Prospieszny and Borodynko, 2006). Further studies revealed that PepMV-PK shows a high nucleotide sequence identity (98%) with the CH2 isolate, which suggests their common origin (Hasiów *et al.*, 2007). In 2007, the presence of an isolate of the US1 strain of PepMV was reported in tomato in the Canary Islands (Spain), located in a different continent to that it where it was originally reported in North America (Alfaro-Fernández *et al.*, 2008a).

Table 3.1. Homology among different complete nucleotide sequences of *Pepino mosaic virus* srains obtained from the GenBank Database.

	EU[a]	PE	CH1	US1	CH2	US2
EU	100	95	82	82	84	81
PE		100	82	82	83	80
CH1			100	98	84	90
US1				100	85	90
CH2					100	91
US2						100

[a]EU (Accession No. AJ606360), PE (Accession No. AM109896), CH1 (Accession No. DQ000984) and CH2 (Accession No. DQ000985) correspond to European, Peruvian, Chilean 1 and Chilean 2 isolates, respectively. Sequences of the isolates US1 and US2 are published under the Accession No. AY509926 andAY509927.

In 2002, a RT-PCR-RFLP assay was proposed as a rapid method to detect and identify new isolates of PepMV. Three different RFLP patterns were identified (P1, P2 and P3 types). Most of the samples analysed were included in the P1 type that corresponded to the tomato strain widely spread throughout

Europe. Some samples showed the P2 type which was identified as the Peruvian isolate, and the third pattern, type P3 that presented one different isolate of PepMV and appeared rarely in the tested samples and almost always in mixed infections with P1 type (Martínez-Culebras *et al.,* 2002). Types P1, P2 and P3 have been suggested to correspond to the EU, PE and US2 strains, respectively (Pagán *et al.,* 2006).

The genetic variability and population structure of PepMV infecting tomato crops in Spain was analysed by sequencing and studying three genomic regions: a part of the RNA-dependent RNA polymerase (RdRp) gene, the triple-gene block (TGB) and the capsid protein (CP) gene. The results showed that the most prevalent genotype in Spain is the EU strain (more than 80% of the population). PE and US2 strains were also detected at a less relative frequency and were always found in mixed infections with the EU strain. Some recombinant isolates were also reported (Pagán *et al.,* 2006). However, in North America all the major genotypes of PepMV (EU, US1, US2 and CH2) were identified, although the EU was also the predominant strain (Ling *et al.,* 2008).

The low sequence homology observed between different PepMV genotypes has not been correlated to different symptomatology in infected plants. However, co-infection with several genotypes resulted in more severe PepMV symptoms and revealed the presence of PepMV recombinant (Hanssen *et al.,* 2008). In this sense, the incorporation of routine detection techniques that permits not only to detect the virus but also identifies the corresponding isolates of PepMV is desirable. Different approaches have been used to detect and identify the virus: immunosorbent electronic microscopy (ISEM) (van der Vlugt *et al.,* 2000), enzyme-linked immunosorbent assay (ELISA) (Jordá *et al.,* 2001a) and different molecular methods including reverse transcriptase-polymerase chain reaction (RT-PCR) (Mumford and Metcalfe, 2001; van der Vlugt *et al.,* 2000) or one-step immunocapture real-time TaqMan RT-PCR assay

designed to use two primers targeting a conserved region of the TGB2 gene plus a single TaqMan™ probe that covered all strains of PepMV (Ling *et al.,* 2007). In order to identify the exact PepMV strain present in one sample, those molecular methods had to be followed by DNA sequencing. However, so far there is only a methodology based on a single RT-PCR-RFLP assay adjusted for simultaneous detection and identification of three (Martínez-Culebras *et al.,* 2002) or five (Hanssen *et al.,* 2008; here after RT-PCR-Hanssen) PepMV strains. The development of multiplex RT-PCR has been used successfully for the routine diagnosis of plant viruses (see James *et al.,* 2006 for review; Ferrer *et al.,* 2007; Nie and Singh, 2000; Sánchez-Navarro *et al.,* 2006; Uga and Tsuda, 2005). This diagnostic method allows the simultaneous detection and identification of different viruses with less time and cost waste. Furthermore, a multiplex RT-PCR assay has been developed to identify different strain types of a single virus (Lorenzen *et al.,* 2006; Ratti *et al.,* 2005; Rigotti and Gugerli, 2007) and even different species of a virus (Martínez-Culebras *et al.,* 2001). In the present work we have developed a one-step multiplex RT-PCR reaction plus a restriction analysis that permits the simultaneous detection and identification of five different PepMV isolates (here after RT-PCR-*Sac*I). The use of the multiplex reaction in routine diagnosis has revealed that mix infection of PepMV strains is a common situation in the field.

MATERIAL AND METHODS

Virus sources and RNA preparations

Tomato PepMV isolates from different geographical origins were included as positive controls in the assay: DSMZ (German Collection Micro-organism and Cell Cultures, GMBH, Baunschweig, Germany) PV-0632 from Italy, DSMZ PV-0674 from United Kingdom, DSMZ PV-0716 from Italy and DSMZ PV-0730 from the Netherlands. A typical PepMV isolate from Peru (DSMZ PV-0554) obtained from *S. muricatum* was also studied. The new Polish PK-isolate, was kindly provided by Dr. H. Prospieszny (Institute of Plant Protection, Poznań, Poland), was included in the assay. Eleven tomato samples infected with a well characterized PepMV isolate were also analysed in this study: ten isolates belonging to our virus isolates collection that were previously analysed: Mu 00.2; Mu 00.3; Mu 00.4; Mu 00.5; CI 01.1; CI 01.2; CI 01.3; Al 01.2; Ba 03.1 (Pagán *et al.,* 2006); PepMV-Can1 isolate (Alfaro-Fernández *et al.,* 2008) and Sp-13 isolate (GenBank accession number AF484251), was kindly provided by Dr. M. Aranda, (CEBAS-CSIC, Murcia, Spain). Fourty-two tomato samples with typical symptoms of PepMV were collected from the major tomato production areas in Spain. Infected leaves were previously tested by DAS-ELISA with specific antisera against PepMV (DSMZ GMBH, Baunschweig, Germany) according to the manufacturer´s instructions to verify the virus infection. ELISA readings were considered positives when the absorbance of sample wells was at least three times greater than the mean absorbance reading of three healthy controls. Samples were analysed from 0.05 g of infected tissue per mL, which corresponded to the original sample (undiluted) in the sensitivity assay explained below.

Total nucleic acid extraction was performed from 0.1 g of leaves using the silica capture extraction protocol (MacKenzie *et al.,* 1997). The extracted nucleic acids were stored at -80 ºC until use.

Primer design

The characterization of specific region of the PepMV genome used to differentiate all genotypes was performed firstly, by the sequence alignment of PepMV isolates representatives of the different genotypes by the CLUSTAL X programme, and secondly, by the design of strain-specific primers by the OLIGO programme. The isolates of PepMV used for the sequence alignment were: Chilean isolates, CH1 (GenBank accession number DQ000984) and CH2 (GenBank accession number DQ000985); Peruvian isolates (PE), SM-74 (GenBank accession number AM109896) and LP-2001 (GenBank accession number AJ606361); European isolates (EU), Sp-13 (GenBank accession number AF484251), LE-2000 (GenBank accession number AJ606359) and LE-2002 (GenBank accession number AJ606360) and the North American (US) isolates, US1 (GenBank accession number AY509926) and US2 (GenBank accession number AY509927). A region of the RNA polymerase gene was selected to identify the different PepMV isolates. Three specific sense primers: PepMV-DEP, PepMV-D1 and PepMV-D2 and a common antisense primer (PepMV-R) were selected to differentiate the three EU/PE, CH1/US1 and CH2/US2 PepMV groups, respectively (Figure 3.1). The selected primers amplified three amplicons of 980, 703 and 549 base pairs corresponding to the EU/PE, CH1/US1 and CH2/US2 PepMV groups, respectively. In addition, the specific PepMV isolate of the EU/PE and CH2/US2 groups could be discriminated by digesting the amplicons with the *Sac*I enzyme (Table 3.2 and Figure 3.1). The expected amplicons of each PepMV group together with the nucleic acid fragments obtained after the incubation with the *Sac*I enzyme and the sequence

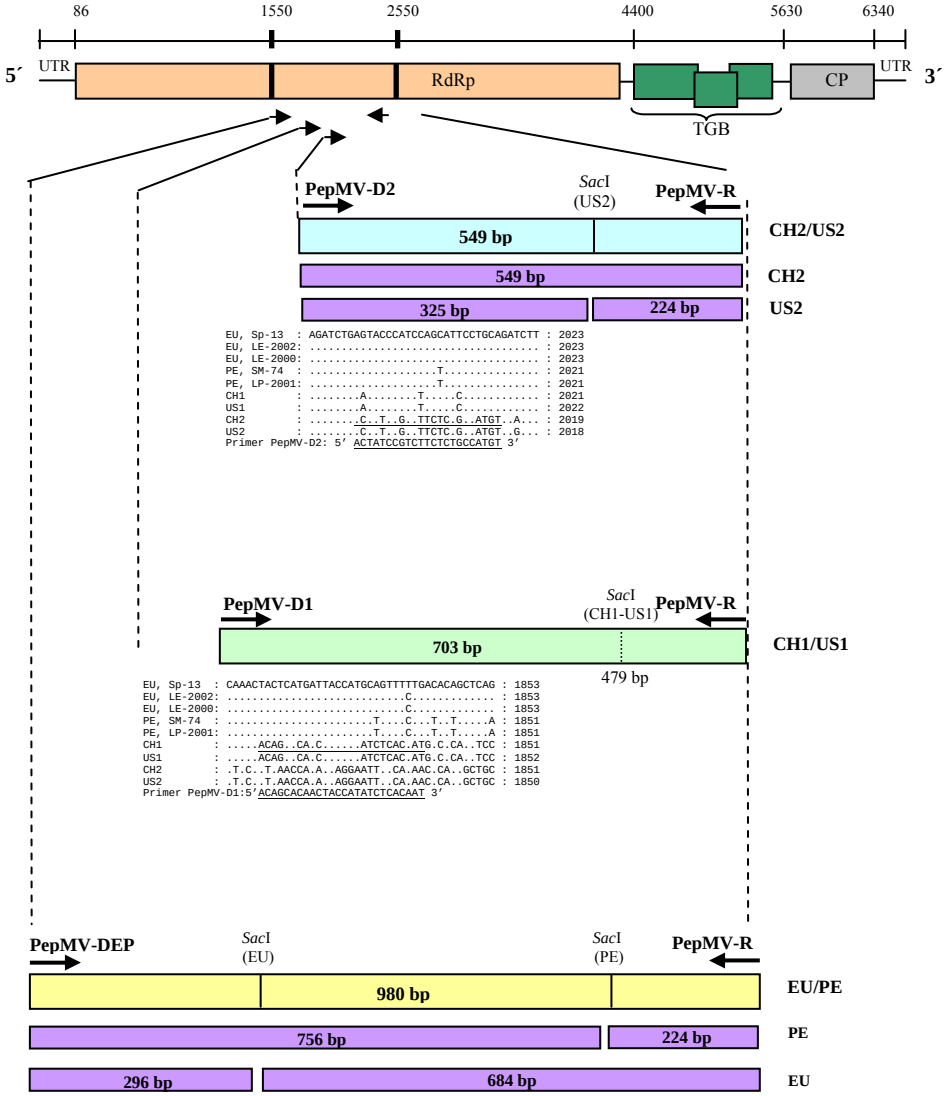
alignment of the different primers with the isolates of PepMV used, are indicated in Figure 3.1.

Table 3.2. PepMV genotypes by *Sac*I restriction endonuclease digestion of multiplex RT-PCR products.

	PepMV amplified fragment					
	549[a]		**703**		980	
	CH2	US2	CH1	US1	EU	PE
cDNA fragments after *SacI* digestion	**549**[b]	**325**	**479**	**479**	296	**756**
		224	224	224	**684**	224

[a]Base pairs of cDNA fragments are based on theoretical digests of reference sequences retrieved from GenBank
[b]Underlined numbers represent cDNA fragments representative of the corresponding PepMV genotype.

Figure 3.1. Schematic representation of the PepMV genome with the localization of the four primers used in the multiplex RT-PCR and the amplified PCR fragments. The sequence of the direct primers PepMV-D1, PepMV-D2 and PepMV-DEP together with the reverse primer PepMV-R is underlined in the alignment performed with Clustal X using PepMV isolates representative of all genotypes. Sequences included in the alignment belong to European (EU) strains (Sp-13, GenBank accession number AF484251; LE-2002, GenBank accession number AJ606360; LE-2000, GenBank accession no. AJ606359), Peruvian (PE) strains (SM-74, GenBank accession number AM109896; LP-2001, GenBank accession number AJ606361), Chilean 1 (CH1) strain (GenBank accession number DQ000984), US1 strain (GenBank accession number AY509926), Chilean 2 (CH2) strain (GenBank accession number DQ000985) and US2 strain (GenBank accession number AY509927). Dots indicate identical nucleotides to the Sp-13 isolate, and numbers on the right represent the position of the last nucleotide in the PepMV genome. The amplified PCR products obtained with the different direct and reverse primers combinations together with the resultant DNA fragments after the *Sac*I digestion is schematized. Numbers inside of the schematic boxes represent the base pairs (bp) size. The coding sequences corresponding to the RNA–dependent RNA polymerase, triple gene block and the coat protein are indicated as RdRp, TGB and CP, respectively. Numbers on the top of the schematic PepMV genome correspond to the nucleotide sequence. Untranslated regions are indicated as UTR.


86
1550
2550
4400
5630
6340
5´
UTR
RdRp
CP
UTR
3´
TGB
PepMV-D2
SacI
(US2)
PepMV-R
549 bp
CH2/US2
549 bp
CH2
325 bp
224 bp
US2
EU, Sp-13 : AGATCTGAGTACCCATCCAGCATTCCTGCAGATCTT : 2023
EU, LE-2002: ..... : 2023
EU, LE-2000: ..... : 2023
PE, SM-74 : .....T..... : 2021
PE, LP-2001: .....T..... : 2021
CH1 : .....A.....T.....C..... : 2021
US1 : .....A.....T.....C..... : 2022
CH2 : .....C..T..G..TTCTC.G..ATGT..A... : 2019
US2 : .....C..T..G..TTCTC.G..ATGT..G... : 2018
Primer PepMV-D2: 5' ACTATCCGTCTTCTCTGCCATGT 3'
PepMV-D1
SacI
(CH1-US1)
PepMV-R
703 bp
CH1/US1
479 bp
EU, Sp-13 : CAAACTACTCATGATTACCATGCAGTTTTTGACACAGCTCAG : 1853
EU, LE-2002: .....C..... : 1853
EU, LE-2000: .....C..... : 1853
PE, SM-74 : .....T....C..T..T.....A : 1851
PE, LP-2001: .....T....C...T..T.....A : 1851
CH1 : .....ACAG..CA.C.....ATCTCAC.ATG.C.CA..TCC : 1851
US1 : .....ACAG..CA.C.....ATCTCAC.ATG.C.CA..TCC : 1852
CH2 : .T.C..T.AACCA.A..AGGAATT..CA.AAC.CA..GCTGC : 1851
US2 : .T.C..T.AACCA.A..AGGAATT..CA.AAC.CA..GCTGC : 1850
Primer PepMV-D1:5'ACAGCACAACTACCATATCTCACAAT 3'
PepMV-DEP
SacI
(EU)
SacI
(PE)
PepMV-R
980 bp
EU/PE
756 bp
224 bp
PE
296 bp
684 bp
EU
EU, Sp-13 : TGAACAGCCTCAATGTTCATCTTCTAGCAGTCATATAC : 1591
EU, LE-2002: .....C... : 1591
EU, LE-2000: .....C... : 1591
PE, SM-74 : .....A..... : 1589
PE, LP-2001: .....A..... : 1589
CH1 : G..T.T...AG....C....ACG....A.ACTCCG.T. : 1589
US1 : G..T.T...AG....C....ACG....A.ACTC.G.T. : 1590
CH2 : G..TGTA...G..A....CGGCA...ATGAAACAT.G. : 1590
US2 : G..TGTA...G..A....CGGCA...ATGAAACAT.G. : 1589
Primer PepMV-DEP: 5'TCAATGTTCATCTTCTAGCAGTC 3'
EU, Sp-13 : AAGTGTATATCATGAGTCCAACAATGAAGCATACATT : 2550
EU, LE-2002: ..... : 2550
EU, LE-2000: ..... : 2550
PE, SM-74 : ..... : 2548
PE, LP-2001: ..... : 2548
CH1 : .....T..... : 2548
US1 : .....T..... : 2549
CH2 : ...C..G.....A..T.....G..... : 2546
US2 : .....T..... : 2545
Primer PepMV-R: 3'ATAGTACTCAGGTTGTTACTTCG 5'

For the amplification of the coat protein gene (CP), three different sense primers that specifically targeted on EU/PE group (sPepMVCP EU: 5´ TGT TCA CAA AAA TCA ACT TCA A 3´), CH1/US1 group (sPepMVCP CH1/US1: 5´ CTT TGA GCA CTT CAC AAT TAA G 3´) and CH2/US2 group (sPepMVCP CH2/US2: 5´ CTA TGG AAA ACC AAC CTA CAG C 3´) in combination with the common reverse primer described by Pagán *et al.* (2006), were designed in order to analyse and check the results obtained with the multiplex RT-PCR-*Sac*I.

Reverse transcription-polymerase chain reaction (RT-PCR) and restriction digestion

RT-PCR reaction was performed using the SuperScript III one step RT-PCR system with Platinum Taq DNA polymerase kit (Invitrogen Life Technologies. Barcelona, Spain). The reaction was carried out with a mixture of all the primers listed in Figure 3.1 at a final concentration of 0.25 pmol/µl and the primers of the internal control Rbcl gene (0.05 pmol/µl) corresponding to partial sequence of the ribulose 1.5-biphosphate carboxylase chloroplast gene (Sanchez-Navarro *et al.,* 2006). The PCR programme consisted of an initial incubation at 50 ºC for 30 min followed by 2 min at 94ºC and 40 cycles of 94 ºC for 15s, 50 ºC for 30s and 68 ºC for 1 min. A final incubation at 68 ºC for 10 min was introduced to finish the incomplete PCR fragments. The amplified PCR products were analysed on 1.2% agarose/TAE gels stained with ethidium bromide. To confirm the viral-strain origin, amplified products of each group were purified with High Pure PCR Product Purification Kit (Roche Diagnostics, Mannheim, Germany) and directly sequenced. Ten microliters of the PCR reaction were directly digested with the *Sac*I enzyme (MBI Fermentas, Vilnius, Lithuania) in a total volume of twenty microliters, following the manufacturer's

instructions. All the digestion reaction was analysed in a 5% TAE polyacrilamide gel and stained with ethidium bromide.

Dot-blot hybridization

Dot-blot hybridization was used to compare the sensitivity and the end point detection limit with the multiplex RT-PCR detection method as described by Sanchez-Navarro *et al.* (1998). Total RNA extractions of three field samples were five-fold diluted using total RNA extracted from healthy tissue. The undiluted sample corresponded to 0.05 g of infected tissue per ml. One microliter of the non-diluted RNA extraction and of each dilution was first denatured with formaldehyde and then directly applied to nylon membrane (Más *et al.*, 1993). Analysis of total nucleic acids by non-isotopic dot-blot hybridization was performed as described previously by Sánchez-Navarro *et al.* (1998) using a dig-RNA probe complementary to a fragment of RdRp of PepMV.

RESULTS

Detection of groups of PepMV isolates with the multiplex RT-PCR

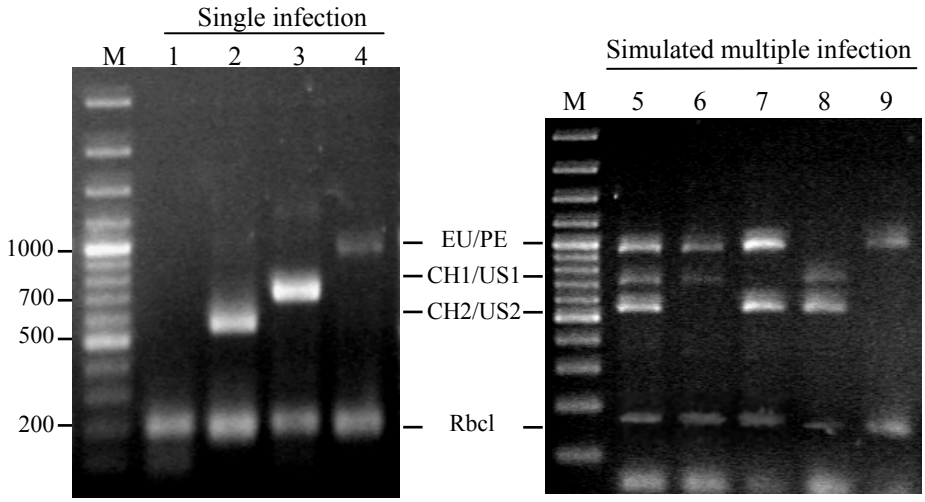
A multiplex RT-PCR reaction with a cocktail of primers PepMV-DEP, PepMV-D1, PepMV-D2 and PepMV-R (Figure 3.1), including the primers to amplify the internal control, was first used to analyse different tomato tissues infected with the PepMV isolates DSMZ PV-0632, PepMV-Can1 and DSMZ PV-0730 representative of the CH2/US2, CH1/US1 and EU/PE groups, respectively. Electrophoretical analysis revealed three PCR products of 549 bp, 703 bp and 980 bp that correspond to the three PepMV groups, plus the internal

control (Figure 3.2a, lanes 2, 3 and 4). In a second step, we analyzed the capacity of the multiplex reaction to amplify the corresponding PCR fragments in a mixed infection. To do this, we obtained simulated multiple infections by combining the total RNA of samples analyzed in lanes 2, 3 and 4 of Figure 3.2a. All simulated infections carrying two or three isolates of the three PepMV groups were clearly detected (Figure 3.2a, lanes 5-8). In addition, we analyzed a sample carrying the two PepMV isolates of the EU/PE group. We observed a single PCR amplicon of the expected size (980 bp, Figure 3.2a, lane 9) that rendered the corresponding fragments for the two isolates when it was digested with the *Sac*I enzyme (see below). All analyzed samples showed the internal control amplification fragment with the expected size of 186 pb. No extra bands interfering with the specific virus DNA fragment were detected. In addition to the previously analysed PepMV isolates originating from Spain (PepMV-Can1), Italy (DSMZ PV-0632) and the Netherlands (DSMZ PV-0730) (Figure 3.2a), we decided to use the multiplex RT-PCR to analyze several isolates of PepMV from Peru (DSMZ PV-0554), United Kingdom (DSMZ PV-0674), Italy (DSMZ PV-0716) and Poland (PK), together with the Spanish EU isolates Mu 00.2, CI 01.1, Ba 03.1 and Sp-13 (Pagán *et al.* 2006). All the isolates rendered a unique DNA fragment corresponding to either the EU/PE or the CH2/US2 groups (Figure 3.2b, lanes 1-8). With the exception of the Spanish PepMV-Can1 isolate, the rest of isolates originating from Spain, Peru, The Netherlands, and Great Britain were classified in the EU/PE group, whereas isolates from Italy and Poland were in the CH2/US2 group. All the EU or CH2 isolates previously classified by Pagán *et al.* (2006) or Hasiów *et al.* (2007) rendered the expected amplicon of 980 and 549 bp, respectively.

Identification of the specific PepMV isolate by restriction analysis

The EU, PE, CH2, CH1/US1 or US2 isolates of PepMV could be identified by direct treatment of the multiplex RT-PCR reaction with the *Sac*I enzyme (Figure 3.1 and Table 3.2). To observe the different DNA fragments obtained in a multiple infection we performed the multiplex RT-PCR analysis plus the *Sac*I digestion of two simulated samples containing total RNA of samples infected with PE, EU and CH2 or EU, CH2 and CH1/US1 isolates (Figure 3.3a and b, respectively). Figure 3.3 shows the analysis of the *Sac*I digestion in which the representative DNA fragments of the PE, EU, CH2 and CH1/US1 are indicated. Furthermore, we decided to identify the isolate of PepMV present in all the samples analyzed in Figure 3.2. Figure 3.3c shows the analysis of RT-PCR-*Sac*I. All the samples contained a unique isolate corresponding to the EU (samples: DSMZ PV-0674, DSMZ PV-0730, Mu 00.2, CI 01.2, Ba 03.1, Sp-13), CH2 (samples: DSMZ PV-0632, DSMZ PV-0716 and PK), PE (sample DSMZ PV-0554) or CH1/US1 (sample PepMV-Can1). All the PepMV isolates previously classified as EU (Pagán *et al.*, 2006) rendered the expected fragment of 684 bp (Figure 3.3c, lanes 8-11). All the analyzed samples rendered one of the predicted DNA fragments for the corresponding PepMV isolate.

(a)


Single infection
M 1 2 3 4
Simulated multiple infection
M 5 6 7 8 9
1000
700
500
200
EU/PE
CH1/US1
CH2/US2
Rbcl

(b)

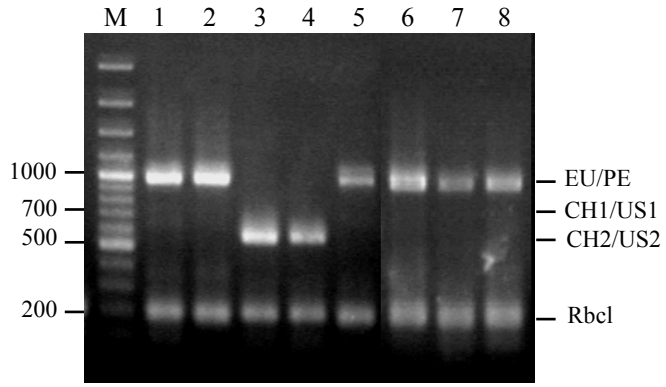

M 1 2 3 4 5 6 7 8
1000
700
500
200
EU/PE
CH1/US1
CH2/US2
Rbcl

Figure 3.2. Analysis by one-step multiplex RT-PCR of tomato samples infected with PepMV genotypes representative of the EU/PE, CH1/US1 or CH2/US2 groups. The multiplex RT-PCR analysis was performed by using a cocktail of primers listed in Figure 1 plus two primers target to the ribulose 1.5-biphosphate carboxylase chloroplast gene (0.05 pmol/µl) as an internal control (Sanchez-Navarro *et al.,* 2006). **(a)** Analysis of tomato samples infected with PepMV isolates representatives of CH2/US2 (lane 2, DSMZ PV-0632) , CH1/US1 (lane 3, PepMV-Can1) or EU/PE (lane 4, DSMZ PV-0730) groups; lane 1 corresponds to healthy tomato. **(b)** Analysis of simulated multiple infections created by mixing single infection extracts of samples analyzed in lanes 1-3. Lane 5: triple infection; lanes 6-8: double infection carrying the three possible combinations; lane 9: double infection of EU (DSMZ PV-0730) and PE (DSMZ PV-0554) isolates. **(c)** Analysis of tomato samples infected with PepMV isolates originating from Peru (lane 1, isolate DSMZ PV-0554), United Kingdom (lane 2, isolate DSMZ PV-0674), Italy (lane 3, isolate DSMZ PV-0716), Poland (lane 4, isolate PK) and Spain (lanes 5-8, isolates Mu 00.2, CI 01.2, Ba 03.1 and Sp-13). The amplicons corresponding to the EU/PE, CH1/US1 and CH2/US2 PepMV groups plus the internal control (Rbcl) are indicated. Lanes M: 100 bp molecular weight marker.

Sensitivity of one-step multiplex RT-PCR-*SacI* and comparison with one-step single RT-PCR, dot-blot hybridization and ELISA

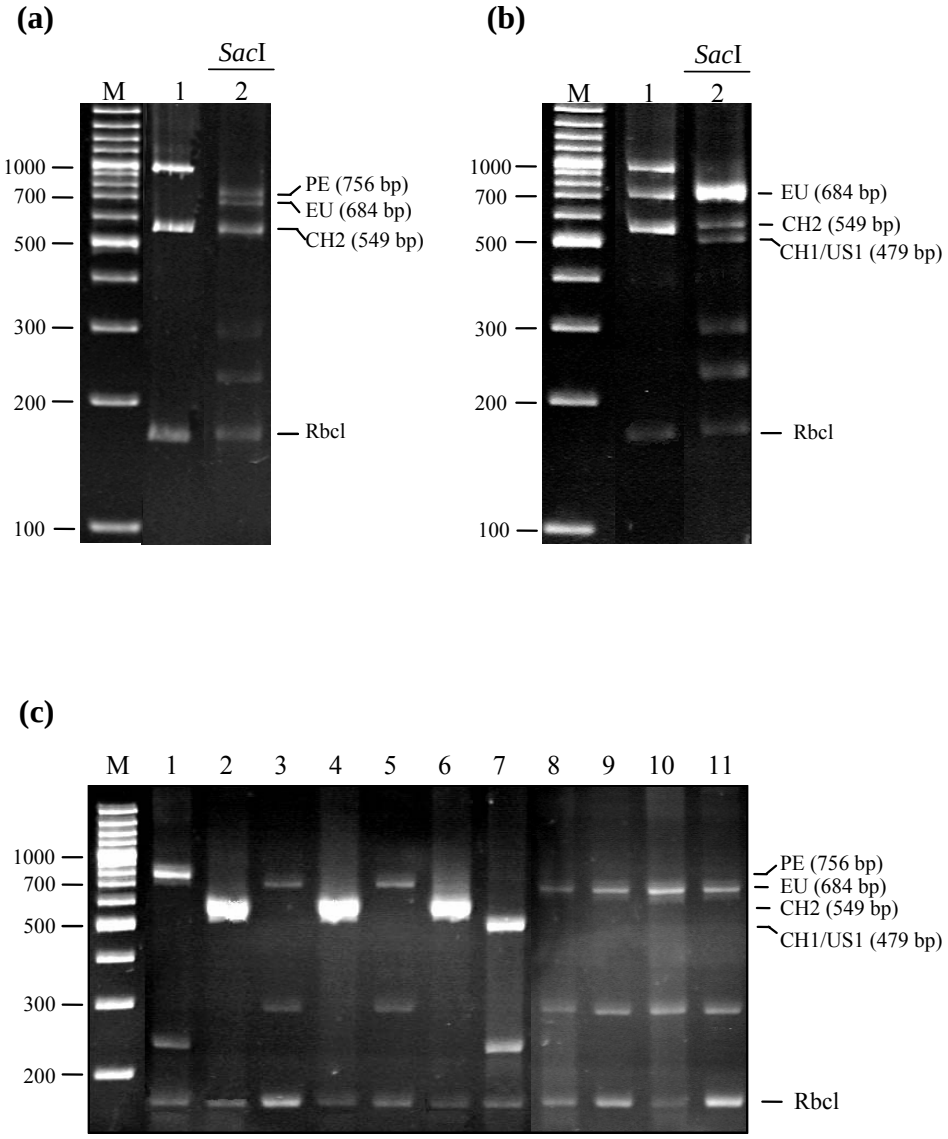
The simultaneous amplification of several amplicons together with the use of a cocktail of primers could affect the detection limit of the multiplex RT-PCR reaction. To analyse how both aspects influence the sensitivity of the multiplex reaction we have analyzed, by either multiplex or single RT-PCR, a serially diluted tomato sample infected with two isolates of PepMV assigned to the EU/PE or CH2/US2 groups. The dilutions were performed by using healthy tomato extract in order to reduce the virus titer. To have a direct comparison between the main virus detection techniques, the same or comparable extract dilutions, in terms of grams of infected tissue per mL, were analyzed by dot-blot hybridization and ELISA, respectively. Figure 3.4 shows the detection limit obtained with all the techniques. The end point dilution limit observed in the multiplex RT-PCR was 5^{-7} (0.64 g/mL) and 5^{-6} (3.2 g/mL) for the EU/PE or CH2/US2 isolates, respectively (Figure 3.4a).

Figure 3.3. Restriction analysis of the multiplex RT-PCR products. Simulated triple infection extracts created by mixing total RNA extracted from tomato samples infected with PepMV isolates EU, PE and CH2 **(a)** or EU, CH2 and CH1/US1 **(b)** were subjected to multiplex RT-PCR and direct *Sac*I restriction digestion. Lane 1, undigested sample; lane 2, *Sac*I digestion products. **(c)** *Sac*I restriction patterns exhibited by amplified PCR products of tomato samples analyzed in Figure 2. Lane 1: DSMZ PV-0554; lane 2: DSMZ PV-0632 ; lane 3: DSMZ PV-0674 ; lane 4: DSMZ PV-0716 ; lane 5: DSMZ PV-0730 ; lane 6: PK ; lane 7: PepMV-Can1 ; lane 8: Mu 00.2 ; lane 9: CI 01.2 ; lane 10: Ba 03.1; lane 11: Sp-13. The DNA fragments representative of each PepMV isolate are indicated (see Table 2). Lanes M: 100bp molecular weight marker. Numbers represent the base pairs (bp) of the DNA fragment. The analyses were performed in 5% TAE poliacrylamide gels stained with ethidium bromide.

The same detection limits were obtained using a single RT-PCR in which we included only the pair of primers required to amplify either the EU/PE or CH2/US2 isolates (Figure 3.4b and c). Apparently, neither the multiple infections nor the presence of a cocktail of six different primers affect the limit detection of the multiplex reaction, regardless of the amount of EU/PE or CH2/US2 isolate present in the infected tissue. When the serial dilutions were analysed by dot-blot hybridization or ELISA, PepMV was detected up to the 5^{-2} dilution (2 mg/mL; Figure 3.4d and e), representing a detection limit 3125 (5^5 times) less sensitive than the multiplex RT-PCR.

Analysis of field samples and previously characterized PepMV isolates by both multiplex RT-PCR-*SacI* and RT-PCR-Hanssen, dot-blot hybridization and ELISA tests.

The robustness of the multiplex RT-PCR-*Sac*I to be used in routine diagnosis was analyzed by performing the analysis of several PepMV isolates from si different countries previously described (samples 1-17) plus 42 field tomato samples harvested in three different Spanish regions (samples 18-59).


(a)
SacI
M
1
2
1000
700
500
300
200
100
PE (756 bp)
EU (684 bp)
CH2 (549 bp)
RbcI
(b)
SacI
M
1
2
1000
700
500
300
200
100
EU (684 bp)
CH2 (549 bp)
CH1/US1 (479 bp)
RbcI
(c)
M
1
2
3
4
5
6
7
8
9
10
11
1000
700
500
300
200
PE (756 bp)
EU (684 bp)
CH2 (549 bp)
CH1/US1 (479 bp)
RbcI

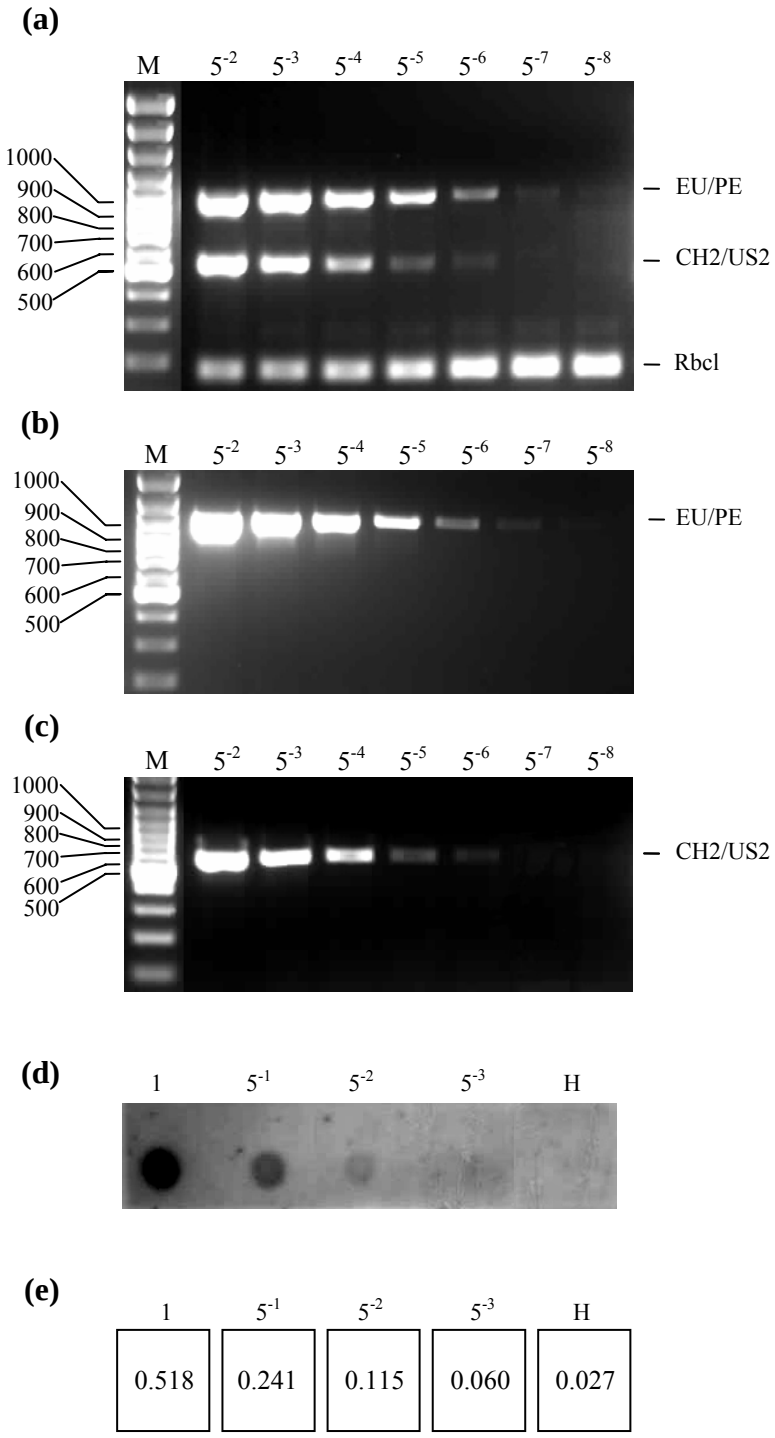

(a)
M
5-2
5-3
5-4
5-5
5-6
5-7
5-8
1000
900
800
700
600
500
EU/PE
CH2/US2
Rbcl
(b)
M
5-2
5-3
5-4
5-5
5-6
5-7
5-8
1000
900
800
700
600
500
EU/PE
(c)
M
5-2
5-3
5-4
5-5
5-6
5-7
5-8
1000
900
800
700
600
500
CH2/US2
(d)
1
5-1
5-2
5-3
H
(e)
1
5-1
5-2
5-3
H
0.518
0.241
0.115
0.060
0.027

Figure 3.4. Comparison of the sensitivity detection limit for PepMV detection by multiplex or single RT-PCR, nonisotopic molecular hybridization and DAS-ELISA. Total RNA extracted from a tomato sample infected with two PepMV isolates representative of the EU/PE and CH2/US2 groups was fivefold serially diluted using total RNA extracted form healthy tomato. Fivefold serially dilutions were analyzed by multiplex RT-PCR **(a)**, single RT-PCR using the specific primers for the EU/PE **(b)** or CH2/US2 **(c)** PepMV genotypes, and nonradioactive molecular hybridization **(d).** In **(e),** comparable fivefold dilutions series in phosphate buffer of the same tomato infected sample were analyzed by ELISA. Each point is the mean of three replications. The last positive signal for the EU/PE or CH2/US2 isolates was 5^{-7} (1:78125) in a and b or 5^{-6} (1:15625) in a and c, respectively. In d and c, the last dilution with a positive signal corresponded to 5^{-2} (1:25). The original dilution (1) corresponded to a tissue concentration of 0.05 g/mL. The PCR products corresponding to the EU/PE, CH2/US2 PepMV isolates or the internal control (Rbcl) are indicated. H, healthy tomato plant. Numbers at the top indicate the dilution performed on the original undiluted sample. Lanes M: 100 bp molecular weight marker.

Total RNA, extracted as described above, was used to perform multiplex RT-PCR and dot-blot hybridizations analysis. Comparable extracts in terms of grams of tissue per mL were analyzed by ELISA procedure. In addition, the multiplex RT-PCR-*Sac*I was compared with the RT-PCR-Hanssen. Briefly, this methodology consisted in the amplification by RT-PCR with two pairs of primers common to all the genotypes of two different fragments of PepMV genome: a part of the RpRd and the complete CP gene. Later, an RFLP analysis with two and four restriction endonucleases for RdRp and CP amplified PCR products, respectively, was performed and theoretically five different genotypes could be distinguished. The comparison between both methods is detailed in Table 3.3.

Table 3.3. Comparative analysis for the presence of PepMV performed by DAS-ELISA, Molecular Hybridization (MH), multiplex RT-PCR with *SacI* restriction (RT-PCR-*Sac*I) and RFLP-PCR method described by Hanssen *et al.* (2008) (RT-PCR-Hanssen) to previously described PepMV isolates and field tomato samples.

	Sample code	Area	Variety	ELISA	MH	RT-PCR-*Sac*I			RT-PCR-Hanssen		
1	PV-0632	Italy	Unknown	+	+	CH2	-	-	nd[a]	-	-
2	PV-0674	Great Britain	Unknown	+	+	-	-	EU	-	-	EU
3	PV-0716	Sardinia, Italy	Unknown	+	+	CH2	-	-	nd[a]	-	-
4	PV-0730	The Netherlands	Unknown	+	+	-	-	EU	-	-	EU
5	PV-0554	Peru	*S. muticatum*	+	+	-	-	PE	-	-	PE
6	PK	Poland	Unknown	+	+	CH2	-	-	CH2[b]	-	-
7	Mu 00.2	Murcia, Spain	Unknown	+	+	-	-	EU	-	-	EU
8	Mu 00.3	Murcia, Spain	Unknown	+	+	-	-	EU	-	-	EU
9	Mu 00.4	Murcia, Spain	Unknown	+	+	-	-	EU	-	-	EU
10	Mu 00.5	Murcia, Spain	Unknown	+	+	-	-	EU	-	-	EU
11	CI 01.1	Canary Islands, Spain	Unknown	+	+	-	-	EU	-	-	EU
12	CI 01.2	Canary Islands, Spain	Unknown	+	+	-	-	EU	-	-	EU
13	CI 01.3	Canary Islands, Spain	Unknown	+	+	-	-	EU	-	-	EU
14	Al 01.2	Alicante, Spain	Unknown	+	+	-	-	EU	-	-	EU
15	Ba 03.1	Barcercelona, Spain	Unknown	+	+	-	-	EU	-	-	EU
16	Can-1	Tenerife, Spain	Unknown	+	+	-	CH1/US1[e]	-	-	CH1/US1	-
17	Sp-13	Spain	Unknown	+	+	-	-	EU	-	-	EU
18	9394	Arico (Tenerife, Spain)	Unknown	-	-	CH2	-	EU	CH2	-	EU
19	9405	Arico (Tenerife, Spain)	Mariana	+	+	-	CH1/US1[e]	EU		nd	EU
20	9415	Arico (Tenerife, Spain)	Unknown	-	-	-	CH1/US1[d]	EU		nd	EU
21	9416	Arico (Tenerife, Spain)	Unknown	-	-	-	-	EU	-	-	EU
22	9423	Arico (Tenerife, Spain)	Mariana	+	+	-	CH1/US1[d]	EU		nd	EU

Table 3.3. *(Continuation)*

	Sample code	Area	Variety	ELISA	MH	RT-PCR-*Sac*I			RT-PCR-Hanssen		
23	9432	Arico (Tenerife, Spain)	Boludo	+	+	-	-	EU[e]	-	-	EU
24	9433	Arico (Tenerife, Spain)	Boludo	-	-	-	-	-	-	-	-
25	9435	Arico (Tenerife, Spain)	Boludo	+	+	-	-	EU[f]	-	-	EU
26	9453	Agüimes (Gran Canaria, Spain)	Boludo	-	-	CH2	-	EU	CH2	-	EU
27	9454	Agüimes (Gran Canaria, Spain)	Boludo	-	-	CH2	-	EU	CH2	-	EU
28	9494	S. Lucía (Gran Canaria, Spain)	Unknown	+	+	-	CH1/US1[e]	EU*		nd	EU
29	9495	Los abrigos (Tenerife, Spain)	Unknown	+	+	-	CH1/US1[e]	EU		nd	EU
30	9503	Los abrigos (Tenerife, Spain)	Unknown	+	+	-	CH1/US1[e]	EU*		nd	EU
31	9506	Los abrigos (Tenerife, Spain)	Unknown	+	+	-	CH1/US1[c,e]	EU		nd	EU
32	9507	Abades (Tenerife, Spain)	Dorothy	-	-	-	-	-	-	-	-
33	9509	Abades (Tenerife, Spain)	Dorothy	-	-	-	-	-	-	-	-
34	9511	Abades (Tenerife, Spain)	Dorothy	-	-	CH2	-	EU	CH2	-	EU
35	9516	Tenerife, Spain	Dorothy	+	+	-	-	EU	-	-	EU
36	9527	Tenerife, Spain	Unknown	-	-	-	CH1/US1[e]	EU		nd	EU
37	9533	Tamaimo (Tenerife, Spain)	Boludo	-	-	CH2	CH1/US1	EU	CH2	CH1/US1	EU
38	9537	Tamaimo (Tenerife, Spain)	Mariana	-	-	-	-	-	-	-	-
39	9538	Tamaimo (Tenerife, Spain)	Mariana	-	-	CH2	-	EU	CH2	-	EU
40	9539	Tamaimo (Tenerife, Spain)	Mariana	-	-	-	-	-	-	-	-
41	9551	Tamaimo (Tenerife, Spain)	Maya	-	-	-	-	-	-	-	-
42	9636	Águilas (Murcia, Spain)	Boludo	-	-	CH2	-	EU	CH2	-	EU
43	9641	Mazarrón (Murcia, Spain)	Unknown	+	+	CH2[e]	-	-	CH2	-	-
44	9645	Mazarrón (Murcia, Spain)	Unknown	+	+	CH2	-	EU	CH2	-	EU
45	9646	Mazarrón (Murcia, Spain)	Unknown	+	+	CH2	-	EU	CH2	-	EU
46	9647	Mazarrón (Murcia, Spain)	Unknown	+	+	-	-	EU	-	-	EU
47	9648	Mazarrón (Murcia, Spain)	Thomas	+	+	CH2	-	EU	CH2	-	EU

Table 3.3. *(Continuation)*

Sample code		Area	Variety	ELISA	MH	RT-PCR-*Sac*I			RT-PCR-Hanssen		
48	9649	Mazarrón (Murcia, Spain)	Thomas	+	+	CH2[e]	-	EU[e]	CH2	-	EU
49	9653	Mazarrón (Murcia, Spain)	Thomas	+	+	CH2	-	EU	CH2	-	EU
50	9654	Mazarrón (Murcia, Spain)	Pitenza	+	+	CH2	-	EU	CH2	-	EU
51	9658	Mazarrón (Murcia, Spain)	Thomas	+	+	CH2	-	EU	CH2	-	EU
52	9659	Mazarrón (Murcia, Spain)	Thomas	+	+	CH2	-	EU	CH2	-	EU
53	9661	Mazarrón (Murcia, Spain)	Thomas	+	+	CH2	-	EU	CH2	-	EU
54	9662	Mazarrón (Murcia, Spain)	Unknown	+	+	-	-	EU	-	-	EU
55	9665	Mazarrón (Murcia, Spain)	Unknown	-	+	CH2	-	EU	CH2	-	EU
56	9674	Lorca (Murcia, Spain)	Flortyl	+	+	CH2	-	EU	CH2	-	EU
57	9679	Lorca (Murcia, Spain)	Velasco	-	-	CH2	-	-	CH2	-	-
58	9680	Lorca (Murcia, Spain)	Unknown	-	-	CH2	-	EU	CH2	-	EU
59	9682	Lorca (Murcia, Spain)	Flortyl	+	+	CH2	-	EU	CH2	-	EU
Partial positive results of the previously characterized PepMV isolates (samples 1-17)				17	17	3	1 17	12/1[g]	1	1 17	12/1[g]
Partial positive results of the field samples (18-59)				23	24	22	9 36	34	22	1 36	34
Total positive results				40	41	25	10 53	46/1[g]	23	2 53	46/1[g]

nd: pattern not described by Hanssen *et al*. (2008).
[a] Amplified fragment from the CP gene, directly sequenced with the primers described by Pagán *et al*. (2006).
[b] Result confirmed by RT-PCR for the CP gene with specific primer for CH2/US2 isolates sPepMVCP CH2/US2 and common reverse primer (Pagán *et al.,* 2006).
[c] Samples tested p ositive to CH1/US1 isolates analysed by multiplex RT-PCR, however that isolate was not determined when analysed with Hanssen *et al.* (2008). Result was confirmed by RT-PCR for the coat protein gene with specific primer for CH1/US1 isolates sPepMVCP CH1/US1, and common reverse primer (Pagán *et al,*. 2006), cloned and sequenced.
[d] Samples tested positive to CH1/US1 isolates analysed by multiplex RT-PCR, however that isolate was not determined when analysed with Hanssen *et al.* (2008). Result was not determined with the analysis by RT-PCR for the coat protein gene with specific primer for CH1/US1 isolates sPepMVCP CH1/US1 and common reverse primer (Pagán *et al*., 2006).
[e] PCR products amplified with the mixture of all the primers of the multiplex RT-PCR, purified and directly sequenced.
[f] Result confirmed by RT-PCR for the CP gene with specific primer for EU isolates sPepMVCP EU and common reverse primer (Pagán *et al.*, 2006).
EU*: Amplified fragment of 980 bp not digested with *Sac*I enzyme. Sequenced and compared with isolates of GenBank Database being grouped with the EU isolates.
[g] Number of EU/number of PE isolates.

Regarding the PepMV isolates previously characterized (samples 1-17, Table 3.3), all diagnosis techniques were able to detect PepMV. Both RT-PCR-*Sac*I and RT-PCR-Hanssen identified the same PepMV isolates except for the samples DSMZ PV-0632 and DSMZ PV-0716. The PepMV isolates present in both samples were identified as CH2 by RT-PCR-*Sac*I meanwhile the RT-PCR-Hanssen rendered a restriction pattern of the CP amplified product that was not associated with any of the described PepMV isolates. The analysis of the nucleotide sequence of the CP-amplified products showed a change at nucleotide 5711 (in a CH2 genome) responsible of the unclassified restriction pattern (GenBank Accession number DQ000985). However, the CP sequences of PepMV isolates DSMZ PV-0632 and DSMZ PV-0716 showed 98% nt identity with CH2 and US2 isolates (GenBank accession numbers DQ000985 and AY509927).

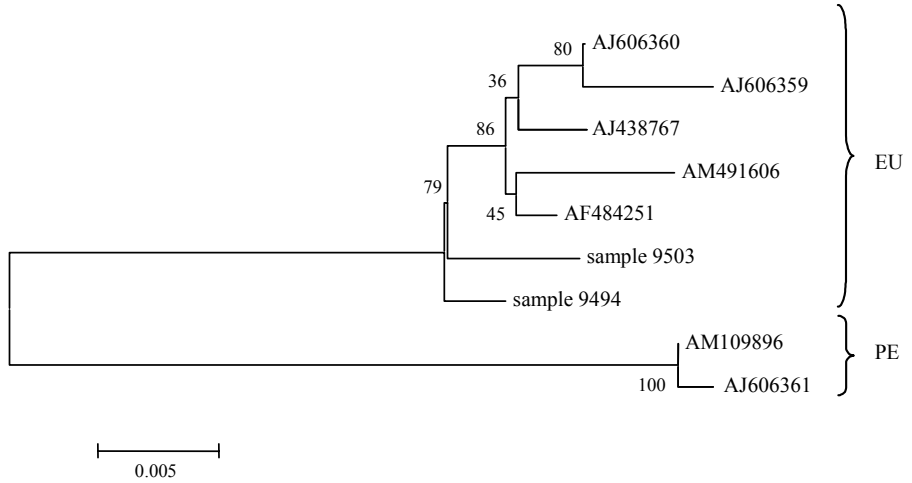
Regarding the field tomato samples we obtained 23, 24 or 36 samples out of 42 positive by ELISA, dot-blot hybridization and multiplex RT-PCR, respectively (Table 3.3; samples 18-59). All positive samples detected by ELISA and dot-blot were also detected by multiplex RT-PCR-*Sac*I. Considering the total number of positive samples detected by multiplex RT-PCR-*Sac*I, the percentage of positives detected by ELISA and dot-blot corresponded to the 63.8 and 66.6% respectively. The capacity of the multiplex reaction to discriminate among the five PepMV genotypes allows us to identify the corresponding isolates present in the infected samples. Thus, 8 samples out of 36 were single infected meanwhile 27 and 1 sample(s) were double- and triple-infected, respectively. The majority of the single-infected samples presented the EU isolate (6 out of 8) the remainder being infected with the CH2 (2 out of 8, respectively). For the double-infection we found two different combinations: CH2 with EU (19 out 27) or CH1/US1 with EU (8 out 27). Interestingly, the two double infection combinations corresponded to two different geographic regions

in which the presence of CH2 and EU isolates was representative of the Murcia area, whereas the combination CH1/US1 with EU was specific of the Canary Islands (Gran Canaria and Tenerife). One sample from Tenerife (Canary Islands) presented a PepMV infection with three isolates that corresponded to CH2, CH1/US1 and EU genotypes. All positive samples rendered the expected DNA fragment after the *Sac*I digestion during the identification of the corresponding PepMV genotype except two samples from Gran Canaria and Tenerife islands (sample numbers 9494 and 9503). In both cases, the amplified fragment of 980 bp characteristic of the EU/PE group was not digested with the *Sac*I enzyme. The PCR products were sequenced and compared to PepMV isolates representative of both EU and PE genotypes, showing 98-97% nt identity with isolates of the European tomato strain published in the GenBank database (Accession numbers AF484251, AJ606360). Figure 3.5a shows the phylogenetic analysis in which both isolates grouped with the EU isolates. To confirm the correct classification of PepMV isolates, PCR products of isolates classified to the three different genotypes found in the field samples, were sequenced and compared with the PepMV isolates published in GenBank (Table 3.3). Two EU isolates (sample numbers 9432 and 9649) showed 98% nt identity with isolates of the European tomato strain (GenBank accession numbers AF484251, AJ606360 and AJ438767). Two isolates (sample number 9506 and PepMV-Can1 isolate), that were classified as CH1/US1, showed 99% nt identity with the US1 (GenBank accession number AY509926) and CH1 (GenBank accession number DQ000984) sequences, respectively. Two isolates (sample numbers 9641 and 9649) classified as CH2 showed 99-98% nt identity with a recently published Belgian isolate (GenBank accession number EF599605), CH2 and PK isolates (GenBank accession numbers DQ000985 and EF408821).

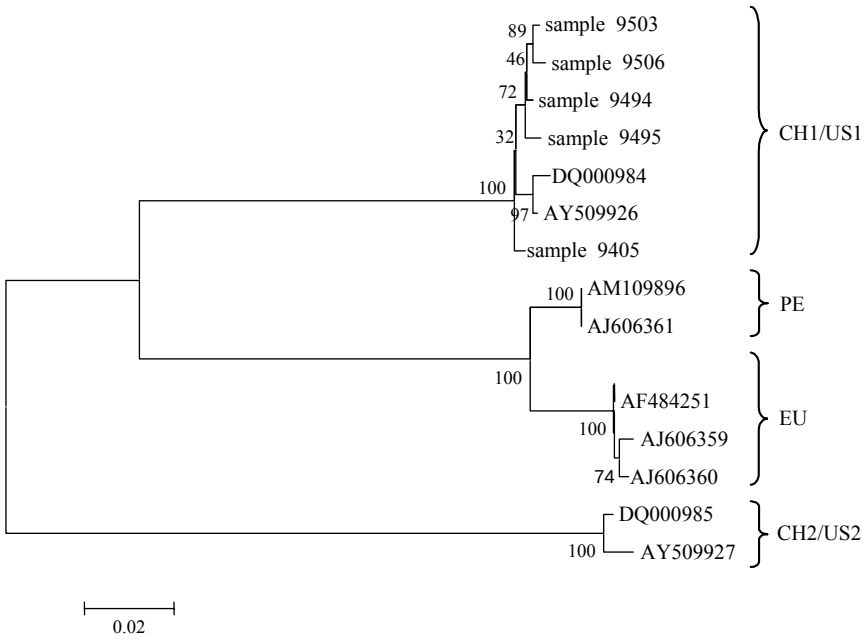
Finally, all field tomato samples were analysed by RT-PCR-Hanssen method (Table 3.3). Results were similar in 34 out of the 42 samples analysed;

however the CH1/US1 isolate identified by RT-PCR-*Sac*I restriction analysis in 8 out of the 42 samples was not determined by RT-PCR-Hanssen method. In order to check the presence or absence of that isolate in these samples, an RT-PCR with sense specific primers designed for the CP gene of PepMV groups CH1/US1 (sPepMV CP CH1/US1), EU/PE (sPepMV EU) and CH2/US2 (sPepMV CH2/US2) and the common reverse primer described by Pagán *et al.* (2006) was performed. Previous results obtained with single-infected samples representatives of the three PepMV groups (samples 9435 for the EU group, Can-1 for the CH1/US1 and PK for the CH2/US2; Table 3.3) showed the specificity of the sense primers. Only the specific CP amplicon of 770 bp corresponding to the CH1/US1 isolate was obtained in 5 out of those 8 isolates. PCR products were cloned and sequenced. The phylogenetic analysis grouped all amplified CP fragments with the CH1/US1 strain as show in Figure 3.5b. In the rest of the samples (3 out of 8) which resulted positive to CH1/US1 by multiplex RT-PCR-*Sac*I, but were not determined by RT-PCR-Hanssen, there was no amplification with the specific primers for CH1/US1 CP gene (Table 3.3).

(a)


80
AJ606360
36
AJ606359
86
AJ438767
AM491606
79
45
AF484251
sample 9503
sample 9494
EU
AM109896
100
AJ606361
PE
0.005

(b)


89
sample 9503
46
sample 9506
72
sample 9494
32
sample 9495
CH1/US1
100
DQ000984
97
AY509926
sample 9405
100
AM109896
AJ606361
PE
100
AF484251
100
AJ606359
74
AJ606360
EU
DQ000985
100
AY509927
CH2/US2
0.02

◄ **Figure 3.5. (a)** Phylogenetic analysis of the EU/PE isolates lacking the *Sac*I restriction site. The analysis was performed using the nucleotide sequence of 980 nt (nt 1563 – 2543 in the PepMV genome) corresponding to the specific PCR product of the EU/PE genotype of the RdRp gene. Nucleotide sequences corresponded to EU (GenBank accession numbers. AJ606360, AJ606359, AJ438767, AM491606 and AF484251) and PE (GenBank accession numbers AM109896 and AJ606361) genotypes, plus the two tomato samples 9494 and 9503. **(b)** Phylogenetic analysis of the CH1/US1 isolates not determined by RFLP-PCR method described by Hanssen *et al.* (2008). The analysis was performed using the CP nucleotide sequence of 770 nt (nt 5593 – 6369 in the PepMV genome). Nucleotide sequences corresponded to EU (GenBank accession numbers. AJ606360, AJ606359 and AF484251), PE (GenBank accession numbers AM109896 and AJ606361), CH1 (GenBank accession number DQ000984), US1 (Accession number AY509926), CH2 (GenBank accession number DQ000985) and US2 (GenBank accession number AY509927) genotypes, plus the five tomato samples 9405, 9494, 9495, 9503 and 9506. Nucleotide sequences were aligned by with CLUSTAL-X version 1.83 with its default parameters (Thompson *et al.* 1997). The neighbor-joining phylogenetic trees were obtained using the MEGA version 3.1. (Kumar *et al.,* 2004). The statistical reliability of the constructed trees was assessed by the bootstrap method based on 10,000 pseudoreplicates. The number above the nodes indicates the percentage of bootstrap replicates which supported the branching.

DISCUSSION

The increasing number of PepMV genotypes characterised so far together with the observation that mixed infection is a common phenomenon in field samples (herein and Hanssen *et al.,* 2008) that could result in more severe symptoms and/or recombination events, entirely justify the incorporation of a routine detection method that permits the identification of the corresponding PepMV genotype. In the present work we have used the variability observed in the polymerase gene to design specific primers for the CH2/US2, CH1/US1 and EU/PE groups. In addition, the presence or absence of different *Sac*I restriction

sites in the amplified DNA fragments permits the identification of the CH2, US2, CH1/US1, EU and PE isolates.

So far, there is only a methodology available based on a single RT-PCR-RFLP assay that was adjusted to simultaneous detection of the five different genotypes (Hanssen *et al.,* 2008). However, this approach implies the amplification of two different coding sequences (polymerase and coat protein genes) that should be digested by six different restriction enzymes. The new approach presented here significantly reduces the analytical process since it is a multiplex reaction in which the RT-PCR products permit discriminate between EU/PE, CH1/US1 and CH2/US2 PepMV groups and only a *Sac*I endonuclease is required to discriminate between EU-PE or CH2-US2 genotypes. The comparison between both RFLP-PCR methods revealed similar results, however multiplex RT-PCR-*Sac*I resulted more sensitive to identify the CH1/US1 isolate in 8 out 42 field samples. This observation was confirmed in five samples by sequencing the CP gene. This difference could be caused by an unequal concentration between CH1/US1 and EU isolates present in mixed infected samples. Therefore, an RT-PCR with common primers, used in Hanssen *et al.* (2008) method, will amplify more efficiently in mixed infections the isolate present at higher concentration, a disadvantage that could be overcome by using specific primers for each PepMV strains. However, we found three samples that were classified as CH1/US1 plus EU by RT-PCR-SacI and were negative by using the specific CH1/US1 CP primers. This pattern could be explained by variation in the CP gene or by the presence of recombinants isolates between CH1/US1 and EU strains. The analysis of such samples by RT-PCR-Hanssen using both the RdRp and CP genes, detected only the EU strain, indicating the absence of recombinant isolates. This result reinforces the idea that the inability to amplify the CH1/US1 CP gene is due to nucleotide changes. In this sense, nucleotide variations in the CP gene have been found in the DSMZ-PV-0632

and DSMZ-PV-0716 samples, and were responsible for the unclassified CP restriction pattern obtained by RT-PCR-Hanssen.

The advantages mentioned above for the RT-PCR-*Sac*I approach are completed by the incorporation of two primers target to a host mRNA as an internal control, an aspect that avoids the presence of false negatives. However, unlike the rest of PepMV genotypes, CH1 and US1 could not be discriminated by restriction analysis due to the high nucleotide sequence identity among them (98.7%) (Ling, 2007). All together, the direct comparison between both RT-PCR-RFLP and RT-PCR-*Sac*I techniques, presents the new multiplex reaction as a more simple, specific and sensitive methodology, being a clear alternative for the previous RT-PCR-RFLP assay. Other methods have been developed for diagnosis of PepMV infection as the one-step immunocapture real-time TaqMan RT-PCR assay (Ling *et al.* 2007), which is a high sensitive technique capable of detecting all the genotypes of PepMV in one single reaction. The new multiplex RT-PCR-*Sac*I method could be perfectly compatible with the previous Real time RT-PCR since the latter should be the option for routine diagnosis whereas the multiplex RT-PCR is more appropriate technique for epidemiological survey. In addition, the observation of a high percent of PepMV mix infections in the field make the new RT-PCR-*Sac*I assay even more necessary to reduce the risk of severe PepMV symptoms and/or recombination events.

To validate the new approach we have analyzed different aspects that should be taken into consideration before incorporating it in routine diagnosis. First, we have analyzed the specificity of the reaction in either single or multiple infections. All the analyzed samples infected with the previously characterized PepMV isolates rendered the expected DNA fragments with a good discrimination among them, even between the EU and PE isolates that share a nucleotide identity of 95%. No extra bands were observed in single-infected plants indicating the specificity of the selected primers. The same results were

obtained when we analyzed simulated multiple infections. The reliability of the method was demonstrated by sequencing the obtained amplicons that were always in agreement with the corresponding sequences published in GenBank database. We decided to use the new approach to characterize the genotype of previously reported PepMV isolates from different European origins (The Netherlands, Italy, Poland, UK and Spain). All the samples rendered a unique genotype that corresponded to the EU and CH2 isolates, except for the Spanish PepMV-Can1, that was classified as CH1/US1. According to this result, the nucleotide sequence of the amplicon derived from the PepMV isolate PepMV-Can1 presented a nucleotide identity of 99% with the US1 and CH1. A similar result has been reported by using another region of the polymerase gene (Alfaro-Fernández *et al.,* 2008).

Another critical aspect for a detection procedure is the detection limit. Previous results obtained by multiplex RT-PCR assay have shown that a cocktail of seven pairs of primers affect the detection limit (Sánchez-Navarro *et al.,* 2006). Although the multiplex RT-PCR procedure presented herein contains a cocktail of six different primers, no differences in terms of detection limit were observed when a serially-diluted double-infected sample was analyzed by either the multiplex (containing a cocktail of six primers) or the single (containing two primers) RT-PCR. When the similarly diluted samples were analyzed by ELISA or dot-blot hybridization, the detection limit of the multiplex reaction was 3125 (5^5) times higher, a difference that is in the range of previously reported results (Sánchez-Navarro *et al.,* 1998; Saade *et al.,* 2000).

To check for the reliability and the robustness of the new multiplex RT-PCR-*Sac*I developed a total of 42 tomato field samples from three different Spanish regions were analysed using DAS-ELISA, dot-blot hybridization and the multiplex RT-PCR-*Sac*I and RT-PCR-Hanssen. Except for one sample, ELISA and dot-blot hybridization were able to detect the same positive samples

confirming the previous observation that both procedures have a similar detection limit. The multiplex technique was able to not only identify the corresponding PepMV isolates, but also to detect 12 more positives than the serological and dot-blot methods. The multiplex procedure was not able to discriminate between the EU and PE isolates only in two double infected samples, since the amplicon corresponding to the EU/PE group was not digested with *Sac*I enzyme. The characterization of the nucleotide sequence of the amplicon and the posterior phylogenetic analysis revealed that both isolates were grouped to the EU genotypes. Apparently, the presence of a 980 bp or 684 bp fragment after the *Sac*I restriction is representative of the EU genotypes. It should be taken in consideration that both EU and PE isolates share the highest identity percentage (95%) among all PepMV isolates discriminated by the multiplex procedure.

The multiplex RT-PCR-*Sac*I allowed the identification of several double- and triple-infected plants. Interestingly, the majority of positive samples were double-infected (75%), in which the presence of the CH1/US1 with EU or the CH2 and EU genotypes were representative of the Canary Islands and Murcia Region, respectively. Field samples showed different combination of isolates commonly found in Europe. The EU genotype was the most prevalent since it was found in 94% of the infected plants, followed by the CH2 (61%) and CH1/US1 (25%) genotypes. A high percentage of double-infected plants was also detected in Belgium (Hanssen *et al.,* 2008) and Spain (Martínez-Culebras *et al.,* 2002; Pagán *et al.,* 2006), in which the only detected genotypes were CH2 and EU, although CH1/US1 has been recently reported in the Canary Islands, Spain (Alfaro-Fernández *et al.,* 2008a). Apparently, double-infected plants are more frequent than we expected, in which the CH2 and EU genotypes are representative of the European region. Since the EU genotype was the more prevalent PepMV genotype in European tomato crops (Aguilar *et al.,* 2002;

Cotillon *et al.,* 2002; Ling 2007), the question that arises is how and when the CH2 and CH1/US1 genotypes were introduced. In this context, we are performing a survey of tomato samples harvested from 2000.

The multiplex one-step RT-PCR procedure developed in this study represents a significant advance in the diagnosis of PepMV. The multiplex technology reduces costs, time and avoids the use of multiple digestions and/or the cloning and sequencing steps to identify the PepMV isolate. Furthermore, since multiple infections are present in the tomato crops, this technology will facilitate the characterization of the phytosanitary status of the tomato crops and the correlation between tomato disorders and the different PepMV isolate combinations.

ACKNOWLEDGEMENTS

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Capítulo 4

Preliminary attempts at the construction of an infectious GFP mutant clone of *Pepino mosaic virus*[*]

A. Alfaro-Fernández, C. Jordá, R. van der Vlugt

**This chapter is still unpublished, although parts of it will be used for future publications*

ABSTRACT

An infectious full-length cDNA clone of *Pepino mosaic virus* (PepMV) was constructed (pUS2-CSL). A fragment covering the full genome of the isolate US2-CSL (GenBank Accession No. FJ212288) was amplified by reverse transcription polymerase chain reaction using the 5´-end primer containing a T7 RNA polymerase promoter and ligated into the pCR-XL-TOPO vector. RNA transcripts from cloned full-length cDNAs were inoculated to *Nicotiana occidentalis* 37B, resulting in virus infection and causing symptoms indistinguishable from those of the wild-type isolate.

Later, a cDNA fragment encompassing the *Aequorea victoria* green fluorescent protein-encoding gene (GFP) was introduced into this genomic cDNA clone of PepMV located in the intergenic region between the TGB3 and the coat protein gene (pUS2-CSL:GFP). The mutant construction of the full length cDNA clone was verified by complete sequencing. However, none of the RNA transcripts produced in vitro from both full-length cDNA clone of US2-CSL, used as a control, and the mutant cDNA clone US2-CSL:GFP resulted infectious on *N. occidentalis* inoculated plants. Further inoculations are being carried out to verify the infectivity of these constructions.

INTRODUCTION

Pepino mosaic virus (PepMV), a member of the genus *Potexvirus*, family *Flexiviridae*, was first described on pepino (*Solanum muricatum* Ait.) in Peru (Jones *et al.,* 1980). Since the first report of PepMV infecting tomato crops (*Solanum lycopersicum* L.) in 1999 (van der Vlugt *et al.,* 2000), it has spread through the main tomato production areas worldwide.

The PepMV genome consists of a positive single-stranded RNA with a total genome length of approximately 6,400 nucleotides (nts). It has a similar organization as other potexviruses containing five open reading frames (ORFs): ORF 1 encodes the RNA-dependent RNA-polymerase (RdRp) and is involved in RNA replication, three overlapping ORFs (2, 3 and 4) which are involved in cell-to cell movement and form the triple gene block (TGB), and ORF 5 which encodes the coat protein (CP) gene. There are two intercistronic regions located between ORF1 and 2, and ORF4 and 5, respectively, hereafter referred to as intergenic regions IR1 and IR2. Two short untranslated regions (UTR) flank the coding regions in both prime ends of the genome. A poly (A) tail is present in the 3'end of the genomic RNA (Aguilar *et al.,* 2002; Cotillon *et al.,* 2002; Maroon-Lango *et al.,* 2005).

PepMV is characterized by a high level of genetic diversity, as well as high variability on symptom expression in infected plants. At present, five genotypes (EU, LP, CH2, US1, US2) have been described (Ling, 2007). The first differentiated genotype was the EU tomato genotype, isolated from tomato, when compared to the original isolate reported in pepino (LP genotype; Jones *et al.,* 1980) showing differences in indicator plant symptoms and nucleotide sequence (Verhoeven *et al.,* 2003). Later, the US1 and US2 genotypes were identified showing less than 81% overall sequence homology to the EU and LP

genotypes (Maroon-Lango *et al.,* 2005). The CH2 genotype was firstly reported in the USA isolated from Chilean tomato seeds produced in 2003 (Ling, 2007). Some studies revealed that the predominant genotypes in Europe were EU and CH2 (Pagán *et al.,* 2006; Hanssen *et al.,* 2008; Hasiów *et al.,* 2007), however the US1 genotype was also recently reported from the Canary Islands, Spain (Alfaro-Fernández *et al.,* 2008a). Despite the number of studies available, no correlation has been found between different PepMV genotypes and variable phenotypes (Pagán *et al.,* 2006; Hanssen *et al.,* 2008), although the co-infection with both genotypes was reported to result in more severe PepMV symptoms (Hanssen *et al.,* 2008). Moreover, some studies showed that differences in symptom expression could be partially attributed to the isolate, which might be related to minor differences at nucleotide level between isolates (Hanssen *et al.,* 2009b; Hasiów-Jaroszewska *et al.,* 2009b). Some aggressive isolates that produce necrosis symptoms on tomato have been described in Poland (Hasiów-Jaroszewska *et al.,* 2009b), and one of them was use to successfully construct an infectious full-length clone which caused undistinguishable symptoms from those of the wild-isolate (Hasiów-Jaroszewska *et al.,* 2009a).

The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has been used as marker of viral gene expression and offers the possibility of monitoring plant virus infection with non-destructive assay techniques (Baulcombe *et al.,* 1995; Oparka *et al.,* 1995). The insertion and fusion of the GFP gene in the *Potato virus X* (PVX) genome has been widely studied (Baulcombe *et al.,* 1995; Oparka *et al.,* 1995; 1996; Santa Cruz *et al.,* 1996; Toth *et al.,* 2001), and different strategies for the GFP insertion have been developed. No infectious GFP mutant clones have been previously described for PepMV. These could represent an important tool not only to study the pathway of the viral infection, but also for a better understanding of the symptom expression related to the virus spread in the plant tissues. This study describes

the previous construction of an infectious full-length clone of PepMV and the first attempts at the construction of a mutant GFP clone of that PepMV isolate.

MATERIAL AND METHODS

GFP and virus sources. RNA preparations

The plasmid pSC001 containing the GFP gene and the complete sequence of the construct was kindly provided by Dr. Theo van der Lee (Plant Research International, Wageningen, The Netherlands). The GFP gene used in the assay and contained in the afomentioned plasmid was the synthetic gene SGFP created with plant-optimized codon usage and the replacement of the serine at position 65 with a threonine (S65T), to increase the expression efficiency of the fluorescent protein in plants compared to the wild-type GFP from *A. victoria* (Haas *et al.,* 1996; Chiu *et al.,* 1996). The PepMV isolate US2-CSL, kindly provided by Dr. Rick Mumford (Food and Environmental Research Agency, Sand Hutton UK), was used for the construction of the full-length clone and the insertion of the GFP gene. The complete sequence of this isolate is published in the GenBank database under the Accession No. FJ212288 (Adams *et al.,* 2009) and showed 98% nt homology with the PK isolate (Hasiów *et al.,* 2007). Total RNA was extracted with RNeasy Plant Mini Kit (Qiagen) from 0.1 g of PepMV-infected leaf tissues with the isolate US2-CSL.

Full-length cDNA amplification of US2-CSL PepMV isolate and cloning

The whole viral genome was amplified from 1 µl of extracted RNA by reverse transcription polymerase chain reaction (RT-PCR) using SuperScript™ III One-Step RT-PCR System with Platinum® Taq High Fidelity (Invitrogen) following the manufacturer's instructions. The primer set was PepMV-CH2-T7-N: 5' GAG CTC TAA TAC GAC TCA CTA TA**G AAA ACA AAA CAT AAC ACA TAA TAT C** 3' (containing the *SacI* restriction site, the T7 promotor underlined and 26 nt of the PepMV 5'UTR sequence in bold) and PepMV-CH2-dT-N: 5' CCC CGG GTT TTT TTT TTT T**AT TTA GTA GAT TTA GAT ACT AAG GGA** 3' (containing the *NotI* restriction site underlined, the poly-T tail and the PepMV 3'UTR sequence in bold). The RT-PCR program consisted of an initial incubation at 55 ºC for 30 min followed by 2 min at 94ºC and 40 cycles of 94ºC for 15s, 50ºC for 30s and 68ºC for 6.5 min, and a final extension step of 68ºC for 5 min. The obtained full-length PCR product was analyzed on a 1% agarose gel in 0.5x TBE stained with ethidium bromide, and later ligated into the pCR®-XL-TOPO® vector (Invitrogen) according to the manufacturer's instructions. The recombinant plasmid was propagated in One Shot® TOP10 chemically competent *Escherichia coli* cells, verifying the presence of the full-length PepMV DNA insert performing colony PCR assays to 6 different bacterial colonies randomly selected. Two different reactions were carried out using PepMV-specific primers: one with the pair of primers PepMV-59 (5' GAA AAC AAA ACA TAA CAC ATA ATA TC 3') and PepMV-56RC (5' CAC CWA RAT TCT CAA GGG 3'), which amplify a 250 bp fragment of the 5'end of the PepMV genome, and the other with PepMV-US2-37 (5' CAA CCT ACA GCT TCT AAC CC 3') and PepMV-US2-dT (5' TTT TTT ATT TAG TAG ATT TAG ATA CTA AGG 3') which target a fragment of 770bp from the 3'end of the viral genome. Plasmid DNA was isolated following standard protocols from overnight cultures of those colonies which presented

both insert amplifications. The recombinant plasmids were checked by *SacI* digestion. *In silico* restriction analysis of the available sequence of the isolate US2-CSL has shown the absence of a *SacI* restriction site, however it was present in the primer PepMV-CH2-T7-N used for the full-length PCR fragment as explained before. Therefore, the recombinant plasmid (hereafter pUS2-CSL) must present a *SacI* unique restriction site which linearizes it, giving a single expected fragment of 9,900 nt approximately (PepMV genome 6,400 nt plus the vector sequence 3,519 nt).

Transcription and inoculation of systemic plant host

A full-length cDNA clone of US2-CSL was liniarized with *NotI* and phenol/chloroform extracted before being used as a template for transcription. The transcription reaction was performed in the presence of the cap analog (m^7G(5')ppp(5')G) using the RiboMAX™ Large Scale RNA Production System-T7 RNA polymerase kit (Promega). The RNA obtained after transcription was ethanol precipitated and then used to inoculate six plants of *Nicotiana occidentalis* 37B. Each plant of *N.occidentalis* 37B pre-dusted with carborundum was inoculated on three different leaves with 100 µl of the transcript. Plants were grown under greenhouse conditions, and after 14 days, were inspected for the development of local and systemic symptoms. The presence of PepMV was tested by DAS-ELISA 21 dpi (days post inoculation) with specific antisera against PepMV (Prime diagnostics) according to the manufacturer´s instructions and by RT-PCR with specific primers against the virus.

Construction of a mutant infectious clone of PepMV US2-CSL carrying the GFP cDNA

Previous sequencing of a fragment of the pUS2-CSL

Despite of the availability of the sequence of the isolate US2-CSL (GenBank Accession No. FJ212288), a fragment of the pUS2-CSL isolate from the ORF2 until the 3'end of PepMV genome was sequenced in order to identify possible point mutations in the sequence. Sequence analyses were performed using Applied Biosystems 3100 Genetic Analyser with a DYEnamic ET Terminator Cycle Sequencing kit (Amersham). Nucleotide and amino acid sequences data were analyzed ans assembled using DNASTAR software version 8 (Lasergene)

Design of primers and generation of DNA segments

The GFP gene was decided to be inserted in the IR2 (the untranslated region between the TGB3 and the CP gene) without altering the promoter of the sgRNA of the CP, probably located in the 3'end of the TGB3 were the conserved octanucleotide motif (3'CAAUUCAA 5') was identify as in other potexvirus (Lee *et al.,* 2000; Côté *et al.,* 2008). Firstly, *in silico* analysis revealed the presence of *StuI* unique restriction site in the pUS2-CSL located in the ORF2 (position 4592). The strategy for the construction of the mutant clone PepMV-US2-CSL carrying the GFP, shown in Figure 4.1, was the ligation of a big DNA fragment (constituted by the fusion of three different fragments A, B and C) generated by different PCR amplifications into the vector. For the PCR amplification of these fragments, three different primer pairs were designed containing 15-bp overlap with the adjacent segment of the construct (Table 4.1). Fragments A, B and C were generated from 1 µl of the plasmid DNA (pUS2-CSL for fragments A and B, and pSC001 for fragment C) 50 times diluted using Taq DNA polymerase (Roche) with the suitable primers (Table 4.1). A

touchdown-PCR format was used to avoid amplifying non-specific fragments: 94°C for 2 min, 94°C for 1 min, 50°C for 1 min, 72°C for 1 min, followed by 5 cycles at decreasing annealing temperatures in decrements of 0.5°C per cycle, then 33 cycles of 94°C for 1 min, 47°C for 1 min, 72°C for 1 min, and a final extension for 10 min. at 72°C.

Table 4.1. Specific primers of each of three fragments which present 15-bp overlap at the 5' prime end with the adjacent fragment to be joined (bold letters).

Frag-ment	Primer name	Sequence 5'-3'	Sense	Size	Location
A	PepMV ORF2-D	ATAGGTTCACCTAGG^CCTTGTTACTTAG	F	28	4577-4604[a]
	PepMV IR2-SGFP-R	**GCCCTTGCTCACCAT**TTAAATTGTTTG	R	27	5610-5621[a]
C	PepMV IR2-SGFP-D	**ATTCAAACAATTTAA**ATGGTGAGCAAGG	F	28	1-13[b]
	SGFP-PepMV IR2-R	**TTTTCCATAGTTGAG**TTACTTGTACAGCTC	R	30	706-720[b]
B	SGFP-PepMV IR2-D	**GAGCTGTACAAGTAA**CTCAACTATGG	F	26	5622-5632[a]
	TOPO-XL-VECTOR-R	CATGCTCGAGC^GGCCGCCAGTGTGATGG	R	28	358-385[c]

^ Cuts with restriction enzymes
[a] Refers to the original sequence of the isolate US2-CSL Accession no. FJ212288 containing the *StuI* restriction site.
[b] Refers to the sequence of the protein SGFP
[c] Refers to the sequence of the TOPO-XL vector (contains the *NotI* restriction site).

Obtained fragments were visualized and purified from an agarose gel using the crystal violet stain supplied in the TOPO® XL PCR cloning kit (Invitrogen). All PCR fragments theoretically contained a 15-bp overlap with the adjacent fragment. To generate the fused fragment BC and subsequently ABC, two PCR were performed with the suitable pair of primers (Table 4.1, Figure 4.1) with 1µl of the gel-purified fragments B and C for the reaction to obtain the

first fused fragment (BC) and A and BC for the second (ABC) with the SuperScript™ III One-Step RT-PCR System with Platinum® Taq High Fidelity (Invitrogen) following the manufacturer's instructions. Fragments BC and ABC were purified using the High Pure PCR Product Purification Kit (Roche Diagnostics) and directly sequenced and analyzed as explained before.

As shown in Figure 4.1, the vector for the mutant clone of PepMV US2-CSL carrying the GFP cDNA consisted of pUS2-CSL after elimination of the fragment between the restriction sites *StuI* and *NotI* . To this end a double digest with 80 µl of the plasmid DNA was performed with these two endonucleases (Roche diagnostics) according to the manufacturer's instructions. A small volume of the obtained restriction products was first verified in an 1% agarose gel in 0.5x TBE buffer with EtBr. Later, the remaining volume was visualized and purified from a crystal violet stained agarose gel, as explained before.

In-Fusion reaction, transformation and sequencing

One hundred fifty nanograms of restriction enzyme-digested, gel-purified vector were mixed at a molar ratio of 1 vector to 2 of the DNA gel-purified fragment (ABC) in a total volume of 20 µl of In-Fusion™ 2.0 CF liquid PCR cloning kit (Clontech) according to the manufacturer's instructions. The reaction was incubated at 37°C for 15 min. followed by 15 min. at 50°C, and immediately transferred into ice. A volume of 20 µl of TE buffer pH 8.0 was added to the reaction. Volumes of 5 and 2.5 µl were transformed into One Shot® TOP10 chemically competent *E. coli* cells (Invitrogen). For verifying the presence of the insert in the growing colonies, six randomly selected colonies were analyzed by colony PCR assays with specific primers listed in Table 4.2.

Table 4.2. Specific primers of PepMV used for verifying that the colonies contain the correct inserts of the plasmid pUS2-CSL:GFP.

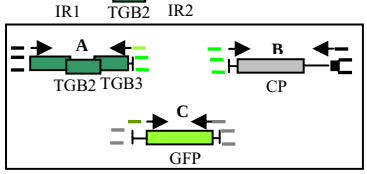
Primer name	Sequence 5'-3'	Sense	Size	Location[a]
PepMV-X	TCACAAACTCCATCAAGG	F	18	4335-4352
PepMV-Y-R	GTATTCCACTGTGTCGTCTTG	R	21	4859-4879
PepMV-Z	GCCGTAATACTCACCAGCATC	F	21	5404-5424
Pep-US2 36RC	AAGCTGTAGGTTGGTTTTCC	R	20	5651-5632
PepUS2-37	CAACCTACAGCTTCTAACCC	F	20	5643-5661
PepMV-dT	TTTTTTATTTAGTAGATTTAGATACTAAGG	R	30	-

[a] Location referred to the genome of the isolate US2-CSL (Accession No. FJ212288)

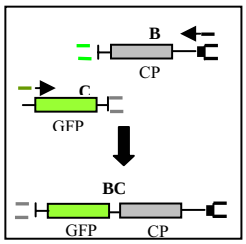
The plasmid DNA was isolated following standard protocols from overnight cultures of colonies positive for each of the three expected PCR products. Recombinant plasmids pUS2-CSL:GFP were checked by single- and double-digestion with the endonucleases *SacI* and *StuI*+*NotI*, respectively. The obtained products were visualized in a 1% agarose gel compared to the same restriction assays performed to the original plasmid pUS2-CSL. One mutant full-length cDNA clone of PepMV pUS2-CSL:GFP was directly sequenced and the obtained sequence was analyzed as described previously.

Figure 4.1. Diagram of the different steps performed for the construction of the GFP mutant clone of the US2-CSL PepMV isolate. The primers used are codified as: 1, PepMV ORF2-D; 2, PepMV IR2-SGFP-R; 3, PepMV IR2-SGFP-D; 4, SGFP-PepMV IR2-R; 5, SGFP-PepMV IR2-D; and 6, TOPO-XL-VECTOR-R.

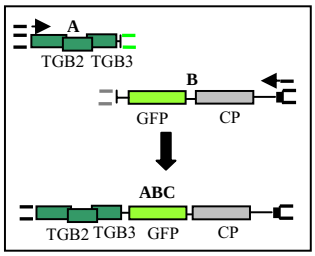
1. PCRs of single fragments A, B and with 15 bp overlaps with the adjacent segment



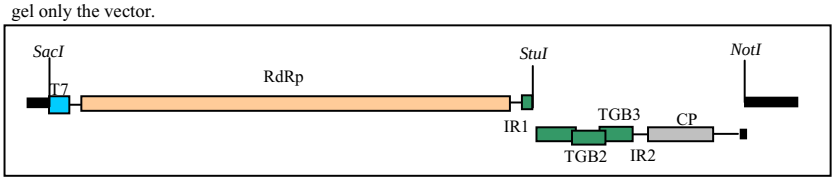
2. PCRs with fragments B and C



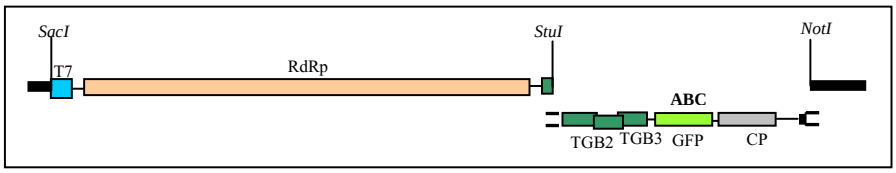
3. PCRs with fragments BC and A



4. Double digestion of the plasmid pUS2-CSL with the restriction enzymes *StuI* and *NotI*, cutting form the gel only the vector.



5. In-Fusion™ 2.0 CF liquid PCR cloning kit (Clontech) reaction to join the vector with the ABC fragment.



6. Transformation in *E.coli* One Shot ® TOP 10 chemical competent cells and plasmid isolation

Transcription and inoculation of systemic plant host

Transcription of the cDNA of the mutant clone pUS2-CSL:GFP was performed as explained before for the pUS2-CSL after its linearization with *NotI* and purification by phenol/chloroform extraction. The obtained RNA transcripts were inoculated to five *N. occidentalis* 37B plants, pre-dusted with carborundum as detailed above. RNA transcripts of pUS2-CSL were simultaneously generated and similarly inoculated to act as a control. Plants were grown under greenhouse conditions, and after 14 days, were inspected for the development of local and systemic symptoms. The presence of PepMV was tested by DAS-ELISA 21 dpi and RT-PCR with specific primers for PepMV.

RESULTS

Sequencing of a fragment of pUS2-CSL PepMV

The obtained sequence from the pUS2-CSL PepMV corresponded to a fragment from the 4430 to 6397 nt, position referred to the original isolate US2-CSL (Accession number FJ212288). Alignments between US2-CSL GenBank accession no. FJ212288 and the pUS2-CSL clone revealed 25 point mutations at nucleotide level in the coding regions amplified. These point mutations caused a change in the predicted aa sequence in 10 of the cases in three coding regions (ORF2, 4 and 5).

Transcription of US2-CSL PepMV isolate and infectivity in susceptible host

The RNA transcripts derived from the full-length cDNA of the US2-CSL PepMV isolate were infectious. All the inoculated plants of *N. occidentalis* 37B

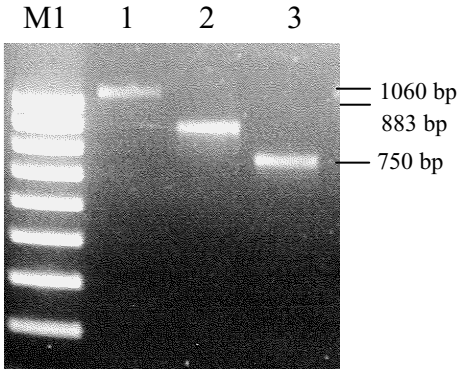
developed systemic symptoms 7-10 days after inoculation, indistinguishable to those developed by the wild-type isolate. Presence of PepMV was confirmed serologically and molecular with primers specific for US2-CSL.

Construction of the mutant clone of PepMV US2-CSL carrying the GFP cDNA

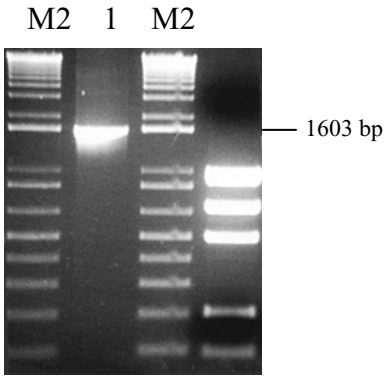
Each of the PCR assays to amplify the three single fragments: fragment A, containing from the *StuI* restriction site of PepMV to the IR2 site where the GFP would be inserted; fragment C, the second consisting in the GPF gene; and fragment B, containing the rest of the IR2 to the *NotI* site with the pair of primers containing the 15-bp overlap with the adjacent fragment (Table 4.2) resulted in the expected fragments (1060 bp, 883 bp and 750 bp, respectively) as shown Figure 4.2a. The PCR of the fragments which joined the fragments B and C (fragment BC), and after that, the fragments A with the obtained fragment BC (fragment ABC) also gave the expected size, 1603 bp and 2633 bp, respectively (Figure 4.2b and 2c). Sequencing of those two fragments revealed that the junctions between the GFP gene and the PepMV in both ends were correct, in the expected position and orientation, and the sequences presented 100% identity with the previously sequenced US2-CSL fragment.

The double digestion of the pUS2-CSL with *NotI* and *StuI* resulted in two fragments of 1883 bp and 8076 bp (Figure 4.3a, lanes 1-3). After isolation from a crystal violet stained agarose gel the largest fragment was used as the cloning vector for the ABC fragment (segment of PepMV pUS2-CSL from the *StuI* until *Not I* restriction containing the GFP inserted in the IR2) using the In-Fusion™ 2.0 CF liquid PCR cloning kit (Clontech). Transformation of 5 µl and 2.5 µl of the In-Fusion reaction product resulted in 37 and 160 colonies, respectively.

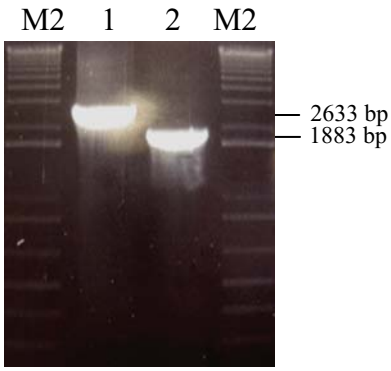
a)


M1
1
2
3
1060 bp
883 bp
750 bp

b)


M2
1
M2
1603 bp

c)

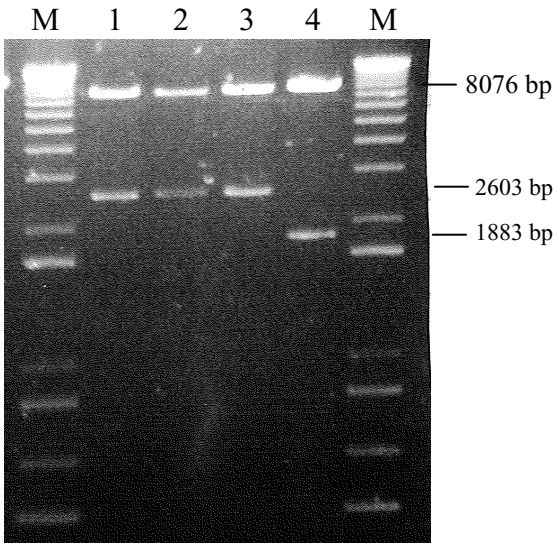

M2
1
2
M2
2633 bp
1883 bp

◄ **Figure 4.2. (a)** Amplification by PCR of the single fragments: A, corresponding to the segment of PepMV from the *StuI* restriction site to the IR2 site where the GFP would be inserted (lane1); B, PepMV fragment containing the rest of the PepMV from the IR2 to the *NotI* site (lane 2), and C corresponding to the GFP gene (lane 3). Lane M1: 100 bp molecular weight marker (BioRad). (**b)** PCR amplification fragment BC with the specific primers PepMV IR2-SGFP-D and TOPO-XL-VECTOR-R (lane 1). Lane M2: 1 kb marker (Invitrogen). (**c)** Fragment ABC obtained after PCR amplification with specific primers PepMV ORF2-D and TOPO-XL-VECTOR-R of the single fragment A and the joined fragment BC (lane 1) and on the pUS2-CSL.

Four out of six randomly selected colonies reacted positive in US2-CSL specific PCR assays. The plasmids isolated from those colonies showed the expected restriction products after digestion with *SacI* (Figure 4.3b, lane 4), and double digestion with *NotI* and *StuI* (Figure 4.3a, lane 4). Complete sequence analysis of the PepMV US2-CSL:GFP revealed the correct insertion of the carrying the GFP cDNA insert .

RNA transcripts derived from the cDNA of the US2-CSL:GFP PepMV isolate were inoculated to *N. occidentalis* 37B plants. However none of the inoculated plants 37B developed any systemic symptoms up to 30 days after inoculation and tested negative in all the serological and molecular analyses. However, the same negative results were obtained with the with the RNA transcripts derived from the cDNA of the US2-CSL PepMV isolate, generated as a control in a parallel transcription reaction.

a)



b)

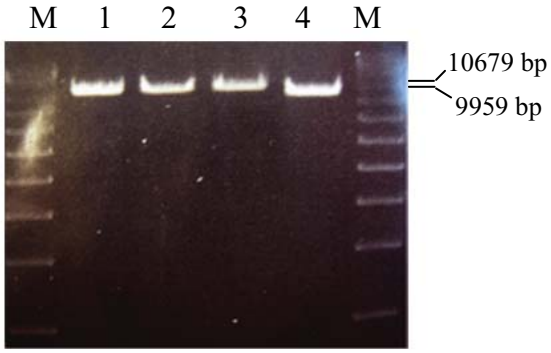


Figure 4.3. Restriction analysis of isolated plasmids containing the GFP mutant clone of the PepMV US2-CSL isolate (lanes 1-3) and the clone PepMV US2-CSL (lane 4) consisting in a (**a)** double digestion with *NotI* and *StuI* and (**b)** single digestion with *SacI*. Lane M: 1 kb marker (Invitrogen).

DISCUSSION

The infectivity of *in vitro* transcripts of the full-length cDNA clone of US2-CSL PepMV isolate was investigated. Inoculated transcripts caused symptom expression on *N. occidentalis* 37B plants similar to those of the wild-type virus, and the virus was detected by RT-PCR in the systemic leaves showing the transcripts to be infectious. The production of infectious RNA transcripts from full-length clones has been proved to be of great importance for studying viral gene functions and also has a potential use as foreign gene expression vectors. For this reason, many infectious full-length clones of different species of viruses have been produced as, e.g., *Potato virus X* (PVX; Choi and Ryu, 2008), *Chrysantemum virus B* (CVB, Okhawa *et al.,* 2008), *Tobacco mosaic virus* (TMV, Choi *et al.,* 2009), and even a necrotic phenotype of a CH2 isolate of PepMV (Hasiow-Jarowzeswska *et al,* 2009a). The production of infectious clones of PepMV will allow detailed analysis of gene functions of this virus in plants by site-directed mutagenesis and recombination, among them, the genomic region responsible for viral infectivity and biological properties could be elucidated. However, until now no correlation has been established between genome sequences of PepMV and their biological properties (Pagán *et al.,* 2006; Hanssen *et al.,* 2008).

In this study the aim of the construction of the infectious full-length cDNA clone of PepMV was to use it as a first step for the introduction of the cDNA fragment encompassing the *A. victoria* green fluorescent protein-encoding gene (GFP). GFP has been widely used as a marker to study cellular and molecular aspects of virus replication, cell-to-cell spread of infection and the interactions between viral proteins and host components. The fusion of GFP to viral proteins has led to new insights into the temporal and spatial distribution of gene products, their functions, and the mechanisms by which they act, especially

in elucidating the mechanisms of virus movement in infected plants (Baulcombe *et al.,* 1995; Lazarowitz and Beachy, 1999). Numerous constructs of PVX the GFP gene have been studied: PVX expressing free GFP to enable viral spread to be monitored; PVX expressing a GFP-coat protein fusion that assembles into fluorescently labelled virus particles, enabling their subcellular localization to be determined; PVX expressing free GFP but with a coat protein deletion to determine the role of the CP in viral movement; PVX vector expressing a fusion of GFP to the MPs of *Tobacco mosaic virus* (TMV) and *Cucumber mosaic virus* (CMV) to determine the localization of these MPs during viral spread or PVX vector expressing GFP targeted to the endoplasmic reticulum (ER) to allow the ER network to be imaged (Baulcombe *et al.,* 1995; Oparka *et al.,* 1996; Santa Cruz *et al.,* 1996; Oparka *et al.,* 1997). Some of these studies have determined some functions of the PVX proteins, specifically the TGB1 protein gating plasmodesmata, regulating virus translation and constituting a RNA silencing inhibitor (Voinnet *et al.,* 2000; Senshu *et al.,* 2009) and the CP is required for cell-to cell viral movement, although it is not involved in plasmodesmal gating (Oparka *et al.,* 1996) .

The RNA transcripts of the pUS2-CSL:GFP construct produced in the present study failed to be infectious on *N. occidentalis* 37B inoculated plants so far. The reason for this is not yet known because not only those of the mutant construction pUS2-CLS:GFP failed to be infectious, but also those of the pUS2-CSL control which was successful in previous studies. New transcripts are being studied in this moment to verify the results obtained in this study.

If the GFP construct is really not infectious there could be several reasons. One could be the strategy of GFP introduction which lacks of duplicated promoter for the coat protein subgenomic RNA (sgRNA) normally introduced upstream of the GFP gene in PVX constructs (Baulcombe *et al.,* 1995). Another reason might be the influence on the infectivity of the polyA tail

as has been reported for PVX (Kavanagh *et al.,* 1992). PVX containing eight adenines at the 3´-end of its RNA showed relatively very low infectivity but a transcript containing eighty adenines showed 50% of infectivity compared to the viral RNA. Therefore, the poly adenine tailing seem to be essential for transcript infectivity. Choi and Ryu (2008) suggested that, as cDNAs with long 3-adenine tails were thought to be unstable during the cloning process in common *Escherichia coli* strains, a polyadenilation treatment of the clones right after confirmation of transcript production on agarose gel should be performed. In our study, no treatment was performed to the studied clones and sequencing indicate that there are not many adenines in the polyA tail, however in previous assays with the US2-CSL cDNA clone no poliydenylation treatment was required to get infectious transcripts and nearly 100% of all transcript inoculated plants became infected.

This study represents the first attempts to construct an infectious GFP mutant clone of a PepMV isolate. The introduction of the GFP gene in the PepMV genome was achieved by fusion PCR methods which consist as explained before in generating a single amplicon from three fragments that contain a small overlapping region of 15 bp, which was engineered into the fragments by the PCR-primers. This method is a modified version of previously described PCR based fusions of overlapping DNA fragments (Hobert, 2002; Charlier *et al.,* 2003), with a shorter region of overlapping between fragments (15bp instead of 24bp). The advantages of this method are the independency of the presence of restriction sites, so it can be performed at any location, and considerable time is saved compared to the traditional cloning approaches, however additive round of PCR reactions do increases the chances of introducing point mutations in the amplicons (Boulin *et al.,* 2006). Moreover, the use of In-Fusion™ assembly for the desired vector and the fused fragment containing the GFP gene was successful as demonstrated by sequencing. The

favourable results obtained with both techniques could be applied in further recombination studies with different strains to identify the responsible part of the genome in symptom expression or introduction of foreign (reporter) genes into the PepMV genome.

The construction of mutant clones with different fluorescent protein markers has been successfully used to identify synergistic effect between different *Potyvirus* and PVX, while co-infections with differently labelled *Potyvirus* population appeared non-synergistic (Dietrich and Maiss, 2003) or even in cross-protection studies with two strains of *Zucchini yellow mosaic virus* (ZYMV) carrying two different markers (Lin et *al.,* 2007). Mutant clones of different PepMV strains or isolates labelling in which each genotype carries a different tag will be an important tool for monitor the accumulation and decline of the same virus in, for example, cross-protection studies.

Capítulo 5

Transmission of *Pepino mosaic virus* by the fungal vector *Olpidium virulentus*

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ABSTRACT

Transmission of *Pepino mosaic virus* (PepMV) by fungal vector *Olpidium virulentus* was studied in two experiments. Two characterized cultures of the fungus were used as stock cultures for the assay: culture A was from lettuce roots collected in Castellón (Spain), and culture B was from tomato roots collected in Murcia (Spain). These fungal cultures were maintained in their original host and irrigated with sterile water. The drainage water collected from irrigating these stock cultures was used for watering PepMV-infected and non-infected tomato plants to constitute the acquisition-source plants of the assay, which were divided into six different plots: plants containing fungal culture A (non-infected and PepMV-infected); plants containing fungal culture B (non-infected and PepMV-infected); PepMV-infected plants without the fungus; and plants non-infected either with PepMV and the fungus. Thirty-six healthy plants grouped into six plots, which constituted the virus acquisition-transmission plants of the assay, were irrigated with different drainage waters obtained by watering the different plots of the acquisition-source plants. PepMV was only transmitted to plants irrigated with the drainage water collected from PepMV-infected plants whose roots contained the fungal culture B from tomato with a transmission rate of 8%. No infection was detected in plants irrigated with the drainage water collected from plots with only a fungus or virus infection. Both the virus and fungus were detected in water samples collected from the drainage water of the acquisition-source plants of the assay. These transmission assays demonstrated the possibility of PepMV transmission by *O. virulentus* collected from tomato crops.

INTRODUCTION

Pepino mosaic virus (PepMV) was first reported in pepino (*Solanum muricatum* Ait.) in Peru (Jones *et al.,* 1980). In 1999, this virus was detected in protected tomato crops (*Solanum lycopersicum* L.) in the Netherlands (van der Vlugt *et al.,* 2000) and it was now been reported in the main tomato production areas of Europe and America (EPPO, 2009). PepMV is a member of the genus *Potexvirus*, family *Flexviiridae* which has filamentous particles with a normal length of 508 nm (Jones *et al.,* 1980). This virus produces various symptoms that depend on the virus isolate, tomato cultivar, temperature and light intensity. Typical leaf symptoms generally include yellow or light-green mosaic, interveinal single yellow spots, and bubbling or other leaf distortions such as filiform. Fruits typically show marbling, an alteration of the fruit colour, resulting in uneven ripening which considerably lowers their market value (Jordá *et al.,* 2000a).

PepMV is readily transmitted mechanically and is spread between plants easily by contaminated tools, hands, clothing, and by direct plant-to-plant contact (Wright and Mumford, 1999). Furthermore, bumble bees, such as *Bombus terrestris* L., *B. canariensis* Pérez and *B. impatiens* (Cresson), which are commonly used as pollinators in tomato greenhouses, have also been implicated in the experimental spread of PepMV between tomato plants through the direct injuring of flowers or through fertilization with infected pollen (Lacasa *et al.,* 2003; Shipp *et al.,* 2008). However, long-distance dissemination of this virus possibly occurs by the transfer of young infected plants from the nursery to the grower, through infected grafts, cuttings or fruits, and even through the seed-to-seedling transmission (Córdoba-Sellés *et al.,* 2007b). PepMV transmission has been studied in a recirculating hydroponic system where the transmission rate was quite high and its spread could remained unnoticed due to the lack of

symptoms in the infected tomato plants (Fakhro *et al.,* 2005, Schwarz *et al.,* 2007).

Simultaneously with the first outbreak of PepMV infection in Spain (Jordá *et al.,* 2001a), a new syndrome associated with the presence of the virus occurred in Murcia which was referred to as 'tomato collapse' because of the wilting of the affected plants. The first symptom was slight reversible wilting which occurred at midday to later become irreversible, and the plant died. This syndrome was reproduced under controlled conditions in different assays which demonstrated that PepMV, together with the fungal vector *Olpidium brassicae* (Wor.) Dang sensu lato (sl), were involved in the 'tomato collapse' syndrome (Córdoba *et al.,* 2004b). Other authors suggested that 'tomato collapse' could be associated with necrosis of the vascular system caused by PepMV accumulation; however collapse was not reproduced in that study so Koch´s third postulate was not fulfilled (Soler-Aleixandre *et al.,* 2005).

O. brassicae sl, a member of the Chytridiales order, is a root-infecting parasitic fungus involved in the transmission of several plant virus, for example, *Lettuce big-vein virus* (LBVV, *Varicosavirus*) (Campbell, 1996). This fungus has three developmental stages during its life cycle: zoospores, zoosporangia and resting spores. The fungus survives from crop to crop as resting spores that produce zoospores. A zoospore encysts on the epidermal cells of host roots. The thallus becomes embedded in the host cytoplasm and later develops into either a thin-walled zoosporangium or a thick-walled stellate resting spore. When mature, zoosporangia release zoospores through exit tubers and the life cycle is repeated (Campbell, 1996; Temmink and Campbell, 1968).

Host specialisation is an important characteristic of *O. brassicae* sl, and different strains or isolates of the fungus have been widely described, such as the crucifer strain, which requires zoospore mating to develop resting spores, or the non-crucifer strain, which does not require sexual mating for resting spores

formation (Campbell and Sim, 1994; Koganezawa *et al.,* 2005). Given these differences and the molecular analysis of the complete rDNA-ITS regions of the fungus, the crucifer and non-crucifer strains of *O. brassicae* sl are now considered different species. A new species named *Olpidium virulentus* (Sahtiyanci) Karling was proposed for the non-crucifer strain of the fungal vector (Koganezawa *et al.,* 2005), which was later confirmed by Sasaya and Koganezawa (2006). This new nomenclature of the *Olpidium* species will be used in this study.

The confirmed relationship of *O. virulentus* and PepMV in the syndrome referred to as `tomato collapse`, the ability of *O. virulentus* to infect different plants species and to transmit several plant viruses, together with the need for detailed research into putative ways of PepMV transmission, led to this study being developed. Our main objective was to evaluate the possibility of PepMV transmission to healthy tomato plants by fungal vector *O. virulentus*.

MATERIALS AND METHODS

Fungal vector cultures

Two different cultures of the fungal vector *O. virulentus* were used in the test: Culture A and Culture B, which were previously characterised molecularly by Herrera-Vásquez *et al.* (2009b). These cultures were obtained from soil samples collected from lettuce crops (Culture A), showing typical symptoms of LBVV, and from tomato crops (Culture B), with collapse symptoms, where the fungus had been previously detected. No other micro-organisms were present in these soil samples. The characteristics of fungal vector cultures A and B are provided in Table 5.1. Routine maintenance of stock culture B and vector transmission experiments were performed with tomato

plants (cv. Marmande) grown under stringent sanitary conditions and planted in a three-times sterilised (120ºC for 30 min) mixture of sand and peat (1:3) substrate. Plants were grown in 30 cm-diameter plastic pots and were kept in growth chambers at 26ºC/22ºC (day/night) with a 12-h photoperiod and 60% relative humidity. Stock culture A was routinely maintained in lettuce plants to keep the fungus in the roots of its original host. To avoid possible contamination with other *Olpidium* spp. isolates, all the plants were watered with sterile water during the assay. Pots which contained fungal vector cultures were placed 5 cm over a tray to collect the drainage water used in the transmission assay and to avoid further contaminations. All the precautions taken were to control *Olpidium* spp. and to prevent accidental spread into uninoculated root systems.

Virus isolates

Three PepMV isolates were used in this work and their characteristics are shown in Table 5.1. Three different zones of the PepMV genome of two isolates, 3672 and 4809, were previously studied: partial RNA-dependent RNA polymerase gene (RdRp), triple gene block gene (TGB), and coat protein gene, (CP), as in Pagán *et al.* (2006), and they were characterised within the European PepMV strain in all the zones studied. The third isolate, 4988, was characterised in this work as previously described (Pagan *et al.*, 2006). The sequence information obtained from the three zones of the genome was submitted to the GenBank database (accession numbers are provided in Table 5.1). The transmission assay was replicated twice in August-November 2005 (Expt. 1) and in November-March 2006-07 (Expt. 2). During Expt.1, all three PepMV isolates were studied. In Expt. 2 however, only isolate 4988 was analysed given the results previously obtained in Expt. 1.

Table 5.1: Characteristics of the *O. virulentus* cultures and PepMV isolates used in the assay.

O. virulentus						PepMV					
Culture	**Origin**	**Original host**	**Collection date**	**Microscopic observation[a]**	**Molecular characterisa-tion**	**Isolate**	**Collection date**	**Origin**	**Tomato cultivar**	**Symptoms[b]**	**Molecular characterisa-tion**
A	Castellón, Spain	Lettuce	2001	rs, zs	EU981901[c]	**3672**	2000	Murcia	Gabriela	W, FM	AM042588[d] AM041933[d] AM313791[d]
B	Murcia, Spain	Tomato	2001	rs, zs	EU981902[c]	**4809**	2001	Murcia	nd	W	AM042568[d] AM041934[d] AM113792[d]
						4988	2001	Las Palmas	Daniela	YM, W	FJ384784[e] FJ384786[e] FJ384785[e]

[a] rs: stellate resting spores, zs: zoosporangia.
[b] FM: Fruit Marbling, W: Wilt, YM: yellow mosaic.
[c] *O. virulentus* isolated molecularly characterized by Herrera-Vásquez *et al.* (2009b). Accession numbers published in the GenBank database.
[d] PepMV isolates molecularly characterised by Pagán *et al.* (2006). Accession numbers published in the GenBank database
[e] Nucleotide sequences obtained in this work as described by Pagán *et al.* (2006) and submitted to the GenBank database.
nd: unknown

Preparation of the acquisition-source plants (P_0)

Tomato cv. Marmande seeds, disinfected with 10% trisodium phosphate for 3h as described by Córdoba-Sellés *et al.* (2007b), were sown in well trays filled with a three-times sterilised (120ºC for 30 minutes) mixture of sand and peat (1:3). At the four leaf stage, plants were transplanted into 30 cm-diameter plastic pots. Until that time, plants were watered with sterile water and kept according to stringent isolation measures to avoid other *Olpidium* spp. contaminations.

Plants were distributed into three plots according to the drainage water applied to them, as described in Figure 5.1. Plot A_0 was constituted by six plants irrigated with the drainage water obtained from culture A of *O. virulentus*. Plot B_0 was constituted by six plants irrigated with the drainage water obtained from culture B of *O. virulentus.* Plot H_0 was constituted by six plants irrigated with sterile water. *O. virulentus* inoculative irrigation of the plants of plots A_0 and B_0 commenced immediately after transplanting. This inoculative irrigation consisted in irrigation with the zoospore suspensions (ranging from $1x10^5$ to $1x10^6$ zoospores per mL, estimated by the method of Campbell (1988)) contained in the drainage water obtained from irrigating the stock-pots cultures A and B of *O. virulentus* with 5L of sterile water. Those stock-pots and plants which constituted plot H_0, were always irrigated with sterile water. P_0 plants were maintained in a growth chamber under controlled temperatures (26ºC/22ºC day/night), and inoculative irrigation (approximately 500mL of drainage water collected from stock-pots A or B) was performed once a week. These irrigations were complemented with other non-inoculative irrigations with sterile water depending on the plants necessities.

Three plants within each plot were inoculated with PepMV to constitute sub-plots AP_0 (adquisition-source plants irrigated with culture A and inoculated

with a PepMV isolate), BP_0 (adquisition-source plants irrigated with culture B and inoculated with a PepMV isolate), and HP_0 (adquisition-source plants irrigated with sterile water and inoculated with a PepMV isolate). The remaining plants in each plot were maintained without PepMV inoculation, thus constituting sub-plots An_0 (adquisition-source plants irrigated with culture A and non-inoculated with any PepMV isolate), Bn_0 (adquisition-source plants irrigated with culture B and non-inoculated with any PepMV isolate), and Hn_0 (adquisition-source plants irrigated with sterile water and non-inoculated with any PepMV isolate).

In Expt. 1, the three PepMV isolates (3672, 4809, 4988; Table 5.1) were inoculated to one plant of sub-pots AP_0, BP_0 and HP_0, as shown in Figure 5.1. In Expt. 2 however, only the 4988 isolate was inoculated to the three plants of these sub-plots. Figure 5.1 represents the design of Expt. 1. Inoculation of the plants belonging to sub-plots AP_0, BP_0 and HP_0 was performed with an inoculum prepared by grinding leaf material from PepMV-infected plants in inoculation buffer (0.01M phosphate buffer pH 7.2 containing 0.2% sodium bisulphite and 0.2% sodium diethyldithiocarbamate DIECA) in 1:4 (wt/v), using carborundum (600 mesh) as an abrasive.

To confirm correct PepMV inoculation in plots AP_0, BP_0 and HP_0, and to ensure non-infection of plots An_0, Bn_0, Hn_0 in both stock cultures A and B, all the plants (P_0) were analysed 15 dpi (days post-inoculation) against PepMV by the double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) using a specific antiserum supplied by DSMZ (Deutsche Sammlung von Mikroorganismen and Zellkulturen GmbH. Braunschweig, Germany) following the manufacturer's instructions. One month after the first inoculative irrigation, presence of the fungus in P_0 roots was verified by observing the samples collected from the secondary roots of the plants P_0, which were previously clarified by following the method described by Jordá *et al.* (2002) using a

Nikon-YS-100 light microscope (Nikon Corporation, Tokyo, Japan). Fungal infection was monitored by quantifying the number of resting spores (rs) and zoosporangia (zs) in these secondary roots samples. Three 3 cm-long slices of secondary roots per plant were randomly visualised and quantified on a 0-3 scale (0= any fungal structure present; 1 = range 0-100 fungal structures, rs or zs; 2 = range 101-1000 fungal structure, rs or zs; 3 = more than 1001 fungal structures, rs or zs). P_0 plants constituted the acquisition-source plants for both the virus and the fungal vector.

PepMV detection in the drainage water of acquisition-source plants (P_0)

The drainage water obtained from irrigating the different sub-plots (AP_0, An_0, BP_0, Bn_0, HP_0, Hn_0) of P_0 was processed in both Expt. as illustrated in Figure 5.2 to check the presence of both the virus and the vector in water samples. P_0 were irrigated with 500 mL of the corresponding irrigation source, as explained before, at 45 dpi (days post inoculation). A volume of 200 mL of drainage water from each sub-plot (AP_0, An_0, BP_0, Bn_0, HP_0, Hn_0) was collected and filtered through musslin. The filtrate was centrifuged at 13000 rpm, 4ºC for 2h in a Sorvall® DuPont Company centrifuge with a RC-5B GSA rotor (Wilmington, DE, USA). Two different fractions were obtained with this centrifugation: a pellet (part A) and a supernatant (part B), which were processed separately as shown in Figure 5.2.

Part A consisted in one obtained pellet which was resuspended in 5 mL of Milli-Q sterile water and centrifuged at 30000 rpm for 150 min in a Beckman Optima™ L-90K ultracentrifuge with an SW-41 rotor (Fullerton, CA, USA). The pellet was resuspended in 1 mL of Milli-Q sterile water. The total RNA extraction procedure with the RNAwiz extraction kit (Ambion, Hungdinton, UK) was performed with 500 µL of the obtained suspension following the manufacturer's instructions. The total DNA of the rest of the suspension (500

μL) was extracted with the EZNA Plant DNA Miniprep kit (Omega, Biotech, Doraville, USA) following the manufacturer's instructions. To detect PepMV in the RNA extracted from the resuspended pellet, reverse transcription-polymerase chain reaction (RT-PCR) was performed using the SuperScript II one step RT-PCR system with the Platinum Taq DNA polymerase kit (Invitrogen Life Technologies. Barcelona, Spain) with specific primers which amplify the complete coat protein gene (CP) of the virus (Pagán *et al.,* 2006). On the other hand, a multiplex PCR (polymerase chain reaction) assay for the simultaneous detection and differentiation of *Olpidium* spp. was performed, as described before, according to Herrera-Vásquez *et al.* (2009b) to detect the fungus in the DNA extracted from the resuspended pellet.

The other part of the process, Part B, consisted in a supernatant which was processed by the concentrating virus method for use in water samples described by Gosálvez *et al.* (2003). The drainage waters of AP_0 and BP_0 were processed together, as were those of An_0 and Bn_0, to constitute a drainage water sample of PepMV-inoculated plants containing the fungal vector (AP_0+BP_0), and virus-free plants containing the fungal vector (An_0+Bn_0), respectively. The drainage water of the plants inoculated with PepMV without the fungal vector (HP_0), along with that of healthy plants (Hn_0), were also included in the assay as controls. The RNA extracts obtained were analysed by RT-PCR against PepMV-CP as described before to detect the presence of the virus in the drainage water of P_0.

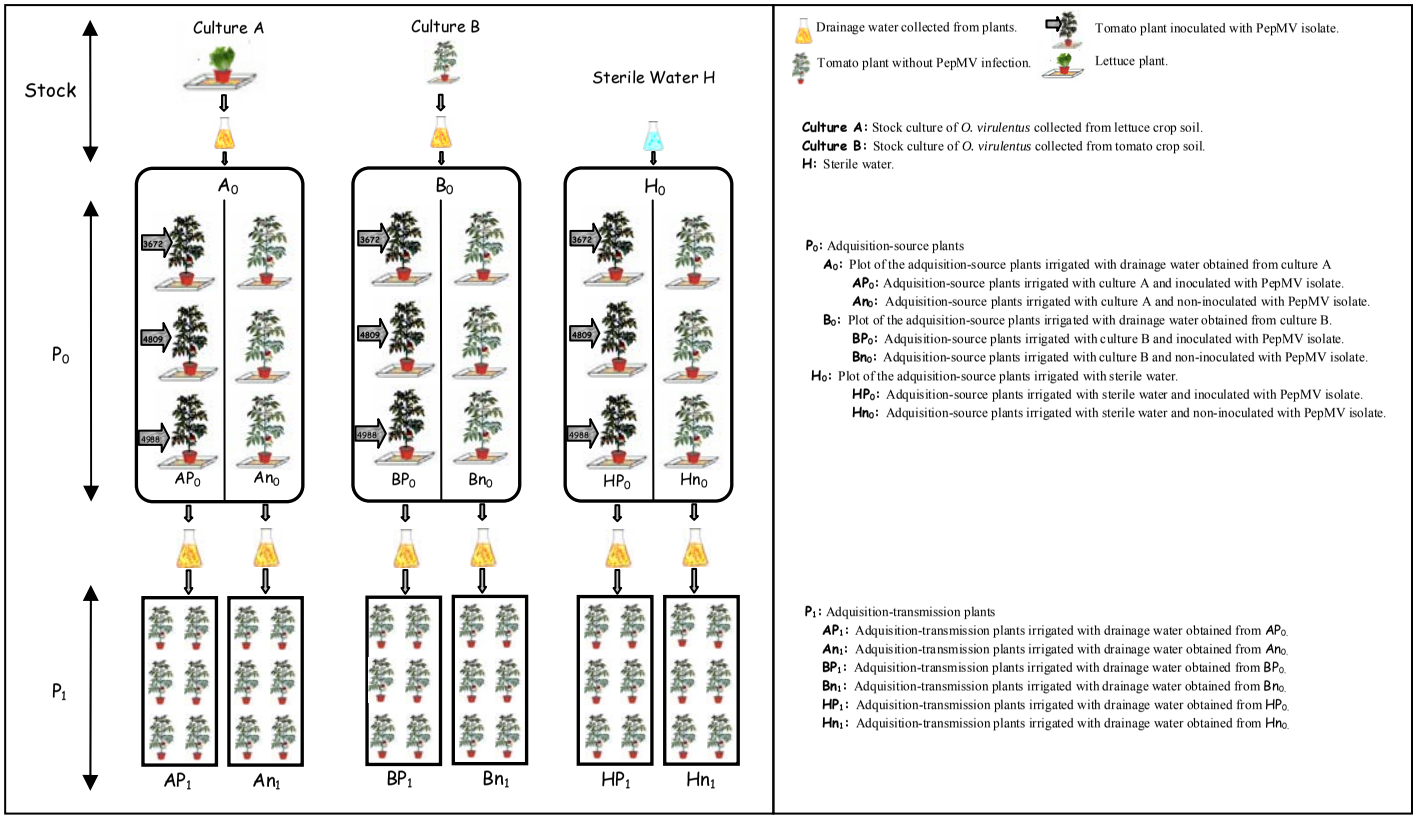

Figure 5.1. Schematic representation of the experimental set-up of the transmission test. The legend of the different images represented is included in the rectangle in the right of the figure.

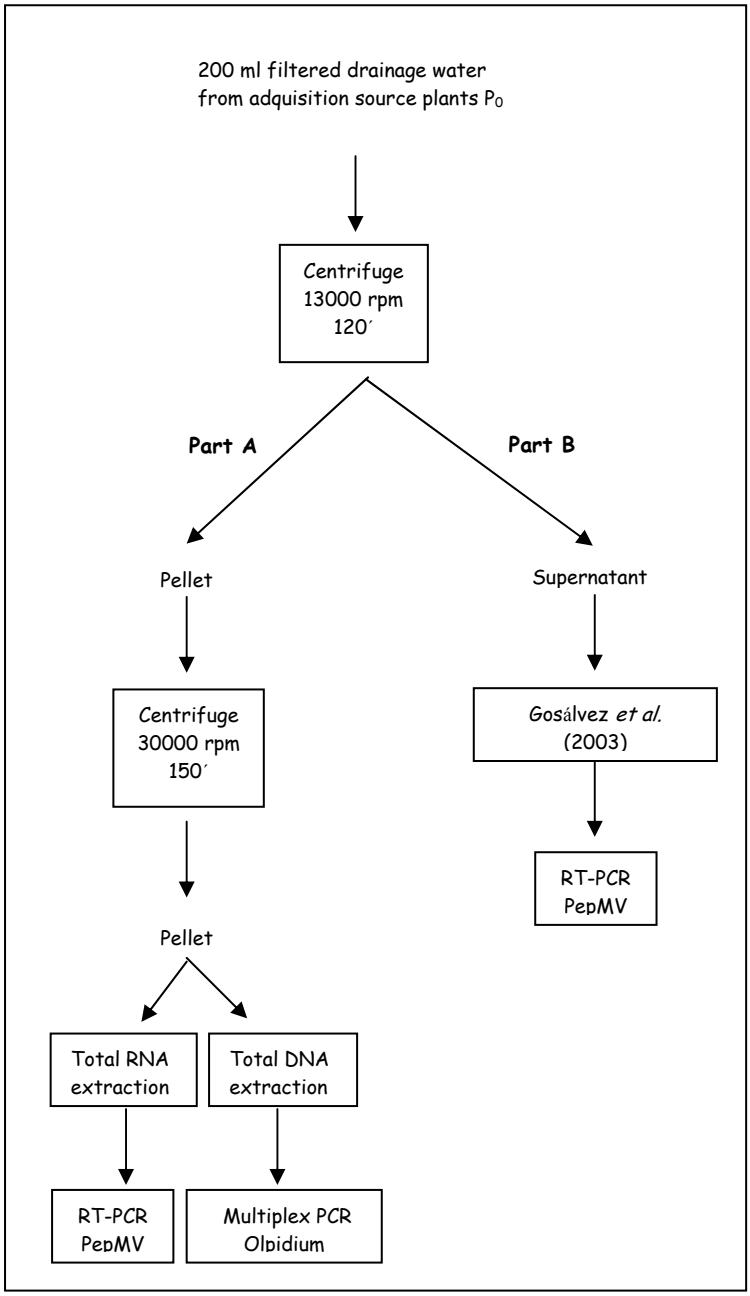

Figure 5.2. Basic steps involved in the extraction procedure of PepMV and *O. virulentus* from the drainage water of the acquisition-source plants (P_0).

All the amplified PCR products were analysed on 1.2% agarose/TAE gels stained with ethidium bromide. The products obtained were compared with a DNA standard marker (GeneRuler^{TM} 100 bp DNA Ladder Plus, MBI Fermentas, Vilnius, Lithuania).

Virus acquisition and transmission by the vector to the acquisition-transmission plants (P_1).

Thirty-six tomato plants, cv. Marmande, were sown and grown in seedbeds until transplanted to 20 cm-diameter plastic pots, as described before. These plants were to constitute the acquisition and transmission plants of the assay (P_1). P_1 plants were classified into six different plots depending on the inoculation source that they would receive (Figure 5.1). P_1 plots were AP_1 (constituted by the six acquisition-transmission plants irrigated with the drainage water obtained from AP_0), An_1 (constituted by the 6 acquisition-transmission plants irrigated with the drainage water obtained from An_0), BP_1 (constituted by 6 the acquisition-transmission plants irrigated with the drainage water obtained from BP_0), Bn_1 (constituted by the six acquisition-transmission plants irrigated with the drainage water obtained from Bn_0), HP_1 (constituted by the six acquisition-transmission plants irrigated with the drainage water obtained from HP_0) and Hn_1 (constituted by the six acquisition-transmission plants irrigated with the drainage water obtained from Hn_0). Inoculative irrigation started immediately after transplanting and was performed at the same frequency as described before for P_0. Each inoculative irrigation consisted in the drainage water obtained from P_0 which had been filtered through Watman No. 4 filter paper to eliminate plant debris from the solution in order to avoid the possible mechanical transmission of the virus. P_1 plants were irrigated with equal volumes (250 mL) of filtered drainage water obtained from irrigating P_0.

Serological analysis of leaf samples by DAS-ELISA was carried out for all the P_1 plants, as described before, at 45 days after beginning the inoculative irrigation. The analysis was repeated with all the P_1 plants every 15 days until the end of the assay. The total RNA of P_1 tomato leaves were extracted and analysed by RT-PCR with PepMV-CP specific primers as described before to confirm the results obtained by the serological analysis.

Observation and monitoring the fungus in the secondary roots by light microscope was performed with all the P_1 in both Expt. 1 and 2, 60 days after transplant and at the beginning of the inoculative irrigation, as described before. In Expt. 2 however, root samples were also molecularly analysed by multiplex PCR, after extracting the total DNAs from 0.05g of root tissues, as detailed before.

Intensive and thorough precautions were taken in all the steps of this study to control *Olpidium,* to prevent accidental spread into uninoculated root systems, and to avoid PepMV plant-to-plant transmission through contact.

RESULTS

Molecular characterisation of the viral isolates

Like the caracterized isolates 3672 and 4809 (Pagán *et al.,* 2006), isolate 4988 (GenBank Accession numbers detailed in Table 5.1) corresponded to the European tomato strain (EU) of PepMV, which in the BLAST analysis, showed a high nucleotide identity with percentages of 99% between them and the EU strain isolates published in the GenBank database (GenBank Accession numbers AJ438767, AJ606360).

Previous analysis performed to the acquisition-source plants (P_0)

In the serological analysis against PepMV performed at 15 dpi to the leaves of P_0, 100% of the inoculated plants with the virus were positive (sub-plots AP_0, BP_0 and HP_0) and negative in all the plants of the rest of the sub-plots (An_0, Bn_0 and Hn_0) (Table 5.2), resulting in successful PepMV inoculation. In Expt. 1, the symptoms observed in the PepMV-inoculated plants depended on the isolate. Isolates 3672 and 4809 were very mild, plants inoculated with isolate 3672 only developed a slight bubbling on the youngest leaves, and isolate 4809 was asymptomatic in all the inoculated plants. However, those plants inoculated with isolate 4988 developed more aggressive symptoms such as dark green mosaic, bubbling and nettle heads in both Expt. 1 and Expt 2. For that reason, only isolate 4988 was used in Expt. 2 for the transmission assay.

A morphological observation of P_0 roots confirmed the presence of *O. virulentus* given the presence of stellate resting spores (Figure 5.4a) in 100% of the plants belonging to plots AP_0, An_0, BP_0 and Bn_0 and which had been irrigated with the stock cultures of *O. virulentus* A and B (Table 5.2). Other fungal structures were also observed in these plants, such as zoosporangia (Figure 5.4a). During Expt. 1, the quantification of the fungal structures was similar in all the plots containing fungal structures. However in Expt. 2, the plots irrigated with the stock culture B of *O. virulentus* generally presented more infection, as revealed the fungal structures (rs and zs) quantification which was codified mainly as 2 (ranged from 101-1000), than those plots irrigated with culture A, which fungal structures (rs and zs) were quantified as 1, ranged 0-100 (Table 5.2).

PepMV detection in the drainage water of acquisition-source plants (P_0)

The total RNA obtained from Part A, used to process drainage water, was seen to be positive via RT-PCR with the specific primers for the CP gene of

PepMV (expected amplicon of 845 bp) in the processed drainage water of plots AP_0 and BP_0, which had been inoculated with the both fungus and the virus in Expt. 1; however only BP_0 was positive in Expt. 2 (Figure 5.3a).

The results of the multiplex PCR analysis for the differentiation of *Olpidium* spp., performed with the DNA extraction obtained in Part A, was positive for *O. virulentus* (expected amplicons of 579 bp) in all the processed drainage water of plots P_0 which had been irrigated with the fungal cultures (AP_0, An_0, BP_0 and Bn_0), as shown in Figure 5.3b.

The RT-PCR assay conducted with the specific primers for the CP gene of PepMV performed with the total RNA extracted from Part B was seen to be positive only in the mixture of drainage water of AP_0+BP_0. The rest of the extracted samples consisting in the drainage water of virus-free plants containing the fungal vector (An_0+Bn_0), the drainage water of plants inoculated with PepMV without the fungus (HP_0), and healthy plants (Hn_0) were negative according to the RT-PCR assay against PepMV (Figure 5.3c).

Virus acquisition and transmission by the vector to the acquisition-transmission plants (P_1).

In Expt.1, one plant of plot BP_1 showed the typical dark green mosaic and bubbling symptoms on the leaves associated with PepMV infection one month after starting inoculative irrigation with the drainage water of the P_0 plants (Figure 5.4b). DAS-ELISA performed with all the P_1 plants proved positive only in this symptomatic plant. RT-PCR analysis further confirmed this ELISA result. This symptom was similar to those which developed in the P_0 plants inoculated with isolate 4988. In Expt. 2, no typical symptoms of PepMV were observed during the assay.

Table 5.2: Serological analysis of the leaves against PepMV and monitoring of *Olpidium* spp. by microscopic observation of the roots performed with the stock fungal cultures and the acquisition-source plants (P_0).

		Expt.1				Expt.2			
	Irrigation source[a]	**PepMV isolate inoculated**	**Plot[b]**	**As-PepMV**	**Monitoring *Olpidium* spp.[c]**	**PepMV isolate inoculated**	**Plot[b]**	**As-PepMV**	**Monitoring *Olpidium* spp.[c]**
Culture A	**H**	none	Culture A	-	rs=2, zs=2	none	Culture A	-	rs=2, zs=2
Culture B	**H**	none	Culture B	-	rs=2, zs=2	none	Culture B	-	rs=2, zs=2
P_0[b]	**A**	3672		+	rs=2, zs=2			+	rs=1, zs=1
		4809	AP_0	+	rs=2, zs=2	4988	AP_0	+	rs=1, zs=1
		4988		+	rs=2, zs=2			+	rs=1, zs=1
		none		-	rs=2, zs=2	none		-	rs=1, zs=1
		none	An_0	-	rs=2, zs=2	none	An_0	-	rs=1, zs=1
		none		-	rs=2, zs=2	none		-	rs=1, zs=1
	B	3672		+	rs=2, zs=3			+	rs=2, zs=3
		4809	BP_0	+	rs=2, zs=2	4988	BP_0	+	rs=2, zs=2
		4988		+	rs=2, zs=2			+	rs=1, zs=1
		none		-	rs=2, zs=2	none		-	rs=2, zs=2
		none	Bn_0	-	rs=2, zs=2	none	Bn_0	-	rs=2, zs=2
		none		-	rs=2, zs=2	none		-	rs=1, zs=1
	H	3672		+	rs=0, zs=0			+	rs=0, zs=0
		4809	HP_0	+	rs=0, zs=0	4988	HP_0	+	rs=0, zs=0
		4988		+	rs=0, zs=0			+	rs=0, zs=0
		none	Hn_0	-	rs=0, zs=0	none	Hn_0	-	rs=0, zs=0

[a]Irrigation performed with three different sources: A= drainage water obtained from the irrigation of fungal culture A; B= drainage water obtained from the irrigation of fungal culture B; H= sterile water.
[b]Plants P_0 (acquisition-source plants) which were grouped into different plots: Culture A= stock culture of *O. virulentus* collected from lettuce crop soil; Culture B= stock culture of *O. virulentus* collected from tomato crop soil; AP_0= acquisition-source plants irrigated with culture A and inoculated with PepMV isolates; An_0= acquisition-source plants irrigated with culture A and non-inoculated with any PepMV isolate; BP_0= acquisition-source plants irrigated with culture B and inoculated with PepMV isolates; Bn_0= acquisition-source plants irrigated with culture B and non-inoculated with any PepMV isolate; HP_0= acquisition-source plants irrigated with sterile water and inoculated with PepMV isolates; Hn_0= acquisition-source plants irrigated with sterile water and non-inoculated with any PepMV isolate.
[c]Monitoring of *Olpidium* spp. structures, resting spores (rs) or zoosporangia (zs), present in the roots of the plants by light microscope observation following the scale: no fungal structure = 0 ; range 0-100 rs, zs = 1; range 101-1000 rs, zs = 2 ; more than 1001 rs, zs= 3.

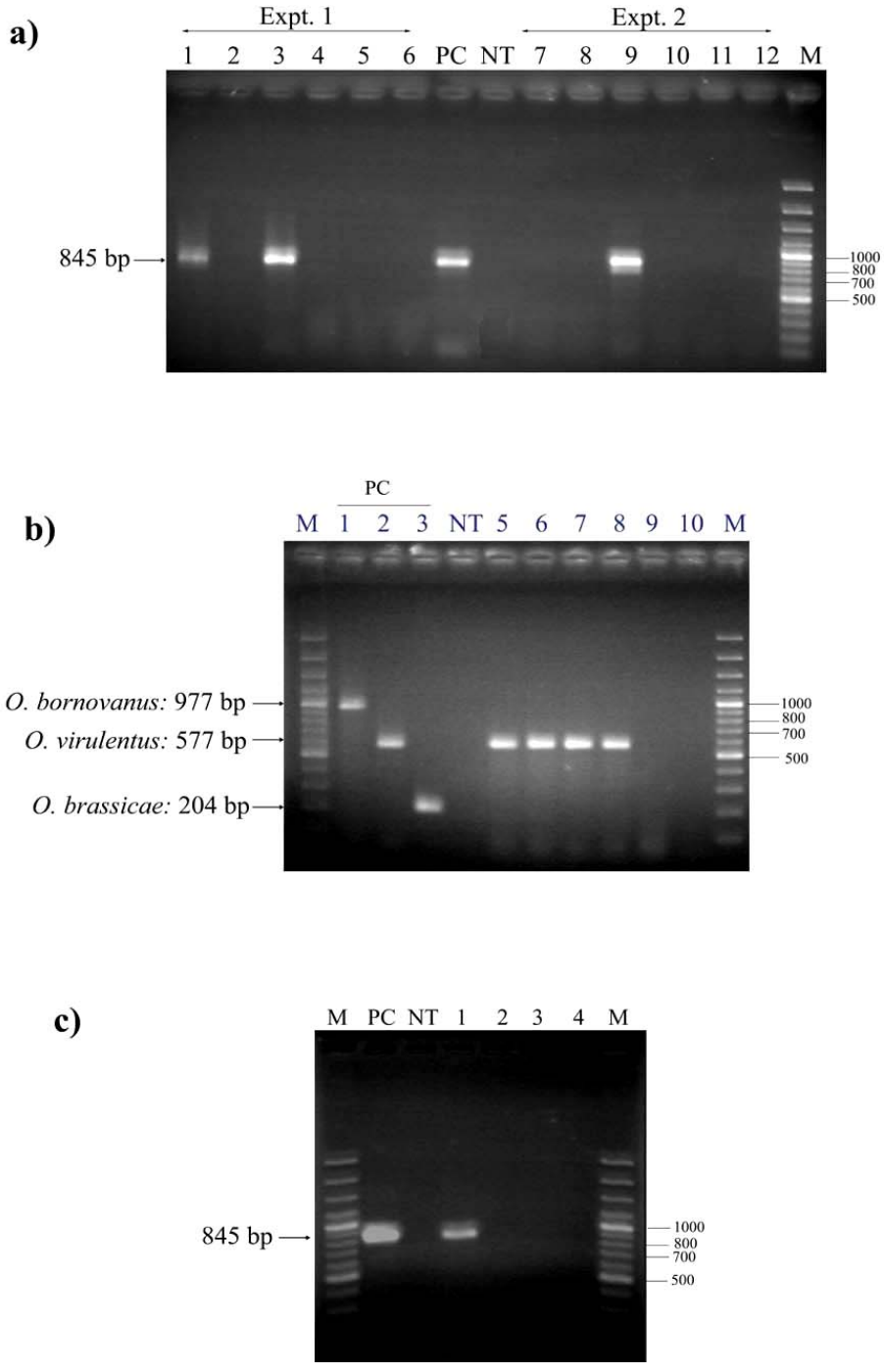
a)
Expt. 1
Expt. 2
1 2 3 4 5 6 PC NT 7 8 9 10 11 12 M
845 bp
1000
800
700
500
b)
PC
M 1 2 3 NT 5 6 7 8 9 10 M
O. bornovanus: 977 bp
O. virulentus: 577 bp
O. brassicae: 204 bp
1000
800
700
500
c)
M PC NT 1 2 3 4 M
845 bp
1000
800
700
500

Figure 5.3. Molecular analysis of water samples collected from the drainage water of the acquisition-source plants (P_0) processed as described in Figure 5.2. **(a)** Analysis by one-step RT-PCR using specific primers for PepMV, CP-D and CP-R described by Pagán *et al.* (2006) performed with the total RNA obtained from part A of the method which corresponded to samples collected from the drainage water of plants AP_0 (Lane 1 and 7), An_0 (Lane 2 and 8), BP_0 (Lane 3 and 9), Bn_0 (Lane 4 and 10), HP_0 (Lane 5 and 11) and Hn_0 (Lane 6 and 12), during Expt. 1 (Lanes 1-6) and Expt. 2 (Lanes 7-12). The expected amplicons corresponding to PepMV positive result are indicated. Lanes PC: positive control infected with PepMV, NT: no RNA template, M: 100 bp molecular weight marker. **(b)** Multiplex PCR analysis using specific primers for the simultaneous detection of *Olpidium* spp. (Herrera-Vásquez *et al.,* 2009b) performed to the total DNA obtained from part A of the method which corresponded to samples collected from the drainage water of plants AP_0 (Lane 5), An_0 (Lane 6), BP_0 (Lane 7), Bn_0 (Lane 8), HP_0 (Lane 9) and Hn_0 (Lane 10). The expected amplicons corresponding to *O. bornovanus, O.virulentus* and *O. brassicae* are indicated. Lanes PC: positive control of each *Olpidium* sp. (lane 1: *O. bornovanus*, lane 2: *O. virulentus*, lane 3: *O. brassicae*), NT: no DNA template, M: 100 bp molecular weight marker. **(c)** Analysis by one-step RT-PCR using specific primers for PepMV, CP-D and CP-R described by Pagán *et al.* (2006) performed with the total RNA obtained from part B of the method which corresponded to samples collected from the drainage water of plants AP_0 and BP_0 mixed (Lane 1), An_0 and Bn_0 mixed (Lane 2), HP_0 (Lane 3) and Hn_0 (Lane 4). The expected amplicons corresponding to PepMV positive result are indicated. Lanes PC: positive control infected with PepMV, NT: no RNA template, M: 100 bp molecular weight marker.

DAS-ELISA performed one month after starting irrigation with the drainage water of the P_0 plants was negative for all the P_1 plants. However, the RT-PCR assay proved positive for one plant of plot BP_1 (Table 5.3). Therefore, the transmission rate of both assays was 8%, and this percentage was calculated as one positive plant of twelve plants which could have been infected (plots AP_1 and BP_1) by PepMV transmission vectored by *O. virulentus*.

The observation of the roots in both experiments revealed the presence of stellate resting spores which are characteristic of *O. brassicae* sl (Figure 5.4a), and zoosporangia were noted in the P_1 plants irrigated with the drainage water of

plots An_0, AP_0, Bn_0 and BP_0. No fungal structure was observed in the P_1 plants irrigated drainage water of plots Hn_0 or HP_0, which were fungus-free plants (Table 5.3). The quantification of the fungal structures in Expt. 1 revealed that the P_1 plants, which had been irrigated with culture B of *O. virulentus,* presented more quantities of fungal structures than those irrigated with culture A, which also occurred in the acquisition-source plants, P_0. In Expt. 2, no differences in the quantification of fungal structures were observed among plots irrigated with culture A or B (Table 5.3).

In Expt. 2, the results of the multiplex PCR assay to differentiate *Olpidium* ssp. performed with the roots of P_1 coincided with the morphological observation of the roots; only those plants irrigated with the drainage water containing fungal cultures (An_1, AP_1, Bn_1 and BP_1) tested positive for *O. virulentus*.

The density of the root system of all the plants irrigated with and without the fungus was compared "*de visu*" at the end of the assay, and a clear difference was observed; those plants irrigated with any of the *O. virulentus* cultures presented a high reduction (15-35%) of root system density than fungus-free plants.

Table 5.3: Results of the analysis performed with the acquisition-transmission plants (P_1) to detect the possible transmission of PepMV and to confirm the presence of *O. virulentus*.

Plot[a]	Expt. 1					Expt. 2					
	PepMV[b]			*Olpidium* spp.		PepMV[b]			*Olpidium* sp.		
	Symptoms observation	DAS-ELISA	RT-PCR	Microscopic observation		Symptoms observation	DAS-ELISA	RT-PCR	Microscopic observation		PCR[d]
				Presence	Monitoring[c]				Presence	Monitoring[c]	
AP_1	0/6	0/6	0/6	6/6	rs.=2, zs=1	0/6	0/6	0/6	6/6	rs=2, zs=2	6/6 (*O.vir*)
An_1	0/6	0/6	0/6	6/6	rs.=2, zs=1	0/6	0/6	0/6	6/6	rs=2, zs=2	6/6 (*O.vir*)
BP_1	1/6	1/6	1/6	6/6	rs.=3, zs=2	0/6	0/6	1/6	6/6	rs=2, zs=2	6/6 (*O.vir*)
Bn_1	0/6	0/6	0/6	6/6	rs=2, zs=2	0/6	0/6	0/6	6/6	rs=2, zs=2	6/6 (*O.vir*)
HP_1	0/6	0/6	0/6	0/6	rs.=0, zs=0	0/6	0/6	0/6	0/6	rs.=0, zs=0	0/6
Hn_1	0/6	0/6	0/6	0/6	rs.=0, zs=0	0/6	0/6	0/6	0/6	rs.=0, zs=0	0/6

[a] Plants P_1 (acquisition-transmission plants) which were grouped into different plots: AP_1= acquisition-transmission plants irrigated with the drainage water collected from the irrigation of AP_0. which were infected with both *O. virulentus* culture A and PepMV; An_1= acquisition-transmission plants irrigated with the drainage water collected from the irrigation of An_0, which were infected only with *O. virulentus* culture A; BP_1= acquisition-transmission plants irrigated with the drainage water collected from the irrigation of BP_0, which were infected with both *O. virulentus* culture B and PepMV; Bn_1= acquisition-transmission plants irrigated with the drainage water collected from the irrigation of Bn_0, which were infected only with *O. virulentus* culture B; HP_1= acquisition-transmission plants irrigated with the drainage water collected from the irrigation of HP_0, which were infected only with PepMV; Hn_1= acquisition-transmission plants irrigated with the drainage water collected from the irrigation of Hn_0, which were free of *O. virulentus* and PepMV infection.

[b] Number of positive plants/total number of plants analysed.

[c] Monitoring of Olpidium spp. structures, resting spores (rs) or zoosporangia (zs), present in the roots of the plants by light microscopic observation following the scale: no fungal structure = 0 ; range 0-100 rs, zs = 1; range 101-1000 rs, zs = 2 ; more than 1001 rs, zs= 3.

[d] *O.vir*= Resulted in the amplicon that correspond to *O. virulentus* (579 bp).

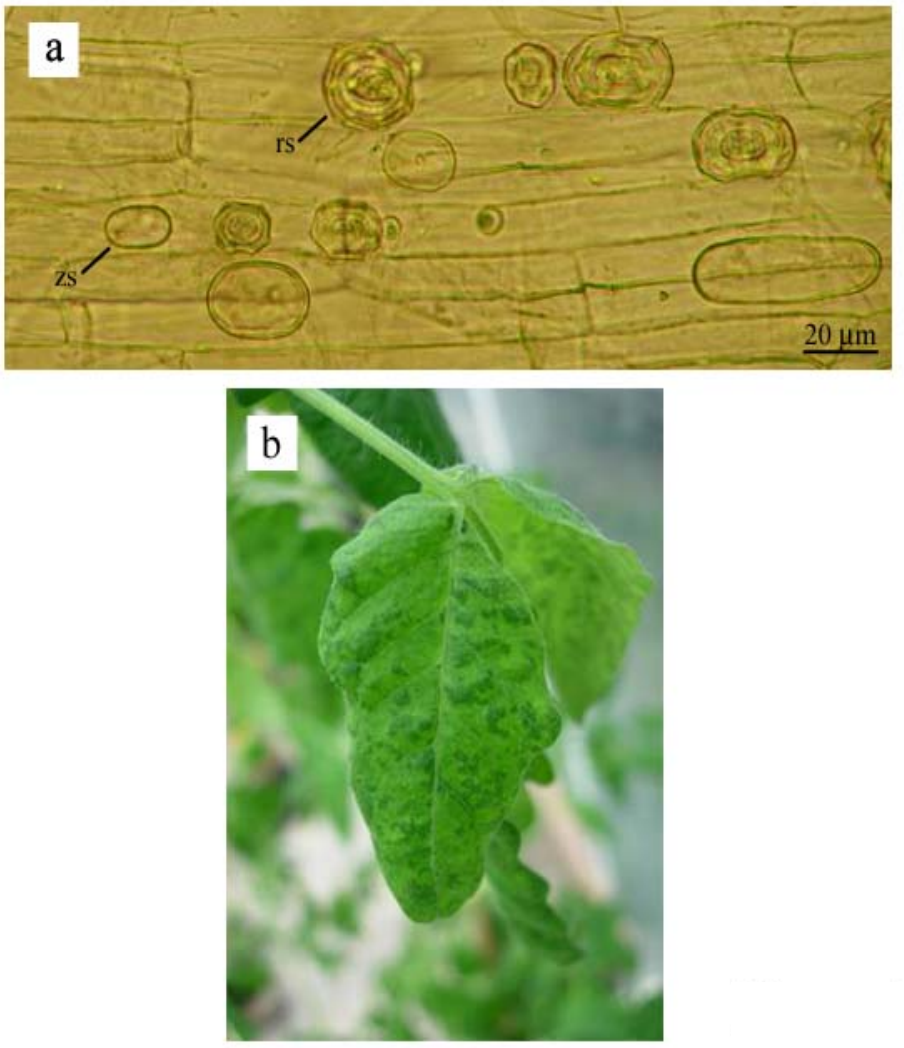

Figure 5.4. (a) Stellate resting spores characteristic of *O. brassicae* sl, and zoosporangia. **(b)** The typical green mosaic and bubbling symptoms on the leaves of one plant from plot BP_1 associated with PepMV infection one month after the beginning of the inoculative irrigation with the drainage water of the P_0 plants during Expt. 1.

DISCUSSION

PepMV, as a member of the genus *Potexvirus*, easily and quickly spreads through tomato crops, causing important economic losses. Some forms of PepMV transmission associated with its mechanical transmission, such as transmission by *Bombus* spp., which carried the virions stuck to their bodies and their contact with plants produced the transmission of the virus (Lacasa *et al.,* 2003), or through infected pollen (Shipp *et al.,* 2008), have been studied. Recently, PepMV seed transmission has been proved (Córdoba-Sellés *et al.,* 2007b) and constitutes a perennation mechanism as well as the long-distance transport of the virus. Some common weed species were also reported to be PepMV infected, which constituted a possible virus reservoir (Jordá *et al.,* 2001b; Córdoba *et al.,* 2004a). Therefore, these facts represent a high risk of PepMV distribution given its easily mechanical transmission. The present study expands the information about the epidemiology of this virus, where PepMV was vector-transmitted into two different replicate assays by the fungal vector *O. virulentus* with a transmission rate of 8%. Another *Potexvirus*, *Potato virus X* (PVX), was reported to be vector transmitted by *Synchitrium endobioticum* (Schilb.) Perc. under experimental conditions; however this form of transmission was not corroborated in later investigations (Šutić *et al.,* 1999).

Throughout this study, apart from the plants infected with both the fungus and virus (AP_0, BP_0 in the assays), all the controls or checks as described by Campbell (1988) were included; fungus alone (An_0, Bn_0 in the assays) to verify that zoospores were produced in virus-free conditions; virus alone (HP_0 in the assays) to check that there was no mechanical transmission or vector contamination; and the usual non-inoculated plants to test that experimental plants were produced and were maintained free of both the virus and the vector (Hn_0 in the assays). These controls were never contaminated during both

experiments (Expt.1 and Expt. 2). Moreover, only those plants irrigated with drainage water containing both the virus and fungal vector succeeded in transmitting PepMV.

Vector-free drainage water obtained from irrigating the plants infected with PepMV (HP_0) did not transmit the virus to healthy plants P_1. This ensures that PepMV was not transmitted in the irrigation water alone without the presence of the vector. This result contrasts with some studies which suggest that PepMV is distributed through a recirculating hydroponic system (Fakhro *et al.,* 2005; Schwartz *et al.,* 2007). In some cases, PepMV infection was delayed because of the pre-infection of *Phytium aphanidermatum* (Edson) Fitzp. (Schwartz *et al.,* 2007). However, the presence of *Olpidium* spp. zoospores in either the recirculating solution or the tomato roots was not checked in those works. In Almería (Spain), *Olpidium bornovanus* (Sahtiyanci) Karling, an efficient vector of *Melon necrotic spot virus* (MNSV), was found in irrigation pools destined to water cucurbit crops, constituted a possible source of MNSV contamination and spread (Gómez and Velasco, 1991). The possibility of *O. virulentus* contamination in the irrigation systems has to be taken into account to avoid possible PepMV transmission which may occur, as this study has evidenced. Although the transmission rate of PepMV by *O. virulentus* was low in this study compared with other viruses vectored by this fungus which presented variable transmission rates, e.g., *Mirafiori lettuce virus* (MiLV) and *Lettuce big vein virus* (LBBV) with 95% (Lot *et al.,* 2002), 40% of *Lettuce ring necrosis virus* (LRNV) (Campbell and Lot, 1996) or 30-40% of MNSV (Tomlinson and Thomas, 1986), PepMV has the extraordinary capability of mechanical spread in tomato crops. The low transmission rate obtained in this study could be a result of the method employed in this assay, which differed from that described by Campbell (1988) who inoculated a zoospore suspension of the fungus mixed with purified virions. In the present study, the intention was

to reproduce the real situation in fields where intensive and reiterated cultures of tomato with recirculating irrigation systems are commonly used. Therefore, the plant which presented PepMV vector transmission was irrigated with the drainage water from plants infected with both the virus and the fungus.

In addition, interaction between PepMV and *O. brassicae* sl has been demonstrated in the syndrome referred to as 'tomato collapse' (Córdoba *et al.,* 2004b). Therefore, not only the potential risk of PepMV transmission by the fungus, but also the manifestation of an aggressive syndrome that causes wilting in tomato crops, meant that suitable control measures had to be taken to avoid fungal spread. *O. brassicae* sl survived in soil as resting spores for 20-22 years, but zoospores and vegetative sporangia were killed by short drying periods (Campbell, 1985). Despite the thermal death point of resting spores of *O. brassicae* s.l. being reported to be near 65ºC for 10 minutes (Campbell and Lin, 1976), the fungus was able to survive in infected soil after composting when high temperatures (50-70ºC) were reached (Bollen *et al.,* 1989). Therefore, all these characteristics have to be considered when controlling fungal spread. Disinfection treatments of the irrigation solution with surfactant agral (alkalyn phenol ethylene oxide) (Tomlinson and Thomas, 1986) and ultraviolet treatment (Campbell, 1996) are two examples of effective measures to control *Olpidium* infections.

Moreover, a method described by Gosalvez *et al.* (2003) for the detection of MNSV in water samples was performed with samples of the drainage water obtained from irrigating the adquisition-source plants (P_0) to detect PepMV in water samples. The virus was detected in the drainage waters obtained from plants that presented both the virus and the fungi (AP_0, BP_0 in the assays). In the first centrifugation (13000 rpm for 120´), all the fungal structures were contained in the pellet because just 1000g (3000-4000 rpm) for 10´ is enough to concentrate *O. bornovanus* zoospores (Tomlinson and Thomas, 1986).

The result of the presence of *O. virulentus* in the pellet obtained from the drainage water was expected. Nonetheless, the virus was only detected in the pellets from the drainage waters obtained from P_0 plants which contained the fungus. Yet the presence of the fungus was needed to detect the virus in the pellet, and even the supernatant was analysed to verify the presence of PepMV in the water tested as only positive in the sample which corresponded to the drainage water from P_0 plants containing both the fungus and the virus. Therefore, the virus itself was not detected in the drainage water, and could not be transmitted to the acquisition-transmission plants (P_1) without the presence of *O. virulentus*.

The transmission of PepMV only occurred in this study with the fungal vector collected from tomato roots, and never with the culture originating from lettuce roots. The host specificity of *O. brassicae* s.l. (even the new renamed species *O. virulentus*) and *O. bornovanus*, which measures reproductive fitness or compatibility between a fungus isolate and a host plant, is one of the widely demonstrated characteristics of these fungal vectors (Campbell and Sim, 1994; Campbell *et al.,* 1995; Koganezawa *et al.,* 2005). The ability to transmit a given virus may be limited to certain host-specific strains of the vector. A high specificity of virus adquisition by the zoospore membranes of the fungus is required for transmission by fungal vectors (Campbell, 1996). Likewise, an inefficient vector requires either more virus or more zoospores, specific hosts, or the combination of all these items for transmission, which occurred with the melon and cucumber strains of *O. bornovanus* that transmitted *Squash necrosis virus* (SqNV) to watermelon, but not to other compatible hosts (Campbell *et al.,* 1995). The inability of zoospores to adsorb virus particles correlated with the failure of the zoospores of some *O. brassicae* strains to transmit *Tobacco necrosis virus* (TNV). Perhaps these zoospores have fewer or none specific sites of adsorption and, therefore, seemed to adsorb fewer particles of some viruses

(Temmink *et al.,* 1970). In this report, the viral transmission was also observed to be restricted to a tomato isolate of *O. virulentus*, although the lettuce isolate, reproduced properly in tomato roots as the quantification results indicated given the plurivorous nature of this species. This is an approach for the evaluation of the specificity between fungus vector and virus, but further studies are required to verify such specificity.

The three PepMV isolates we used in this study were of the European tomato strain. PepMV presents a high molecular variability and different strains of the virus have been described. Our results are preliminary and other assays are being performed to check the ability of other strains of the virus to be vector-transmitted by *O. virulentus*.

Furthermore, *Olpidium* spp. have always been considered fungal vectors that cause conspicuous damage to the roots of the host. However, reduced root density between those plants inoculated and non-inoculated by the fungus was evident, as reported by Campbell and Sim (1994) who observed the browning of roots and reduced development in the stock culture plants inoculated with *O. brassicae*. A reduction in root and shoot growth of 20-50%, and increased respiration of roots, have been attributed to an unidentified *Olpidium* spp. in melons (suspected to be *O. bornovanus*) (Hadar *et al.,* 1992). Similarly, the root systems of infected and non-infected roots resulted in clear developmental differences in the present study.

The importance of constantly inspecting plants at the seedling stage to avoid the early spread of mechanical transmissions of PepMV and to control the presence of the fungal vector in water or soil, which could represent a potential risk for PepMV transmission in hydroponic systems, or even in soil cultures, can never be underestimated or ignored.

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Capítulo 6

Occurrence and geographical distribution of the 'torrado' disease in Spain

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ABSTRACT

In surveys to determine the occurrence and distribution of the 'torrado' disease (Tomato torrado virus, ToTV) in the main Spanish tomato growing areas from 2001 to 2008, a total of 584 samples from symptomatic and asymptomatic plants were collected from 92 greenhouses. The tests showed that 451 plants from 85 greenhouses of different areas were infected with ToTV. The majority of the positive samples showed typical symptoms of the disease. However, plants showing different symptoms of necrosis and even asymptomatic plants were infected with the virus. Co-infection of ToTV with *Pepino mosaic virus* (PepMV) occurred in a large number of samples (60.5%), and several samples were infected with other tomato-infecting viruses, including *Cucumber mosaic virus* (CMV), *Potato virus Y* (PVY), *Tomato spotted wilt virus* (TSWV), *Tomato mosaic virus* (ToMV), *Parietaria mottle virus* (PMoV), *Tomato chlorosis virus* (ToCV) and *Tomato yellow leaf curl virus* (TYLCV). Tomato apex necrosis virus (ToANV) was not detected in any of those samples with similar symptoms to those described for that virus. Additional tests revealed that i) ToTV whitefly transmission is highly efficient and variety-dependent in tomato plants, ii) *Datura stramonium* is another solanaceous species susceptible to this virus and iii) the tissue-printing hybridization is a reliable technique which could facilitate the routine diagnosis and large-scale analysis of ToTV.

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is an important vegetable crop, the world production of which in 2007 was 126.1 MMT (million metric tons). Spain is the eighth largest tomato producer in the world (3.62 MMT), and the second in the European Union after Italy (FAO, 2009). Nearly 85% of Spanish tomato production is located in four southern regions of the country: Andalucía, Extremadura, the Murcia Region and the Canary Islands; tomatoes are mainly produced in greenhouses, except in Extremadura where production is in open fields (MARM, Spanish Ministry of Environment and of Rural and Marine Affairs, 2009). Tomato crops are susceptible to a wide range of diseases, of which virus diseases are difficult to control and can result in substantial crop losses, being one of the main limiting factors in intensive protected tomato production (Jones *et al.,* 1991).

In 2001, several greenhouse-grown tomato crops in the Murcia Region showed either an initial yellowing at the base of the leaflet that later developed into necrotic spots or an extensive necrotic area progressing from the base to the tip. Fruits had necrotic areas that often developed into cracks. These plants generally had overall reduced growth which seriously affected productivity. The disease was named 'torrao' or 'torrado' by the producers, which refers to the general burnt-like appearance of the affected plants (Jordá *et al.,* 2003; Alfaro-Fernández *et al.,* 2006).

In 2007, a new virus named Tomato torrado virus (ToTV) was identified and reported as the causal agent of the 'torrado' disease in tomato samples from the Murcia Region (Spain). This virus was characterized as a Picorna-like virus and is a possible member type of a new genus, Torradovirus (Verbeek *et al.,* 2007a), which also includes two other recently characterized tomato-infecting viruses, Tomato apex necrosis virus (ToANV) and Tomato marchitez virus

(ToMarV). ToANV and ToMarV have been identified in tomato crops in Mexico which showed different necrotic symptoms on leaves and fruits (Turina *et al.,* 2007; Verbeek *et al.,* 2007b). ToTV has isometric particles with a diameter of 28 nm, and its genome is composed of two single-stranded positive-sense RNA molecules which contain three coat protein subunits. This structure resembles those of members of the genera *Sequivirus*, *Waikavirus, Sadwavirus* and *Cheravirus*; however, phylogenetic analyses revealed important differences between ToTV and the viruses of these genera (Verbeek *et al.,* 2007a).

Recently, this virus has been detected in tomato in Poland (Pospieszny *et al.,* 2007, 2009), Canary Islands (Spain; Alfaro-Fernández *et al.,* 2007a), Australia (IPPC, 2008), Panama (Herrera-Vásquez *et al.,* 2009a) and Hungary (Alfaro-Fernández *et al.,* 2009a), as well as on weed hosts in Spain (Alfaro-Fernández *et al.,* 2008b), and it has been included in the EPPO alert list (EPPO, 2009). Three ToTV Polish isolates were biological and molecularly characterized showing them to be closely related (Pospieszny *et al.,* 2009).

The 'torrado' disease has always been associated with large whitefly populations and infestations in the affected greenhouses since the first observation of its symptoms (Jordá *et al.,* 2003). ToTV has been reported to be efficiently transmitted by *Trialeurodes vaporariorum* (Westwood) (Pospieszny *et al.,* 2007, 2009) and *Bemisia tabaci* (Gennadius) (Amari *et al.,* 2008).

Tomato is seriously affected by several viral diseases in which a mixed viral infection may result in synergisms and more severe disease symptoms (García-Cano *et al.,* 2006). In preliminary assays, ToTV was frequently found in tomato samples together with *Pepino mosaic virus* (PepMV), which is widely distributed in the tomato production areas of the country (Alfaro-Fernández *et al.,* 2007b; Verbeek *et al.,* 2007a; Alfaro-Fernández *et al.,* 2009b). However, no data of a mixed infection of ToTV with the other main viruses affecting tomato crops are available.

Despite this disease being present in greenhouse-grown tomatoes in Spain every growing season, there is very limited information about its incidence in Spain. Therefore several surveys have been made in the main tomato production areas in Spain since the outbreak of the disease in 2001. We report here the occurrence and geographical distribution of the 'torrado' disease in Spanish tomato crops. We also assessed the percentage of mixed infections of ToTV with PepMV and whitefly transmission. Finally, we present the useful techniques of tissue-printing and dot-blot hybridization for the detection of ToTV in field samples.

MATERIAL AND METHODS

Surveys and sample collection

Surveys were conducted in greenhouses of commercial tomato crops in 11 different regions of the most important tomato-growing areas in Spain (Figure 6.1). Tomato leaves were collected from 2001 to 2008. A total of 584 plants at different stages of development were surveyed from 92 greenhouses (Figure 6.1, Table 6.1). All the greenhouses contained both symptomatic and asymptomatic plants.

The symptoms in all the collected plants were recorded and marked as symptomatic plants (s) for those plants showing the symptoms currently associated with the 'torrado' disease, plants showing some symptoms of necrosis which could not be clearly associated with the 'torrado' disease (n), and plants without necrotic symptoms (w/s), even though some of them presented another viral symptom (bubbling, mosaic, yellowing, etc.). Samples were maintained at -80ºC or as dried material at room temperature until processed. All the leaves were tested individually by the direct antibody sandwich enzyme-linked

immunosorbent assay (DAS-ELISA), molecular hybridization by dot-blot and reverse transcription-polymerase chain reaction (RT-PCR) against different viruses, as explained below.

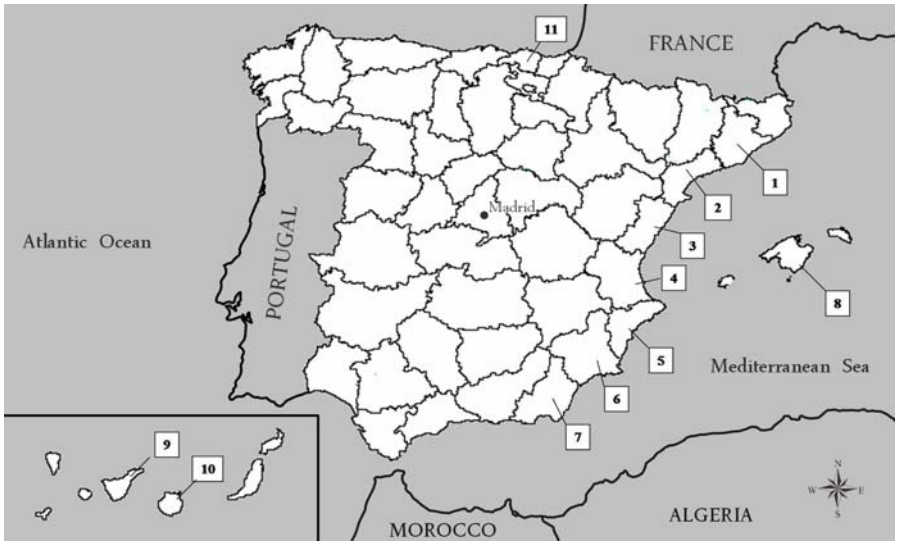


Figure 6.1. Map of Spain showing the location of regions 1 to 11 where greenhouse tomato crops were surveyed during the 2001-2008 growing seasons.

In 2008, four greenhouses in different locations of the Murcia Region were selected. The symptoms of a total of 1527 plants were recorded in randomly selected rows along each greenhouse, which represented 10% of the total growing plants, in order to evaluate the incidence of the virus inside these greenhouses. In these greenhouses, 47 symptomatic or asymptomatic samples were randomly collected to analyze the presence of other viruses (PepMV, ToTV, ToMV, TSWV, CMV and PVY) individually by RT-PCR or DAS-ELISA, as explained below.

Serological assays

The leaf samples surveyed were tested by DAS-ELISA against different viruses depending on the symptoms observed. Samples collected during the earlier growing seasons (2001-2005), when the necrotic symptoms associated with the 'torrado' disease were not identified to any known virus, were serologically tested with antisera against different viruses which generally produce necrosis in tomato plants as: CMV, *Groundnut ringspot virus* (GRSV), *Potato virus X* (PVX), *Tobacco necrosis virus* (TNV), *Tomato bushy stunt virus* (TBSV) and *Tomato chlorotic spot virus* (TCSV) (Loewe Biochemica, Sauerlach, Germany); *Alfalfa mosaic virus* (AMV), *Tobacco mosaic virus* (TMV), *Tobacco streak virus* (TSV), *Tomato black ring virus* (TBRV) and *Tomato ring spot virus* (ToRSV) (Bio-rad Phyto-Diagnostics, Marnes-La Coquette, France); *Pelargonium zonate spot virus* (PZSV) and *Pepper mottle virus* (PepMoV) (DSMZ Deutsche Sammlung von mikroorganismen und Zellkulturen, Braunschweig, Germany). Tests for other viruses commonly infecting tomato crops in Spain were also made depending on the symptoms observed in the sampled plants in all the surveys conducted (2001-2008), such as PVY, ToMV, TSWV (Loewe Biochemica, Sauerlach, Germany) and PepMV, (DSMZ Deutsche Sammlung von Mikroorganismen und Zellkulturen. Braunschweig, Germany). Tissue samples (0.15 g) were homogenized in sample extraction buffer (1:20 w/v). DAS-ELISA was carried out in paired wells using 100 µl of the extracts obtained following the manufacturer's instructions for each specific antiserum supplied. Healthy and virus-infected tomato leaves were included in each ELISA analysis as negative and positive controls, respectively. ELISA reactions were measured spectrophotometrically at 405 nm using a Titertek Multiscan immunoplate reader (Flow Laboratories, Finland). Samples were considered positive if the mean of the absorbance value of the duplicated wells was more than twice that of the corresponding healthy controls.

RNA extraction and molecular hybridization analyses

Total RNA was extracted from 0.1 g of fresh leaf tissue from infected plants using the silica capture protocol (MacKenzie *et al.,* 1997). Total DNA was directly extracted from those samples selected according to the symptoms observed using the E.Z.N.A® Plant DNA Miniprep Kit (OMEGA Biotech, Doraville, USA) following the manufacturer's instructions. The extracted nucleic acids were stored at -80ºC until used.

Non isotopic dot-blot hybridization was used to detect ToTV in all the surveyed samples. One µl of total RNA was firstly denatured with formaldehyde and then directly applied onto a nylon membrane. An analysis of the total nucleic acids was performed by non-isotopic dot-blot hybridization as previously described by Sánchez-Navarro *et al.* (1998) using a dig-RNA probe complementary to a fragment of the polyprotein of ORF2-RNA2 of ToTV. Some samples that showed possible symptoms of PMoV were also analyzed by dot-blot hybridization, as described above, using a dig-RNA probe complementary to a fragment of the coat protein of PMoV provided by Dr. Pallás.

A total of 103 samples, collected from three different fields, were tested by both dot-blot molecular hybridization and tissue-prints of petioles which were cut transversely and pressed directly onto the nylon membrane. Usually two prints were prepared from each sample. After air-drying, the genetic material was cross-linked by UV. The hybridization and detection procedures were conducted as described above.

RT-PCR amplification and sequencing

RT-PCR was used to verify the presence of ToTV following the procedure described by van den Heuvel *et al.* (2006). In order to identify the specific isolate of PepMV in the infected samples, a multiplex RT-PCR assay

was performed as described by Alfaro-Fernández *et al.* (2009b). Briefly, this method identifies which of the five currently described genotypes of PepMV is present in the infected sample: European (EU), Peruvian (PE), Chilean 2 (CH2), US2 and/or CH1/US1 (or four strains: US1/CH1, EU, PE and CH2 strains, this last grouping also US2 genotype; Ling, 2007). Based on the symptoms of the surveyed plants, some other viruses were tested by RT-PCR, such as ToCV, commonly found in Spanish tomato crops, and *Tomato infectious chlorosis virus* (TICV), as previously described (Louro *et al.,* 2000; Vaira *et al.,* 2002, respectively). TYLCV was tested by PCR in several suspected samples as described by Martínez-Culebras *et al.* (2001). The ten samples collected during the first growing season (survey conducted in 2001) were tested by nested-PCR as described by Lee *et al.* (1993) to confirm the presence or absence of phytoplasmas. RT-PCR and PCR analyses were performed using the SuperScript III One Step RT-PCR system with the Platinum Taq DNA polymerase kit (Invitrogen Life Technologies, Barcelona, Spain) and NETZYME® DNA polymerase (NEED S.L., Valencia, Spain), respectively.

For the detection of the recently described ToANV, a pair of specific primers were designed using the published sequence of the RNA2 available in the GenBank database (Accession number EF063642): ToANV-D (5′ GTGCAACTGAGCTTACTGGAG 3′) and ToANV-R (5′ CCACCGAATCCAGATGAACAG 3′), targeting to a fragment of 611 bp of the RNA2 of ToANV. The RT-PCR conditions were an initial incubation at 50ºC for 30 min followed by 2 min at 94ºC and 40 cycles of 94ºC for 15s, 58 ºC for 30s and 68ºC for 45s. A final incubation at 68ºC for 10 min was introduced to finish the incomplete PCR fragments. These samples which presented similar symptoms to those described for ToANV (Turina *et al.,* 2007) were tested against this new virus. A positive control, kindly provided by Dr. Turina, was included in the assay.

All the amplified PCR products were analyzed on 1.2% agarose/TAE gels stained with ethidium bromide. To confirm the correct ToTV amplification, five amplified PCR products corresponding to a fragment of the ORF2 of the RNA2 (van den Heuvel *et al.,* 2006) were purified with the High Pure PCR Product Purification Kit (Roche Diagnostics, Mannheim, Germany) and directly sequenced. These five positive samples were collected in three different areas of Spain over a five-year period of surveys and marked with a three-letter code to indicate their geographic origin (MUR= Murcia, TEN= Tenerife, GNC= Gran Canaria), followed by the collection year: MUR-03, MUR-05, TEN-07, MUR-08 and GNC-08. These samples were also analyzed by RT-PCR and sequenced with the specific primers to the subunit Vp23 of coat protein (CP) gene described by Pospieszny *et al.* (2007). Nucleotide sequences were compared with known sequences deposited in the NCBI database using the Blastn program, and an identity/similarity matrix of the amino acid-analysed sequences was calculated using the Matrix Global Alignment Tool software, version 2.02 (http://bitincka.com/ledion/matgat). Phylogenetic analyses were performed with MEGA (Molecular Evolutionary Genetics Analysis), version 3.1 (Kumar *et al.,* 2004). The robustness of the inferred evolutionary relationships was assessed by 1,000 bootstrap pseudoreplicates.

Mechanical back-inoculation and whitefly transmission

Symptomatic tissues from plants collected in the surveys, which were only positive for ToTV, were used to mechanically inoculate tomato plants cvs Marmande and Boludo. Mechanical transmission was carried out with a sap inoculation of 14 ToTV isolates by grinding the leaves in 0.01 M phosphate buffer, pH 7.4 (1:4 w/v). Extracted sap was rubbed onto 56 healthy tomato plants (28 plants of each cultivar), pre-dusted with Carborundum (600 mesh), at the four-leaf-stage of development. Four plants were inoculated with each ToTV

isolate. Half inoculated plants were placed in growth chambers at 26ºC/22ºC (day/night) with a 12 h photoperiod and 60% relative humidity, while the others were placed in a greenhouse. Plants were inspected for virus symptoms periodically for 8 weeks. All the plants were analyzed by RT-PCR using specific primers to ToTV, as described before.

A viruliferous colony of adults of *T. vaporariorum* was collected in the Murcia Region from a greenhouse infected with ToTV and it was released on 66 healthy tomato plants of cvs Marmande (21 plants), Cedrico (12 plants), Boludo (11 plants), Marglobe (10 plants), and 1123 (12 plants) at the six-leaf-stage of development. We also analysed 6 plants of *Datura stramonium* L., and 4 plants of each species of *N. glutinosa* L., *N. occidentalis* Wheeler and *N. rustica* L. All the plants were placed in muslin-covered shelves inside a growth chamber at 27ºC/24ºC (day/night) with a 12 h photoperiod and 60% relative humidity. Plant symptoms were monitored every two days and sampled 15 and 45 days after whiteflies release. These leave samples were analyzed to ToTV by RT-PCR.

RESULTS

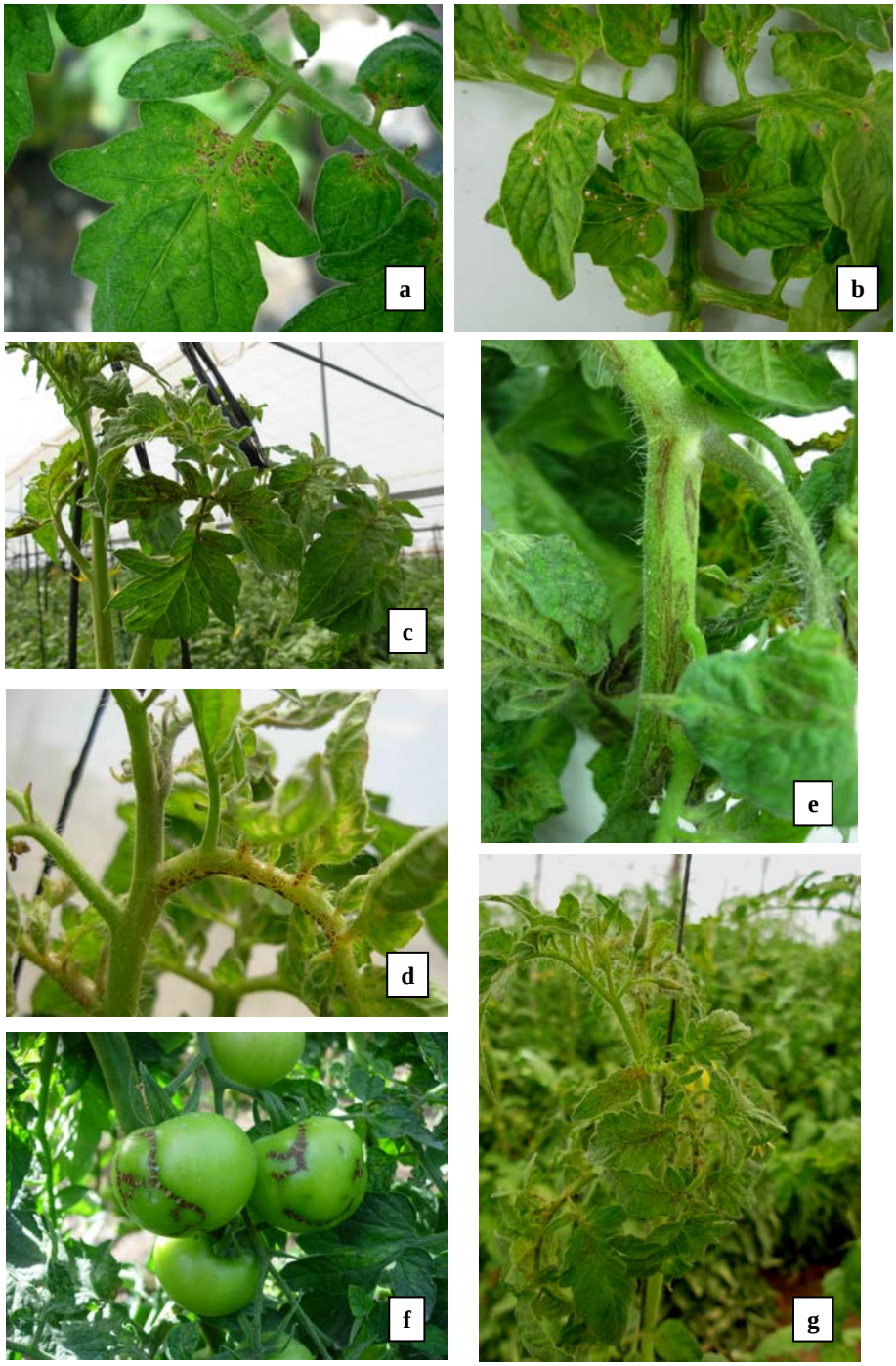
Symptom observation

Plants infected with ToTV showed an initial yellowing in defined areas at the base of the leaflet that developed into necrotic spots (Figure 6.2a), which sometimes abscised, leaving little holes in the leaflet. This symptom is referred to as 'cribado' (Figure 6.2b). Therefore, other plants presented extensive necrotic areas which progressed from the base to tip (Figure 6.2c). On the stems, some plants showed necrotic streaking (Figure 6.2d and 6.2e) and fruits appeared distorted with necrotic lines (Figure 6.2f). Generally, the affected plants had a burn-like appearance, hence the name of this disease, 'torrado', meaning burnt or

roasted (Figure 6.2g). Nevertheless, some plants observed in the surveys which also show symptoms of necrosis were positive for other tomato-infecting viruses, such as PVY (Figure 6.3a and 6.3b), TSWV (Figure 6.3c and 6.3d) or PMoV (Figure 6.3e), but were negative to ToTV. When comparing Figures 6.2 and 6.3, clear differences were observed between the symptoms of necrosis developed by ToTV-infection and the plants infected with other tomato viruses.

Surveys

In the surveys conducted from 2001-2008, 584 samples of tomato from 92 greenhouses in different parts of the country were collected (Figure 6.1). The results of these surveys are summarized in Table 6.1. The surveyed plants had a range of necrotic symptoms which were recorded and classified in three different groups: 105 samples were recorded as plants with necrotic symptoms which could not be clearly associated with 'torrado' disease, 373 showed typical symptoms of the disease, while 106 samples had been collected in the diseased fields but showed no symptoms of necrosis (marked with 'n', 's' and 'w/s' in Table 6.1, respectively). Analysis revealed that 53.3% (56 out of 105), 94.4% (352 out of 373) and 40.6% (43 out of 106) of the 'n', 's' and 'w/s' samples were positive for ToTV, respectively. Of the samples collected, 451 plants from 85 different greenhouses were infected with ToTV. This virus was detected in Mallorca, the Murcia Region, Gran Canaria, Tenerife, Almería, Alicante and Barcelona in the different survey years.


a
b
c
e
d
f
g

◄ **Figure 6.2.** Symptoms observed in the plants showing the 'torrado' disease. Defined areas at the base of the leaflet of the affected tomato leaves showing yellowing and necrotic spots **(a)**, which sometimes abscised, leaving little holes in the leaflet ('cribado') **(b)**. Extensive necrotic areas progressed from the base to the tip of the leaflet **(c)**. Necrotic streaking observed on the stems **(d,e)**. Fruits appeared distorted with necrotic lines **(f)**. Burnt-like appearance of the plants affected with the 'torrado' disease (**g**).

PepMV was usually detected in the surveyed samples, as the 357 positive samples to the virus revealed (61.1%) in which the EU genotype was the most prevalent until 2004 (positive EU samples/positive CH2 samples =2). The incidence of the CH2 genotype later increased significantly (positive EU samples/positive CH2 samples = 0.71; Table 6.1). In all, the EU, CH2 and CH1/US1 genotypes were detected in 214, 272 and 36 of the 357 positive samples of PepMV, respectively. The CH1/US1 genotype was only detected in the Canary Islands (Tenerife and Gran Canaria) mainly in mixed infections with the EU genotype. Besides, 45% of the PepMV positive samples corresponded to a mixed infection between the EU genotype and CH2 (35.2%) or CH1/US1 (9.8%). A double infection between ToTV and PepMV was detected in 273 samples of the 451 infected ToTV plants (60.5%), where the most prevalent PepMV genotype was CH2 (44.3%), followed by the mixed infection of the EU and CH2 genotypes of PepMV (32.6%), the EU and mixed infection of EU and CH1 (13.2% and 9.5%, respectively) and, finally, CH1/US1 (0.4%). No PE genotype was detected in the analysed samples (Table 6.1).

Several samples showing necrotic symptoms (marked with 'n') were negative for ToTV, but positive for other viruses such as CMV (5 plants), PMoV (5 plants) PVX (1 plant), PVY (4 plants), TSWV (12 plants), or TYLCV (1 plant). Nevertheless, some samples infected with ToTV were also positive for TSWV (30 plants), ToMV (7 plants) and ToCV (26 plants). Interestingly, PMoV was only detected in 5 samples collected from other areas of Spain, such as

Vizcaya, Tarragona and Valencia. None of the 40 samples tested for ToANV, resulted in a positive reactions, although they showed similar symptoms to those described for this virus by Turina *et al.* (2007).

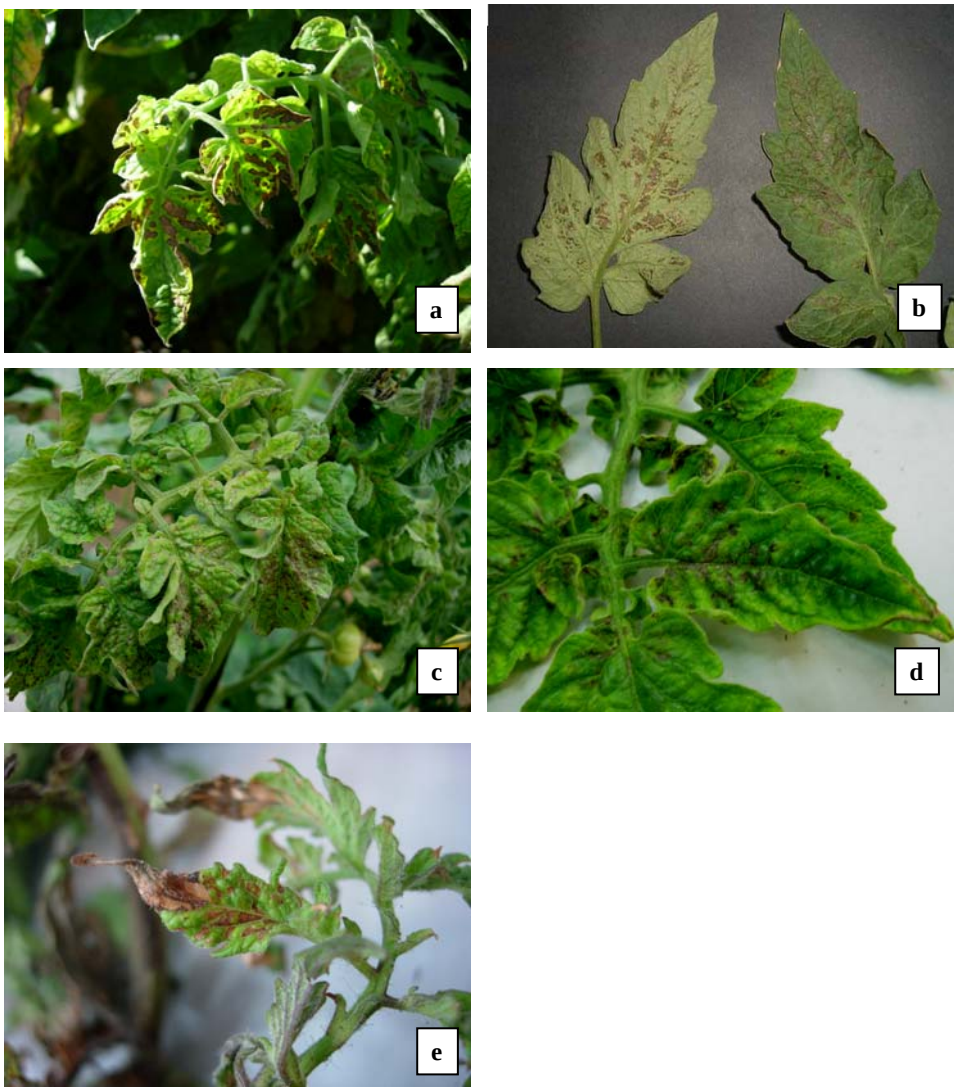


Figure 6.3. Necrotic symptoms observed on the leaves of tomato plants infected with PVY **(a)** and **(b)**, TSWV **(c)** and **(d)** or PMoV **(e)** collected in the different surveys.

To obtain more detailed information of the distribution of the infected plants in greenhouses, we conducted an exhaustive survey of four greenhouses in the Murcia Region in 2008. A total of 1,527 plants were monitored for the presence or absence of symptoms (Table 6.2). These greenhouses revealed a variable rate of symptomatology ranging from 37% to 8.4% of plants showing the typical 'torrado' symptoms. Interestingly, 60% of symptomatic plants were located close to doors, windows or main corridors of greenhouses. The testing of 47 samples collected in the four greenhouses revealed that 25, 2 and 1 sample(s) showing ‘s’, ‘w/s’ and ‘n’ symptomatology, respectively, were positive for ToTV. Almost all the collected samples presented mixed infections of ToTV and PepMV (25 out of the 28 samples found to be positive to ToTV, Table 6.2), while all the samples marked with 'n' (13 samples) were positive for TSWV, although 2 samples presented typical ToTV symptoms and were mix infected with ToTV and TSWV.

Due to the high incidence of ToTV in tomato crops, we tested the heterogeneity of the virus population by determining the nucleotide and amino acid sequences of two regions of different isolates from various regions/greenhouses. The analyzed sequences of the fragments of polyprotein RNA2 (partial MP and Vp23 CP subunit) of five different isolates (MUR-03, MUR-05, TEN-07, MUR-08 and GNC-08) revealed a high nucleotide identity (99-97%) in comparison to ToTV sequences deposited in the GenBank database: ToTV-PAN1 (Accession numbers EU934037 and EU357151 for the partial MP and Vp23 CP subunit, respectively), Isolate Wal´03 (Accession number: EU563947), PRI-ToTV0301 (Accession number DQ388880) and ToTV-CE (Accession number EU476181). However, the percentage of nucleotide identity among ToTV isolates and the two new viruses included in the tentative genus Torradovirus (ToANV and ToMarV) was below 73%. The phylogenetic analysis also confirmed these results (Figure 6.4).

Table 6.1. Results of the surveys performed between 2001 and 2008: location, number of fields and samples surveyed, symptoms observed, and viruses detected.

Year	No. Map[a]	Region	Fields	No. Samples	Symptoms observed[b]			Virus detection						
								ToTV[d]	PepMV					Double infection ToTV+PepMV
					n[b,c]	s[b,c]	w/s[b,c]		Total of positives	EU	CH1/US1	CH2	Mixed infection	
2001	5	Alicante	1	1	1 (0)	0	0	0	1	1	0	0	0	0
	8	Mallorca	1	2	2 (1)	0	0	1 (1)	2	2	0	0	0	1
	6	Murcia	2	9	9 (6)	0	0	6 (2)	1	1	0	1	1	1
2002	6	Murcia	7	20	9 (5)	11 (11)	0	16 (5)	9	8	0	2	1	6
	11	Vizcaya	1	2	0	2 (0)	0	0	0	0	0	0	0	0
2003	10	Gran Canaria	1	2	2 (1)	0	0	1 (1)	1	1	0	0	0	0
	6	Murcia	6	22	9 (4)	12 (12)	1 (1)	17 (6)	12	8	0	10	6	8
	9	Tenerife	4	5	4 (2)	1 (1)	0	3 (2)	3	2	0	1	0	2
	4	Valencia	1	1	1 (0)	0	0	0	0	0	0	0	0	0
2004	7	Almería	1	2	0	2 (2)	0	2 (1)	1	0	0	1	0	1
	3	Castellón	1	1	1 (0)	0	0	0	0	0	0	0	0	0
	10	Gran Canaria	3	9	6 (6)	3 (3)	0	9 (3)	2	2	0	0	0	2
	6	Murcia	5	8	1 (1)	6 (6)	1 (0)	7 (5)	4	4	0	0	0	3
	2	Tarragona	1	2	2 (0)	0	0	0	0	0	0	0	0	0
	9	Tenerife	1	1	1 (0)	0	0	0	1	1	0	0	0	0

Table 6.1. *(Continuation)*

Year	No. Map[a]	Region	Fields	No. Samples	Symptoms observed[b]			Virus detection						
								ToTV[d]	PepMV					Double infection ToTV+PepMV
					n[b,c]	s[b,c]	w/s[b,c]		Total of positives	EU	CH1/US1	CH2	Mixed infection	
2005	10	Gran Canaria	1	10	0	10 (10)	0	10 (1)	1	1	0	1	1	1
	6	Murcia	11	90	11 (9)	75 (73)	4 (2)	84 (11)	50	13	0	47	10	48
	4	Valencia	1	2	2 (0)	0	0	0	0	0	0	0	0	0
2006	6	Murcia	10	135	8(7)	89 (84)	38 (18)	109 (10)	98	54	0	94	50	77
	10	Gran Canaria	4	21	7 (3)	9 (8)	5 (4)	15 (4)	2	2	0	0	0	1
	9	Tenerife	1	2	2 (2)	0	0	2 (1)	2	2	0	2	2	2
2007	5	Alicante	2	12	2 (0)	9 (9)	1 (1)	10 (1)	12	8	0	7	3	10
	7	Almeria	1	1	0	1 (1)	0	1 (1)	0	0	0	0	0	0
	10	Gran Canaria	3	22	0(0)	20 (18)	2 (2)	20 (3)	8	6	3	4	4	8
	6	Murcia	4	52	4 (4)	31 (28)	17 (5)	37 (4)	41	11	0	40	10	31
	9	Tenerife	9	75	11 (1)	47 (44)	17 (7)	52 (7)	46	45	33	7	36	34
2008	1	Barcelona	1	2	2 (2)	0	0	2 (1)	2	0	0	2	0	2
	10	Gran Canaria	1	3	1 (0)	1 (1)	1 (1)	2 (1)	3	0	0	3	0	2
	6	Murcia	7	70	7 (2)	44 (41)	19 (2)	45 (7)	55	42	0	50	37	33
TOTAL			92	584	105 (56)	373 (352)	106 (43)	451 (85)	357	214	36	272	161	273

[a] The number indicates the corresponding region in Figure 6.1.
[b] The symptoms were marked as symptomatic plants (s), for those plants which showed the symptoms currently associated with the "torrado disease"; plants which showed some necrotic symptoms that could not be clearly associated with the "torrado disease" (n), and plants without necrotic symptoms (w/s).
[c] The number represents the plants showing the corresponding symptomatology. The numbers in parentheses correspond to the samples which were positive for ToTV.
[d] Number of positive plants. The number of fields from which the positive samples were collected are shown in parentheses.

Table 6.2. Results of the surveys conducted in 2008 in four greenhouses of the Murcia Region.

Greenhouse	No. of mononitored plants	Plants with symptoms (%)	No. samples collected	Symptoms observed[a]			Virus detection							
							ToTV	PepMV					Double infection ToTV+PepMV	Other analyses[c]
				n[b]	s[b]	w/s[b]		Total of positives	EU	CH1/US1	CH2	Mixed infection		
1	115	37	16	2 (1)	10 (10)	4 (0)	11	16	12	0	14	10	11	TSWV (2)
2	132	37	10	2 (0)	6 (6)	2 (1)	7	7	4	0	6	3	5	TSWV (3)
3	648	8.4	9	1 (0)	8 (8)	0 (0)	8	8	7	0	7	6	7	TSWV (3)
4	632	9	12	8 (0)	1 (1)	3 (1)	2	12	11	0	11	10	2	TSWV (8)
TOTAL	1527	37-8.4	47	13 (1)	25 (25)	9 (2)	28	43	34	0	38	29	25	TSWV (15)

[a] The symptoms were marked as symptomatic plants (s), for those plants which showed the symptoms currently associated with the "torrado disease"; plants which showed some necrotic symptoms that could not be clearly associated with the "torrado disease" (n), and plants without necrotic symptoms (w/s).
[b] Number of samples showing the corresponding symptomatology. The numbers in parentheses correspond to samples which were positive for ToTV.
[c] **Positive analysis to other viruses.**

ToTV studied isolates were grouped in the same cluster and separated from ToMarV and ToANV. Within the ToTV group, the isolate GNC-08 (collected during 2008 in Gran Canaria) was slightly different to the rest in both genome fragments. The percentage of identity of the predicted amino acid sequence for ToTV studied isolates ranged between 98.3-100% and 97.5-100% for the partial MP and Vp23 CP subunit, respectively. This value was slightly lower for the CP subunit, indicating a more variable region as described for other viruses (Aparicio and Pallás, 2002; Fiore *et al.,* 2008; Table 6.3). By contrast, the percentage of identity of those isolates with the PRI-ToMarV0601 isolate was less than 75.2% (Table 6.3).

Comparison between tissue-printing and dot-blot hybridization

The high incidence of ToTV and resulting economical losses in tomato crops are strong arguments for using a powerful detection technique that is capable of testing a large number of field samples in a very short time. Non-radioactive molecular hybridization has proved to be a reliable methodology with a good detection limit, even with the direct application of tissue on the membrane (tissue printing). A previous test of the different parts of the ToTV-infected tomato plant revealed that the petioles of apical leaves are a good tissue to perform the tissue-printing analysis. The analysis of 103 tomato plants by dot-blot and tissue printing molecular hybridization using a riboprobe complementary to a fragment of the ORF2-RNA2, revealed that both hybridization techniques were coincident. Figure 6.5 shows the results of the tissue-printing (5a) and dot-blot (5b) hybridization of samples collected from the three different fields included in the surveys. The specificity of both hybridization techniques was confirmed by the lack of cross-reaction with the healthy controls and with tomato plants infected with viruses other than ToTV.

Figure 6.4. Phylogenetic analysis based on nucleotide sequences of partial movement protein (a) and subunit Vp23 of coat protein **(b)** of analyzed ToTV isolates (MUR-03, MUR-05, TEN-07, GNC-08 and MUR-08) and sequences published in the GenBank Database of ToTV isolates (PRI-ToTV0301, Wal03, ToTV-PAN1, ToTV-CE) and other related viruses (PRI-TMarV0601 and ToANV-VE434). The phylogenetic tree was constructed and visualized with MEGA 3.1 using neighbor-joining algorithm and 1,000 bootstrap values. The number above nodes indicates the percentage of bootstrap replicates which supported the branching. Internal branches with percentage of bootstrap replicates lesser than 50% were not indicated. The accession numbers of the sequences used in the analysis were as follows: ToTV-CE, Accession number EU476182; ToTV-PAN1, Accession numbers FJ357161 and EU934037; PRI-ToTV0301 Accession number DQ388880; Wal03, Accession number EU563947; PRI-ToMarV06031, Accession number NC010988; and ToANV-VE434, Accession number EF06364.

Mechanical back-inoculation and whitefly transmission

Fifty-six tomato plants of cvs Boludo and Marmande were mechanically inoculated with 14 different ToTV isolates. None of the inoculated plants were positive for ToTV 45 days after inoculation. However, whitefly ToTV transmission was observed using a viruliferous colony of *T. vaporariorum* collected from a ToTV-infected greenhouse and different cultivars of tomato, *N. rustica*, *N. occidentalis, N. glutinosa* and *D. stramonium* species.

The percentage of infected tomato plants varied according to the cultivar, and ranged from 67.0% (8 of 12 plants of cv. Cedrico), 54.0% (6 of 11 plants of cv. Boludo), 8.3% (1 of 12 plants of cv. 1123) or 0% for cvs. Marmande (21 plants) and Marglobe (10 plants).We observed typical necrotic spots associated with ToTV-infection on the base of the leaflet in only 5 and 6 infected tomato plants of cvs. Cedrico and Boludo, respectively. The remaining solanaceous species used for whitefly transmission, 33.3% (2 of 6 plants) of *D. stramonium* and 50.0% (2 of 4 plants) of *N. rustica*, *N. occidentalis* and *N. glutinosa* were ToTV-infected. All infected *Nicotiana* species were symptomless; however, infected plants of *D. stramonium* had symptoms which consisted in interveinal yellowing that developed into necrosis, although similar symptoms were also observed in non-infected plants which could be associated with whitefly feeding.

a)


MUR-03
TEN-07
MUR-05
PRI-ToTV0301
MUR-08
Wal03
ToTV-PAN1
10
ToTV-CE
GNC-08
PRI-TMarV0601
0.05

b)

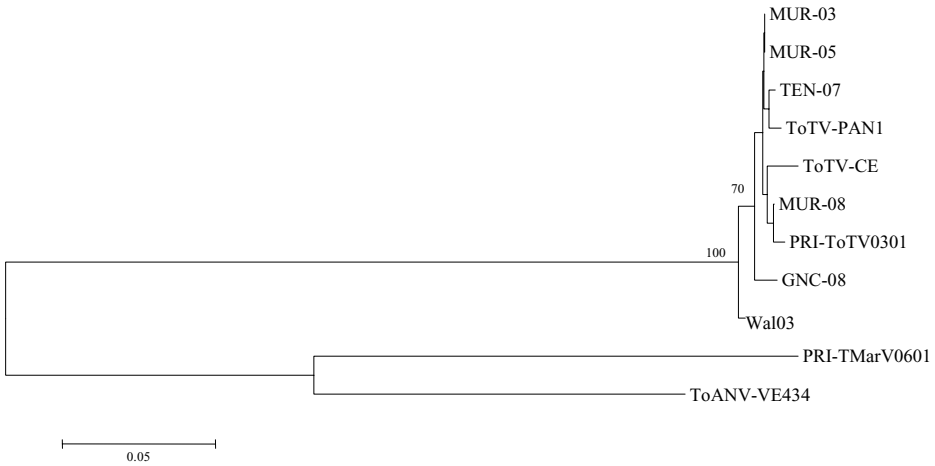

MUR-03
MUR-05
TEN-07
ToTV-PAN1
ToTV-CE
70
MUR-08
PRI-ToTV0301
100
GNC-08
Wal03
PRI-TMarV0601
ToANV-VE434
0.05

Table 6.3. Comparison of amino acid sequences of the partial MP and the Vp23 CP subunit of RNA2 of ToTV isolates studied with other ToTV isolates and ToMarV sequences available in the GenBank database. The identity/similarity matrix was calculated using the Matrix Global Alignment Tool software (http://bitincka.com/ledion/matgat).

	MUR-03																	
	MP	Vp23 CP	MUR-05															
MUR-05[a]	Id. 100 Sim. 100	Id. 100 Sim. 100	MP	Vp23 CP	TEN-07													
TEN-07[a]	Id. 99.3 Sim. 99.3	Id. 99.4 Sim. 100	Id. 99.3 Sim. 99.3	Id. 99.4 Sim. 100	MP	Vp23 CP	GNC-08											
GNC-08[a]	Id. 99.3 Sim. 99.3	Id. 99.4 Sim. 99.4	Id. 99.3 Sim. 99.3	Id. 99.4 Sim. 99.4	Id. 98.6 Sim. 98.6	Id. 98.8 Sim. 99.4	MP	Vp23 CP	MUR-08									
MUR-08[a]	Id. 100 Sim. 100	Id. 100 Sim. 100	Id. 100 Sim. 100	Id. 100 Sim. 100	Id. 99.3 Sim. 99.3	Id. 99.4 Sim. 100.0	Id. 99.3 Sim. 99.3	Id. 99.4 Sim. 99.4	MP	Vp23 CP	PRI-ToTV0301							
PRI-ToTV0301[b]	Id. 100 Sim. 100	Id. 98.8 Sim. 99.4	Id. 100 Sim. 100	Id. 98.8 Sim. 99.4	Id. 99.3 Sim. 99.3	Id. 98.2 Sim. 99.4	Id. 99.3 Sim. 99.3	Id. 98.2 Sim. 98.2	Id. 100 Sim. 100	Id. 98.8 Sim 99.4	MP	Vp23 CP	ToTV-CE					
ToTV-CE[b]	Id. 99.3 Sim. 99.3	Id. 98.1 Sim. 98.8	Id. 99.3 Sim. 99.3	Id. 98.1 Sim. 98.8	Id. 98.6 Sim. 98.6	Id. 97.5 Sim. 98.8	Id. 98.6 Sim. 98.6	Id. 96.9 Sim. 97.5	Id. 99.3 Sim. 99.3	Id. 97.5 Sim. 98.8	Id. 96.9 Sim. 98.1	Id. 96.9 Sim. 98.1	MP	Vp23 CP	Wal03			
Wal03[b]	Id. 99.3 Sim.100	Id. 100 Sim. 100	Id. 99.3 Sim. 100.0	Id. 100 Sim. 100	Id. 98.6 Sim. 99.3	Id. 99.4 Sim.100	Id. 98.6 Sim. 99.3	Id. 99.4 Sim. 99.4	Id. 99.3 Sim. 100	Id. 100 Sim. 100	Id. 100 Sim. 100	Id. 98.8 Sim. 99.4	Id. 98.6 Sim. 99.3	Id. 98.1 Sim. 98.8	MP	Vp23 CP	ToTV-PAN1	
ToTV-PAN1[b]	Id. 100 Sim. 100	Id. 99.4 Sim.100	Id. 100 Sim. 100	Id. 99.4 Sim. 100	Id. 99.3 Sim. 99.3	Id. 100 Sim. 100	Id. 99.3 Sim. 99.3	Id. 98.1 Sim. 98.8	Id. 100 Sim. 100	Id. 100 Sim. 100	Id. 98.1 Sim. 99.4	Id. 98.1 Sim. 99.4	Id. 99.3 Sim. 99.3	Id. 97.5 Sim. 98.8	Id. 99.3 Sim. 100.0	Id. 99.4 Sim. 100	MP	Vp23 CP
PRI-TMarV0601[b]	Id. 75.2 Sim. 88.3	Id. 69.6 Sim. 85.1	Id. 75.2 Sim. 88.3	Id. 69.6 Sim. 85.1	Id. 74.4 Sim. 87.5	Id. 69.6 Sim. 84.5	Id. 75.2 Sim. 89.2	Id. 69.6 Sim. 85.1	Id. 75.2 Sim. 88.3	Id. 69.6 Sim. 84.5	Id. 69.6 Sim. 85.1	Id. 69.6 Sim. 85.1	Id. 74.4 Sim. 87.5	Id. 69.6 Sim. 83.9	Id. 75.2 Sim. 88.3	Id. 69.6 Sim. 85.1	Id. 75.2 Sim. 88.3	Id. 69.6 Sim. 84.5

Id., identity; Sim., similarity

[a]These isolates are marked with a three-letter code indicating their geographical origin (MUR= Murcia, TEN= Tenerife, GNC= Gran Canaria), followed by the collection year.

[b]The accession numbers of the sequences used in the analysis: PRI-ToTV0301 (DQ388880), ToTV-CE (EU476181), Wal03 (EU563947), ToTV-PAN1 (EU934037 and EU357151), PRI-ToMarV (NC010988)

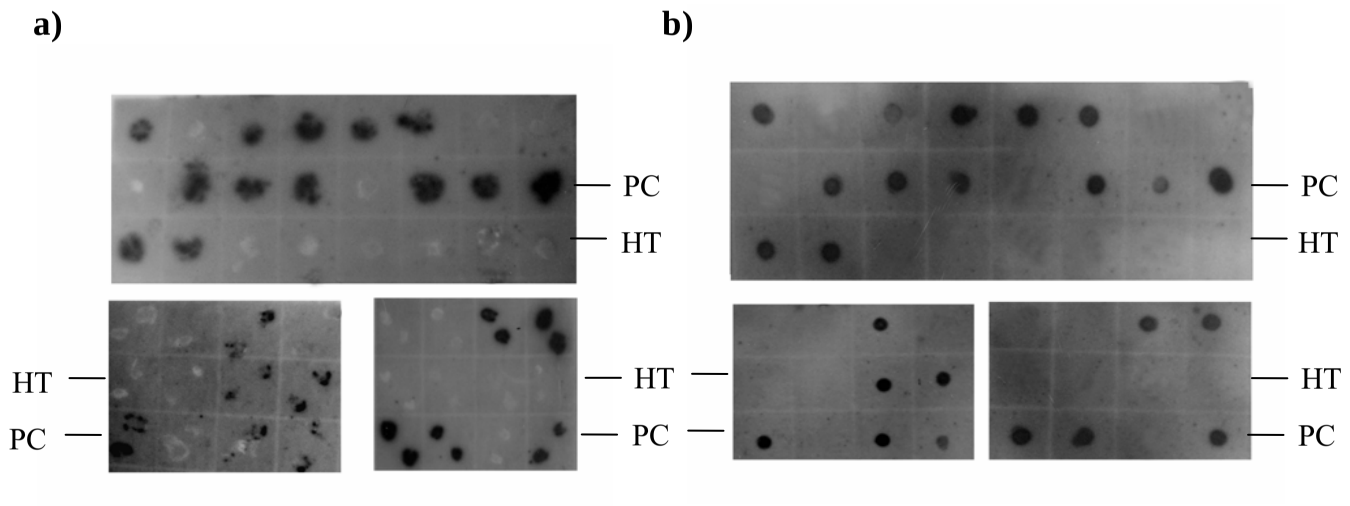


Figure 6.5. Comparative analisis of tissue-printing **(a)** and dot-blot **(b)** molecular hybridization procedures. The tomato plants previously analyzed by dot-blot hybridization (b) were analyzed by tissue printing using petioles which were transversely cut and directly pressed onto the nylon membrane (a). The membranes were crosslinked and hybridized using a specific dig-RNA ToTV probe. The positive and negative controls corresponding to infected or healthy tomato plants are indicated as PC and HT, respectively. Samples infected with viruses other than ToTV are indicated as 1 (TSWV), 2 (ToCV), 3 (TYLCV), 4 (ToMV), 5 (PMoV) and 6 (PVY).

DISCUSSION

Since 2001, the aggressive 'torrado' disease has been reported to affect tomato crops in two of the most important tomato production areas of Spain (the Murcia Region and the Canary Islands; Jordá *et al.,* 2003; Espino *et al.,* 2007), although similar 'torrado' symptoms were previously observed in 1996 and 1997 in the Canary Islands (Gran Canaria and Tenerife; Espino *et al.,* 2007). This symptomatology has been associated with the presence of ToTV (Verbeek *et al.,* 2007a). In the present study, we conducted extensive surveys from 2001 to 2008 in different important tomato production areas of Spain to determine the occurrence and distribution of the 'torrado' disease and its new associated viruses. These surveys reveal that ToTV-infected plants are present in tomato crops in Mallorca, the Murcia Region, Gran Canaria, Tenerife, Almería, Alicante and Barcelona. These seven regions cover 40% of the total Spanish tomato production (MARM, 2009). The incidence of ToTV varied between 58.3% (2001) and 92.2% (2005) of the plants collected from 92 greenhouses in the different tomato production areas, with an average of 77.0% over all the survey years. However, the incidence of the disease recorded in four greenhouses in the Murcia Region in 2008 varied, and could represent a percentage of affected plants of up to 37.0% of all the plants growing in one greenhouse (Table 6.2). Furthermore, an incidence of 4.68% (in Tenerife) and 2.99% (in Gran Canaria) of the total cultivated area was reported in other surveys in the Canary Islands in 2007 (Espino *et al.,* 2007).

To gain a better understanding of the correlation between the 'torrado' symptoms and the presence of ToTV, we visually classified the surveyed plants into three symptomatology categories: 'torrado' disease with typical necrotic symptoms (s), necrotic symptoms whose appearance differs from that of the 'torrado' disease (n) and plants without necrotic symptoms (w/s). Furthermore,

94.4% of 's' plants were ToTV-infected, and 53.3% and 40.5% were 'n' and 'w/s' plants, respectively, were also ToTV-positive. These data indicated the no complete correlation between symptomatology and ToTV infection, and the presence of latent infections that represent a potential risk for virus spread. Several viruses could cause different necrotic symptoms in tomato plants (Córdoba-Sellés *et al.,* 2007a), which make diagnosis of some symptomatic samples difficult. In addition, symptoms and their severity vary with the virus isolate, cultivar, plant stage and environmental conditions in which infection takes place (EPPO, 2004). Our results clearly show that ToTV symptoms are not only related to the 'torrado' symptomatology, but also to other necroses, or even to asymptomatic phenotypes. Thus, some of the symptomatic (s) and necrosis samples (n) were infected with other tomato viruses which commonly induce necrosis, such as TSWV, PVY, PMoV, CMV, ToCV and TYLCV. PMoV, which induced brown patches on tomato fruits and necrotic mosaic on leaves that progressed to apical stem necrosis, is easily confused with CMV, TSWV (Galipienso *et al.,* 2005) or ToTV symptoms (Jordá *et al.,* 2003). Tomato plants infected with PMoV, marked with 'n' in the symptomatology evaluations, were only detected in restricted areas of north and east Spain (Vizcaya, Tarragona and Valencia). These results agree with previous surveys in which PMoV was detected in tomato crops from north-eastern (Cataluña; Aramburu, 2001; Galipienso *et al.,* 2005) or east Spain (Comunidad Valenciana; Aparicio *et al.,* 2008).

The high incidence of ToTV in some areas of Spain may present a potential risk for more aggressive or adaptive viral variants. To analyze the heterogeneity of the ToTV population in Spain, we characterized the nucleotide and amino acid sequences of two regions of the RNA 2 (partial MP and subunit Vp23 of CP) of five Spanish isolates from different geographic areas and collected in different time. This confirmed the high genetic similarity of the

Spanish ToTV isolates to each other as well as their close relationship to ToTV isolates from other countries as Poland (Budziszewskza *et al.,* 2008; Pospieszny *et al.,* 2009) or Panama.

Given the high percentage of ToTV-infected tomato plants and the lack of an available commercial antibody, we explored alternative detection techniques to the standard serological ELISA method to be used to screen a large amount of samples. Molecular hybridization (MH) using non radioactive riboprobes has proved to be an interesting detection technique for carnation (Sánchez-Navarro *et al.,* 1996), stone fruit (Herranz *et al.,* 2005) and tomato crops (Saldarelli *et al.,* 1996). The analysis of the five ToTV Spanish isolates reveals that the fragment which includes the putative MP in the ORF2-RNA2, is a conserved region that represents a good viral portion to be detected by MH. The use of a riboprobe target to that fragment was probed to specifically detect ToTV using total RNA extracted from tomato tissue. The same result was obtained when the samples were applied directly onto the membrane by tissue-printing, thus avoiding the RNA extraction procedure. Tissue printing hybridization proves to be a reliable technique with satisfactory results as a first detection step to determine the phytosanitary status of tomato plants in fields. This technique also saves time and simplifies the handling procedure in large-scale testing (Aparicio *et al.,* 2008; Galipienso *et al.,* 2005). Furthermore, the MH allows the simultaneous detection of different viruses affecting tomato crops by either mixing the corresponding riboprobes in the same hybridization solution (Saldarelli *et al.,* 1996) or using polyprobes (Aparicio *et al.,* 2008). Our results indicate that ToTV is easily detected by MH to allow its inclusion in the previously described simultaneous detection procedures. However, it could prove very interesting to analyze whether the tissue-printing approach allows the multiple detection of the main viruses affecting tomato crops.

Due to the large number of viruses affecting tomato crops, another aspect to consider is the presence of mixed infections. The testing of tomato plants infected with ToTV revealed that a high number of these plants were co-infected with another viruses in which PepMV was the most prevalent (46.7%), followed by ToCV and TSWV (5.7% and 6.7%, respectively). However, other than ToTV and PepMV, only suspicious plants were tested for the remaining viruses, which indicate that the percentage could be even higher. PepMV is widespread in tomato crops in Spain. The surveys from 2000 to 2004 revealed that the PepMV EU strain showed a high incidence in just single (80%), and the presence of the PE or US2 PepMV strains at lower frequency relative to EU (2 and 8 out of 64 isolates respectively; Pagán *et al.,* 2006). Since then, new PepMV isolates have been identified and, four major PepMV strains may now be distinguished EU, PE, CH1/US1, and CH2 (US2 genotype belongs to CH2 strain; Ling, 2007). The distribution and occurrence of some of these strains in other European countries has also been reported and show that tomatoes infected with PepMV present the EU and CH2 genotypes (Hanssen *et al.,* 2008). A recent analysis performed by our group using a specific multiplex RT-PCR that discriminates among the five PepMV genotypes shows how the EU strain is the most prevalent (94.0% of the infected plants), followed by CH2 (61.0%) and CH1/US1 (25.0%) (Alfaro-Fernández *et al.,* 2009b). Our results show the genetic variability of PepMV to be associated with 'torrado' disease symptoms. Then 61.1% of tomato plants were infected with PepMV (357 plants of 584) in which the EU, CH2 and CH1/US1 genotypes represented 13.2%, 44.3% and 0.4%, respectively. Meanwhile, mixed infections of EU-CH2 and EU-CH1/US1 represented 32.6% and 9.5%, respectively. Regarding the different PepMV genotypes, we observed that CH2 has been detected in samples since 2001, and is mainly associated with the Murcia Region. This observation contrasts with previous studies in which the CH2 strain (referred then as US2 strain) was only

found in samples collected in 2004 in the Murcia Region (Pagán *et al.,* 2006). In addition, the incidence of CH2 in the analyzed tomato plants increased from 8.3% (2001) to 73.3% (2008). The fact that the incidence of the EU genotype in single infections decreased from 2001, especially in the Murcia Region, could be interpreted as the CH2 genotype displacing the EU variant. However, more analyses are required to confirm this notion. Unlike PepMV population studies performed by Pagán *et al.* (2006), PE genotype was not detected in any of the tested samples. Nevertheless, the incidence of this genotype in these studies was quite low (4 out of 64 isolates) and was only detected in the Canary Islands in 2001 and 2003, and in 2 isolates from Murcia collected in 2004, studying the TGB region of the PepMV genome (Pagán *et al.,* 2006). All these results revealed that PE genotype was present, but not highly distributed in tomato crops in Spain, although our study only focused on one area of the PepMV genome. Otherwise, no specific PepMV genotype is associated with ToTV infection, which contrasts with previous assays performed in the Murcia Region (Alfaro-Fernández *et al.,* 2007b). Interestingly, the co-infection of ToTV and PepMV is mainly associated with a more aggressive symptomatology (73.3% and 14.3% of the plants infected with both viruses showed symptomatology of 's' and 'n'), indicating a putative synergism. This phenomenon could be explained by the expression of an RNA silencing protein, as previously described (Yang and Ravelonandro, 2002). In this sense, the TGB1 protein expressed by potexvirus is an RNA silencing inhibitor that shows different silencing levels (Voinnet *et al.,* 2000; Senshu *et al.,* 2009). Indeed, we checked the capacity of the TGB1 protein of PepMV to influence ToTV symptomatology.

Unlike mechanical transmission, ToTV is easily and efficiently transmitted by whiteflies (up to 67.0% in some tomato cultivars). In previous tests, the efficiency of the mechanical and approach-grafting inoculation of tomato plants was low, resulting in a few positive plants (1.8%; Alfaro-

Fernández *et al.,* 2006). The higher efficiency of whitefly transmission compared to the mechanical transmission of ToTV was previously reported by Pospieszny *et al.* (2007; 2009). Furthermore, the results obtained in whitefly transmission assays reveal that 66.7%, 54.5% and 8.3% of tomato plants cvs Cedrico, Boludo and 1123 were infected with ToTV. In addition, we observed that tomato plants cv. Marmande were not infected, indicating that ToTV transmission to *S. lycopersicum* is variety-dependent as previously reported Budziszewska *et al.* (2008). It should be noted that there are tomato plants with a natural resistance to ToTV which is conferred by at least an allele of one gene. However, these virus-resistant plants and the method to produce them have been patented (Maris *et al.,* 2007). Some other solanaceous species tested in the assay were ToTV-infected as: *N. glutinosa*, *N. rustica*, *N. occidentalis* and *D. stramonium*. Remarkably, the three *Nicotiana* species had already been reported as virus hosts as well as *D. inoxia* (Verbeek *et al.,* 2007a; Pospieszny *et al.,* 2007; 2009; Amari *et al.,* 2008). However, we report the susceptible response of *D. stramonium* to ToTV infection (33.3%) for the first time.

Therefore several studies have confirmed the vector-assisted transmission of this virus by whiteflies (Pospieszny *et al.,* 2007, 2009; Amari *et al.,* 2008), which means that the distribution of this virus throughout the production areas has been totally determined by both the climate and the vector, although plenty of epidemiological aspects of ToTV still remain unknown. Moreover, ToTV is an emerging disease which has been reported in several countries (Australia, Hungary, Panama, Poland and Spain) and has been recently included in the EPPO alert list due to the risk of spread within the European countries (EPPO, 2009). The efficiently whiteflies transmission of ToTV in tomato cultivation may mean that it becomes a serious agricultural problem for tomato production in forthcoming years. Whitefly populations have increased worldwide since the 1970s, probably due to the combination of different effects,

such as: i) increased use of synthetic organic insecticides with an increased resistance to them, ii) changing climatic conditions, iii) intensified agricultural practices, or iv) international movement of plant materials as part of the nursery and horticultural trade (Wisler *et al.,* 1998a). In Spain, a displacement of *T. vaporariorum* by *B. tabaci* in melon crops cultivated in greenhouses of south-east Spain has been reported to change the incidence of a whitefly-transmitted closterovirus (Berdiales *et al.,* 1999; Célix *et al.,* 1996). Otherwise, the two whitefly species are present in tomato, and *T. vaporariorum* is usually the most important species in north and north-east Spain, whereas *B. tabaci* is predominant in south Spain where it causes severe damage. In a mid-transition area, both species co-exist all year long, especially during winter, and they even co-exist within a plant, exhibiting *T. vaporariorum* with a greater preference for younger leaves than *B. tabaci* (Arnó *et al.,* 2006). Although both species have been reported to transmit ToTV (Pospieszny *et al.,* 2007; Amari *et al.,* 2008), the specificity and the parameters of the whitefly transmission remain unknown. Furthermore, ToTV has also been found in weeds to show that, besides the main crop host, the virus and the vector have alternative hosts for their dissemination. Therefore, weed management demands particular attention to effectively control the spread of ToTV (Alfaro-Fernández *et al.,* 2008b).

Our research provides more current data on the occurrence and distribution of ToTV in Spain, which is usually found in mixed infections with PepMV. The impact on crop productivity and the potential spread of this virus to other growing regions with resident whitefly populations emphasizes the need for further research to develop and implement effective control strategies.

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Capítulo 7

First report of Tomato torrado virus on weed hosts in Spain

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Tomato torrado virus (ToTV) is a recently identified Picorna-like virus that causes 'torrado' disease in tomatoes (Verbeek *et al.,* 2007a). Typical symptoms of 'torrado' disease seen in tomato crops (*Solanum lycopersicum* L. formerly *Lycopersicon esculentum* L.) were initially defined as yellow areas at the base of the leaflet that later developed into necrotic spots that sometimes abscised, leaving holes in the leaflet. Other plants showed extensive necrosis progressing from the base to the tip of the leaflet. Fruits were distorted with necrotic lines on the surface that often cracked. Affected plants had a burnt-like appearance and the production was seriously reduced. These symptoms have been observed in tomato crops in Murcia (Spain) and the Canary Islands (Spain) (Alfaro-Fernández *et al.,* 2007a). To identify possible alternative hosts that may serve as virus reservoirs, samples of 72 different common weed species were collected in greenhouses in Murcia and the Canary Islands where 'torrado' disease symptoms were observed in tomatoes. Forty-seven showed virus-like symptoms and 25 were asymptomatic. Symptoms included mild mosaic, blistering, vein clearing, interveinal yellowing, yellow spots, necrosis, leaf distortion, and curling. Samples were analyzed by one-step reverse transcription (RT)-PCR using primers specific for ToTV to amplify 580 bp of the polyprotein region of RNA2 (van den Heuvel *et al.,* 2006) and dot-blot hybridization with a digoxygenin-labeled RNA probe complementary to the same portion of the ToTV genome. Twenty-two of the 72 weed samples belonging to *Amaranthus* sp. (*Amaranthaceae*); *Spergularia* sp. (*Caryophyllaceae*); *Atriplex* sp., *Chenopodium ambrosioides* L., *Chenopodium* sp., and *Halogetum sativus* (Loef. ex L.) Moq. (*Chenopodiaceae*); *Senebiera didyma* Pers. (*Cruciferae*); *Malva* sp. (*Malvacae*); *Polygonum* sp. (*Polygonaceae*); and *Nicotiana glauca* Graham and *Solanum nigrum* L. (*Solanaceae*) were positive for ToTV by

molecular hybridization (10 samples) and RT-PCR (22 samples, including the samples positive by molecular hybridization). PCR products obtained from *Atriplex* sp. (Canary Islands) and *S. didyma* (Murcia) were sequenced (GenBank Accessions EU090252 and EU090253). BLAST analysis showed 99% identity to ToTV RNA2 sequence (GenBank Accession DQ388880). Two tomato plants were positive for ToTV by RT-PCR after mechanical back-inoculation, although no symptoms were observed. This study showed ToTV infects common weeds present in Spanish tomato crops. Recently, *Trialeurodes vaporariorum* has been reported to transmit ToTV (Pospieszny *et al.,* 2007), although the efficiency of transmission is unknown. The vector-assisted transmission of ToTV could explain the infection of weeds in affected greenhouses. To our knowledge, this is the first report of natural infection of weeds by ToTV.

Capítulo 8

Ultrastructural aspects of tomato leaves single-infected by Tomato torrado virus (ToTV) and co-infected by other tomato viruses

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ABSTRACT

Optical and electron microscopy studies were conducted to know the cytopathology induced in tomato leaves infected by *Tomato torrado virus* (ToTV), a new picorna-like virus associated with the 'torrado' disease. Infected leaves, showing typical 'torrado' disease symptoms, surveyed in commercial greenhouses of the main tomato production areas in Spain were analyzed. The effect of the co-infection of ToTV with other viruses which commonly infect tomato crops was also studied. Ultra-thin sections of ToTV-infected tomato leaves did not present a strong cellular alteration. However, crystalline arrays of isometric virus-like particles (VLPs) of 20-30 nm in the inclusion bodies were observed in phloem parenchyma cells of the infected tissues. Tissues double infected by ToTV and *Tomato chlorosis virus* (ToCV) or *Pepino mosaic virus* (PepMV) presented more severe cellular alterations. The most deleterious consequences for tomato cells were found in triple infections of ToTV, PepMV and *Tomato spotted wit virus* (TSWV), where characteristic cell wall overgrowth was distinguishable, together with a large amount of necrotic cells.

INTRODUCTION

Since 2001, the new tomato disease referred to as 'torrao' or 'torrado' has been affecting several tomato (*Solanum lycopersicum* L.) crops in the most important production areas of Spain: Murcia (South Eastern Spain) and the Canary Islands (Alfaro-Fernández *et al*., 2006; 2007a; 2007b).

The affected plants show an initial plesionecrosis in the basal zone of leaves, close to the central nerve, that later develops into necrotic spots, which are sometimes abscised and leave 'shot holes' in the leaflet (hence the Spanish name 'cribado' meaning 'sieve'). Sometimes affected plants show extensive holonecrosis which progresses from the base to the apical area of the leaf. Longitudinal necrotic lesions also appear on stems. Patterns of necrotic lines are usually observed on forming fruits which often crack with fruit growth, rendering them unmarketable. Affected plants have a burnt-like appearance which seriously endangers production.

The association of this disease with a virus was confirmed by Verbeek *et al*. (2005) who characterized it as a picorna-like virus and proposed the name Tomato torrado virus (ToTV) for the new virus, a member type of a new genus, Torradovirus (Verbeek *et al.,* 2007a), which also includes two other recently characterized tomato-infecting viruses, Tomato apex necrosis virus (ToANV) and Tomato marchitez virus (ToMarV) (Turina *et al.*, 2007; Verbeek *et al.*, 2007b). ToTV was also detected on common weeds present in Spanish tomato crops (Alfaro-Fernández *et al*. 2008b), and it was transmitted to other *Solanaceae* crops such as pepper and eggplant under controlled conditions (Amari *et al*., 2008). ToTV has been demonstrated to be efficiently vectored by two whitefly species, *Trialeurodes vaporariorum* (Pospieszny *et al.*, 2007) and *Bemisia tabaci* (Amari *et al*., 2008).

Tomato crops are seriously affected by several viral diseases in which mixed viral infection may result in synergisms, causing more severe disease symptoms than those produced in single infections (García-Cano *et al.*, 2006). In different surveys conducted since the first outbreak of the disease, ToTV has been seen to affect tomato by single infections, although a high percentage of samples contained double infections with *Pepino mosaic virus* (PepMV), which is highly distributed in tomato production areas of the country (Alfaro-Fernández *et al.*, 2007b). In addition, other viruses have been detected in some of the samples surveyed, and might induce symptoms in tomato plants by interfering or contributing to the 'torrado' disease symptoms. Furthermore, necrosis symptoms are also produce by other viral agents when infecting tomato crops, which are not easily distinguishable of "Torrado" disease symptoms (Córdoba-Sellés *et al.,* 2007a). To date however, little is known about either the cytopathological effects of ToTV in single viral infections or the possible synergistic interactions at macroscopic and microscopic levels between this virus and others which are commonly detected and affect Spanish tomato crops.

The aim of this study is to evaluate the microscopic effect of ToTV in plants affected by the 'Torrado' disease. To this end, we present the comparative studies of the cytopathology induced by the viral entity ToTV in single infections, and the ultrastructural effect of the mixed infection of this virus with other related viruses commonly found in Spanish tomato crops by optical and electron microscopy.

MATERIAL AND METHODS

Plant material

Leaf samples of the different tomato plants showing typical symptoms of 'Torrado' disease were collected in commercial greenhouses from areas of Murcia (Mazarrón and Águilas), Almería and the Canary Islands (Las Palmas), where the incidence of disease has been widely reported. A total number of 32 samples from plants developed approximately six weeks after transplant, was surveyed, labeled and transported to the laboratory under cold conditions. Leaf samples collected from the terminal leaflet from the first fully developed leaf reckoned from the plant apex of virus-infected tomato plants were used for the comparative and synergism studies. The characteristics of the collected samples are summarized in Table 8.1. Tomato plants cv. Boludo, grown in a growth chamber under controlled conditions (18-25ºC), 16 h daylight and 60% relative humidity, were used as healthy controls. To compare mixed infections of ToTV with the most commonly detected viruses PepMV and *Tomato chlorosis virus* (ToCV) in plants affected by the 'Torrado' disease (see below), single infections of the last two viruses were also studied in mechanical-inoculated and whitefly-infected tomato plants maintained under controlled conditions as described before. Mechanical transmission of two isolates of PepMV, belonging to genotypes Chilean 2 (CH2) and European (EU) respectively, was carried out with sap inoculation by grinding the leaves in 0.01 M phosphate buffer, pH 7.4 (1:4 w/v). The extracted sap was rubbed onto healthy tomato plants at 4 leaf-stage of development pre-dusted with carborundum (600 mesh). For ToCV transmission, a viruliferous colony of adults of *Trialeurodes vaporariorum* was released on healthy tomato plants at four-leaf-stage of development. After 48h inoculation access period, plants were treated against whiteflies and kept in a whitefly-free growing chamber. One month after inoculation, PepMV and ToCV

inoculated-plants were analysed to verify the infection as described below, and sampled for further observation.

Serological and molecular diagnostics

Serological analyses were performed with the collected leaf samples by double antibody-sandwich enzyme-linked immunosorbent assay (DAS-ELISA) using polyclonal antisera against *Cucumber mosaic virus* (CMV), *Potato virus Y* (PVY), *Tomato mosaic virus (ToMV), Tomato spotted wilt virus* (TSWV) (Loewe Biochemica), and *Pepino mosaic virus* (PepMV) (DSMZ Deutsche Sammlung von mikroorganismen und Zellkulturen), as recommended by the manufacturer. Healthy tomato leaves of Boludo cv. were included as a negative control. Positive controls consisted in virus-infected tomato leaf samples. Absorbance values (A405nm) were measured in a Titertek Multiskan immunoplate reader. Samples were considered positive to the analysis when the mean absorbance of duplicate wells was more than twice the mean absorbance of the corresponding healthy controls.

For the molecular analysis, total RNA extraction was performed from 0.1 g of fresh leaf tissue from infected plants using the silica capture protocol (MacKenzie *et al*., 1997). Total DNA was directly extracted using the E.Z.N.A® Plant DNA Miniprep Kit (OMEGA Biotech) following the manufacturer's instructions.

For the detection of ToTV and *Parietaria mottle virus* (PMoV), RNA extractions were tested using non isotopic dot-blot hybridization, performed as described previously by Sanchez-Navarro *et al*. (1998). Two digoxygenin-labeled RNA probes complementary to a fragment of the polyprotein of the RNA2 for ToTV based on published primers (van den Heuvel *et al.*, 2006) and a fragment of the coat protein of PMoV (kindly provided by Dr. Pallás) were used for the analysis.

To confirm the results obtained by the ToTV analysis, samples were also analyzed by one-step reverse-transcription polymerase chain reaction (RT-PCR) with the SuperScript III Platinium Taq kit (Invitrogen Life Technologies) and with specific primers targeted to the subunit Vp23 of the coat protein of the RNA2 of the virus (Pospieszny *et al.,* 2007). RT-PCR assays were also performed to verify the presence of other viruses with specific primers for both *Tomato chlorosis virus* (ToCV, Louro *et al.,* 2000) and *Tomato infectious chlorosis virus* (TICV, Vaira *et al.,* 2002), and by the multiplex RT-PCR procedure for the simultaneous detection of PepMV isolates, as described by Alfaro-Fernández *et al.* (2009b). PCR was performed to confirm possible *Tomato yellow leaf curl virus* (TYLCV, Martínez-Culebras *et al.,* 2001) infection. The amplified PCR products were separated by electrophoresis on 1.2% agarose in 1x TAE buffer (40 mM Tris-acetate and 1mM EDTA at pH 8.0), and were stained with ethidium bromide. Fragment sizes were determined by comparison with a 100 bp DNA standard marker (GeneRuler^{TM} DNA Ladder Plus, MBI Fermentas).

Optical and Electron Microscopy analysis

Then 1cm x 1cm pieces from the plesionecrotic areas of one leaf per plant were obtained and cut into smaller pieces measuring 1mm x 1cm, and were maintained in fixative (glutaraldehyde 2.5% in 0.1M buffer phosphate pH 7.2) while cutting to avoid additional stress to cells. They were immersed in the same fixative for 16-24h. Pieces were washed three times (1h) in buffer and kept at 4ºC until the electron microscopy (EM) process. The material was fixed for 2h in 2% osmium tetroxide and washed again in buffer. Then, the tissue was dehydrated in an ethanol series, from 30% to 100%, and uranyl acetate was added in the 70% phase. Finally, they were embedded in Araldite resin. Semi-thin and ultra-thin sections for optical and electron microscopy studies,

respectively, were obtained, placed onto slides and stained with Richardson's blue, or they were placed on carbon-coated cupper grids (200 mesh) and contrasted with uranyl acetate (10 min) and lead citrate (Reynolds' solution, 2 min). Semi-thin sections were examined with an optical microscope while ultra-thin sections were observed with a TEM 910 Zeiss microscope.

RESULTS AND DISCUSSION

Results of the serological and molecular analyses

The results of the serological and molecular analyses revealed that ToTV was present in single infections, although mixed infections with other tomato viruses was common in commercial greenhouses (Table 8.1). In addition to the infection of plants with ToTV, 21.88% of the analyzed samples were infected with TSWV, 3.13% with CMV, 15.63% with ToMV, 62.50% with ToCV, 6.25% with TYLCV and 43.75% with PepMV. The multiplex RT-PCR presented greater advantadges in the PepMV diagnosis compared to ELISA, because this procedure was able to identify the corresponding PepMV genotype present in the sample, and also to detect a higher number of positives (14 positives) than the serological method (10 positives). The obtained result of the higher sensibility of this molecular method compared to the ELISA is in accordance to those reported in previous assays for PepMV (Alfaro-Fernández *et al.,* 2009b) or other viruses (Sánchez-Navarro *et al.,* 1998). Of the 14 PepMV-positive samples, 4 belonged to the European genotype (EU), while 10 corresponded to the Chilean 2 genotype (CH2).

Of the total number of samples, single infection of ToTV was detected in 5 samples, and 12 samples presented a double infection of ToTV and ToCV, PepMV or TSWV. Nine of the samples presented a triple infection of ToTV and

PepMV/ToCV, ToMV/ToCV, PepMV/TSWV or ToCV/TYLCV. Multiple infections were even detected in 6 of 32 samples, of which four (ToTV and PepMV/ToCV/TSWV or PepMV/ToCV/ToMV or PepMV/ToMV/TSWV) or five (ToTV and PepMV/ToCV/TSWV/CMV) viruses were positively tested. Tomato crops are usually affected by several viral diseases in which mixed infections might result in synergism which, in some cases, has been reported to breakdown the resistance of the cultivar against one of the viruses involved (García-Cano *et al.*, 2006). While observing the symptoms, TSWV and TYLCV induced more severe macroscopic symptoms. However, further studies have to be performed to quantify the macroscopic effect of such mixed infections.

The large number of samples infected with ToTV and ToCV (20 of 32) was predictable since both are efficiently transmitted by *B. tabaci* and *T. vaporariorum* (Wisler *et al*., 1998b; Pospieszny *et al.*, 2007; Amari *et al*., 2008), and large populations of whiteflies often co-infect tomato fields worldwide. In addition since the first outbreak of ToCV in Spain, which affected tomato crops with an incidence of more than 30% of symptomatic plants in individual fields (Navas-Castillo *et al.*, 2000), this virus has commonly infected tomato crops during different growing seasons (EPPO, 2005).

On the other hand, PepMV was detected in almost half of the studied samples (14 of 32). This result was expected due to this virus is endemically present in Spain where it was first reported in 2000 (Jordá *et al.*, 2001a) and has been frequently detected in co-infection with ToTV in field samples (Alfaro-Fernández *et al.*, 2007b).

Table 8.1. Results of the serological and molecular analyses performed to the tomato leaf samples studied.

Sample	Origin	Variety	Serological analysis					Molecular analysis						Type of infection[b]
			PepMV	TSWV	PVY	CMV	ToMV	PepMV[a]	ToTV	ToCV	TICV	PMoV	TYLCV	
6269	Las Palmas	Ukn[c]	+	+	-	+	-	+ EU	+	+	-	-	-	M
6295	Murcia	Boludo	+	-	-	-	-	+ EU	+	+	-	-	-	T
6296	Murcia	Boludo	+	-	-	-	-	+ EU	+	+	-	-	-	T
6339	Almeria	Ukn	-	-	-	-	-	-	+	+	-	-	+	T
6343	Almeria	Ukn	-	+	-	-	-	+ CH2	+	+	-	-	-	M
7436	Murcia	Ukn	-	-	-	-	+	-	+	+	-	-	-	T
7444	Mazarrón	Ukn	-	-	-	-	-	-	+	-	-	-	-	S
7445	Mazarrón	Ukn	+	-	-	-	-	+ EU	+	-	-	-	-	D
7446	Mazarrón	Ukn	-	+	-	-	-	+ CH2	+	-	-	-	-	T
7449	Mazarrón	Ukn	-	+	-	-	-	-	+	-	-	-	-	D
7450	Mazarrón	Ukn	+	+	-	-	-	+ CH2	+	-	-	-	-	T
7454	Mazarrón	Ukn	+	+	-	-	+	+ CH2	+	-	-	-	-	M
7456	Mazarrón	Ukn	+	+	-	-	+	+ CH2	+	-	-	-	-	M
7530	Aguilas	Raferter	-	-	-	-	-	-	+	+	-	-	-	D
7533	Aguilas	Raferter	-	-	-	-	-	-	+	-	-	-	-	S
7534	Aguilas	Raferter	-	-	-	-	-	-	+	+	-	-	-	D
7544	Aguilas	Raferter	-	-	-	-	-	-	+	+	-	-	-	D
7545	Aguilas	Raferter	-	-	-	-	-	-	+	+	-	-	-	D
7547	Aguilas	Ukn	-	-	-	-	-	-	+	+	-	-	-	D
7821-1	Mazarrón	Ukn	-	-	-	-	-	-	+	-	-	-	-	S
7821-2	Mazarrón	Ukn	-	-	-	-	+	+ CH2	+	+	-	-	-	M

Table 8.1. *(continuation)*

Sample	Origin	Variety	Serological analysis					Molecular analysis						Type of infection[b]
			PepMV	TSWV	PVY	CMV	ToMV	PepMV[a]	ToTV	ToCV	TICV	PMoV	TYLCV	
7822	Mazarrón	Ukn	-	-	-	-	-	-	+	+	-	-	-	D
7825	Aguilas	Boludo	-	-	-	-	-	-	+	+	-	-	-	D
7826	Aguilas	Boludo	-	-	-	-	-	-	+	-	-	-	-	S
7827	Aguilas	Boludo	-	-	-	-	+	+ CH2	+	+	-	-	-	M
7828	Aguilas	Boludo	-	-	-	-	-	-	+	+	-	-	-	D
7829	Mazarrón	Corly	+	-	-	-	-	+ CH2	+	-	-	-	-	D
7830	Mazarrón	Corly	+	-	-	-	-	+ CH2	+	+	-	-	-	T
7831	Mazarrón	Corly	+	-	-	-	-	+ CH2	+	+	-	-	-	T
487/08	Aguilas	Boludo	-	-	-	-	-	-	+	-	-	-	-	S
491/08	Aguilas	Boludo	-	-	-	-	-	-	+	+	-	-	-	D
498/08	Aguilas	Boludo	-	-	-	-	-	-	+	+	-	-	+	T
TOTAL POSITIVE RESULTS			10	7	0	1	5	14	32	20	0	0	2	5(S) 12(D) 9(T) 6(M)
PERCENTAGE OF POSITIVES			31.25	21.88	0	3.13	15.63	43.75	100	62.50	0	0	6.25	

[a]Genotype of PepMV detected in the multiplex RT-PCR assay (Alfaro-Fernández *et al.*, 2009b): EU= European genotype; CH2=Chilean 2 genotype.
[b]Referring to the viruses detected in the sample and codified as: S= single infection, only ToTV was detected; D= double infection, ToTV and other viruses were positive; T=Triple infection, ToTV and two viruses were positive; M=Multiple infection, more than 3 viruses tested positive in the sample.
[c]Ukn= Unknown.

Cytopathology of a single infection of ToTV

Interestingly, the tomato tissue of single-infected samples with ToTV alone, which had been analyzed by optical (Figure 8.1b) or electronic microscopy, did not appear to be over disrupted or have necrotic cells. All the semi-thin sections observed under optical microscopy (Figure 8.1) were compared with healthy leaf sections (Figure 8.1a), although this sample presented a larger size in the intercellular spaces due to it was cultivated in a growing chamber, and the tissues appeared to be more swollen. Under EM, crystalline inclusions of virus-like particles (VLPs) of 20-30 nm were only observed in phloem parenchyma cells (Figure 8.2a, b). The presence of crystal-like arrays of viral particles in infected tomato leaf tissues was considered as a characteristic feature of ToTV (Pospieszny *et al.,* 2009). No crystals outside the vascular tissue were observed. Cells of ToTV-infected tissue displayed cytoplasmic vesiculation (Figure 8.2c), and the sieve elements exhibited no abnormal presence of the p-Protein (Figure 8.2c). Only a few of the observed samples presented hypertrophy of the mitochondria and strong cytoplasm disorganization, but the mitochondria and chloroplasts usually appeared normal (Figure 8.2c). The results of this work reveal that a single infection of ToTV induces a cellular alteration more at the phloem level than in other kinds of tomato leaf tissues (Table 8.2). This vascular tissue was considered as the translocation route used by this virus in tomato (Sánchez-Pina *et al.,* 2008). Generally chloroplasts showed no observable differences with healthy tissues (Figure 8.2c).

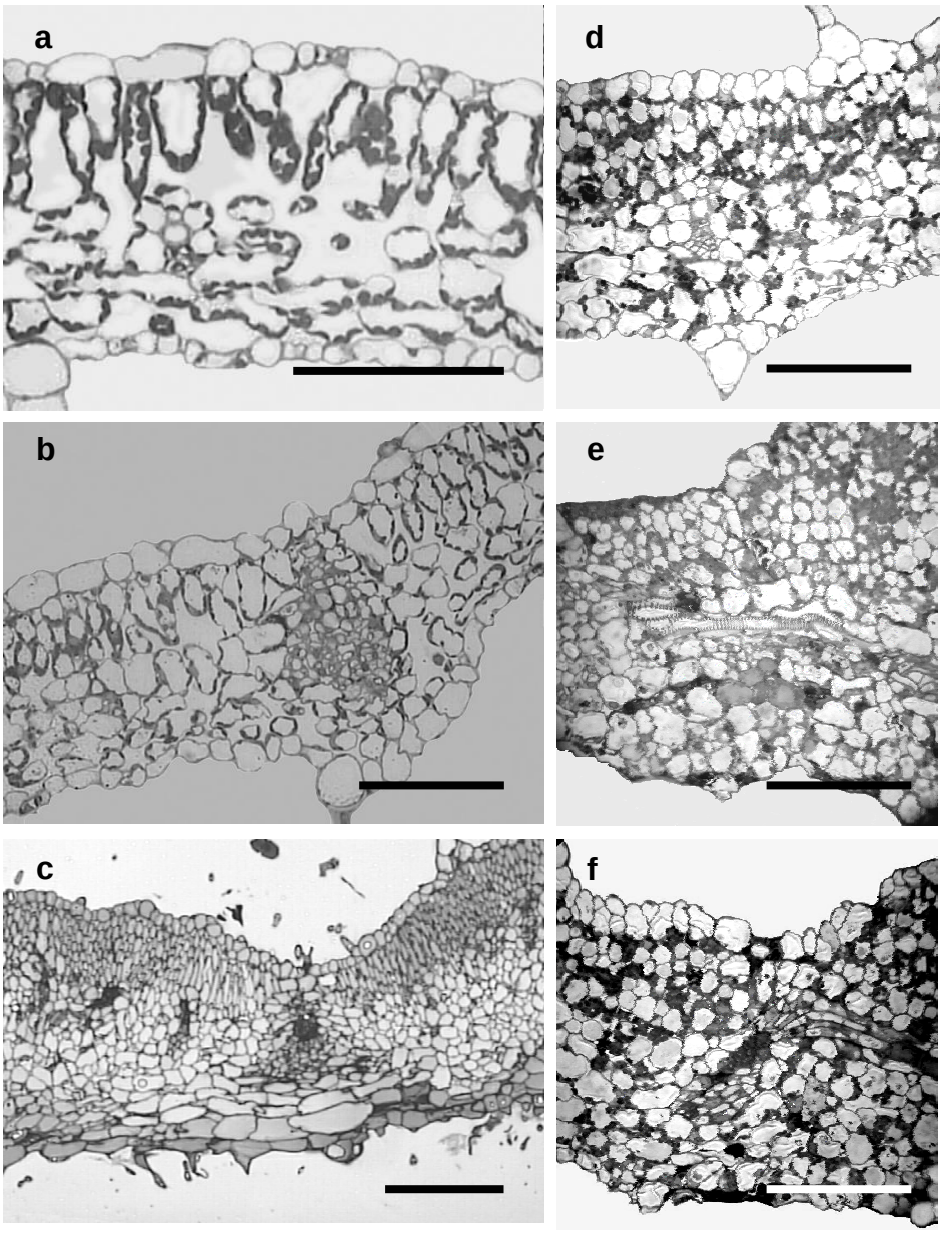


Figure 8.1. Light micrographs showing semi-thin sections of healthy **(a)** and virus-infected tomato leaves **(b-f)** with *Tomato torrado virus* (ToTV) **(b)**, *Tomato chlorosis virus* (ToCV) **(c)**, *Pepino mosaic virus* (PepMV) Chilean 2 genotype in a single infection **(d)**, and a co-infection ToTV/PepMV **(e)** or ToTV/PepMV/*Tomato spotted wilt virus* (TSWV) multiple infection **(f)** (bars= 42µm).

Table 8.2. Main alterations induced in tomato leaf vascular tissues by the different virus infections studied.

Virus infection	Cytoplasm vesiculation			Cell wall overgrowth			Virus-like particles (VLPs)		Chloroplasts		Mitochondria	
	CC[a]	PhP	BS	CC	PhP	BS	Crystals	Aggregates	H	OG	H	P
ToTV[b]	+[c]	+	±	-	-	-	+	-	-	-	±	-
ToCV	+	±	-	+	+	-	-	+[1]	-	±	-	+
PepMV	+	+	+	-	-	-	.	+[1]	+	+	+	+
ToTV + ToCV	+	+	±	++++	++	-	+	+	-	+	+	+
ToTV + PepMV	+	+	+	-	-	-	+	+	+	+	+	+
ToTV + PepMV + ToCV	+	+	+	+++	++	-	+	+	+	+	+	+
ToTV + PepMV + TSWV	+	+	+	++	++	++	-	+	+	+	+	+
ToTV + ToCV + TYLCV	+	±	±	++	++	-	+	+	±	±[2]	+	+

[a] Letters indicate the cells of the vascular tissues observed (CC= companion cell, PhP= phloem parenchyma cell, BS= bundle sheet cell) or alteration observed (H= hypertrophy, OG= Osmiophilic globules, P= proliferation/increase in number).
[b] ToTV: *Tomato torrado virus*, ToCV: *Tomato chlorosis virus*, PepMV: *Pepino mosaic virus*, TSWV: *Tomato spotted wilt virus*, TYLCV: *Tomato yellow leaf curl virus.*
[c] += indicates that the given feature was seen in more or less quantities for the respective viral infection; ±= indicates that the observation is not conclusive.
[1] Forming masses of flexuous VLPs.
[2] Chloroplast also showed an abnormal starch accumulation.

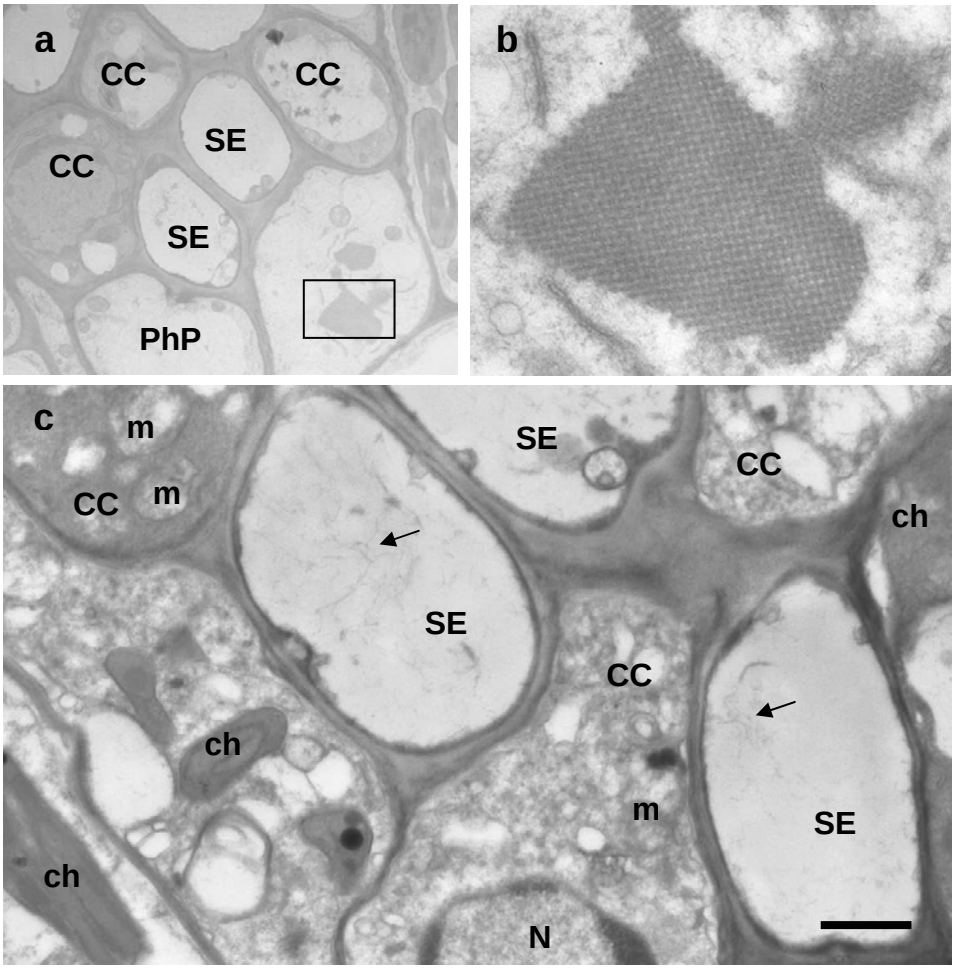
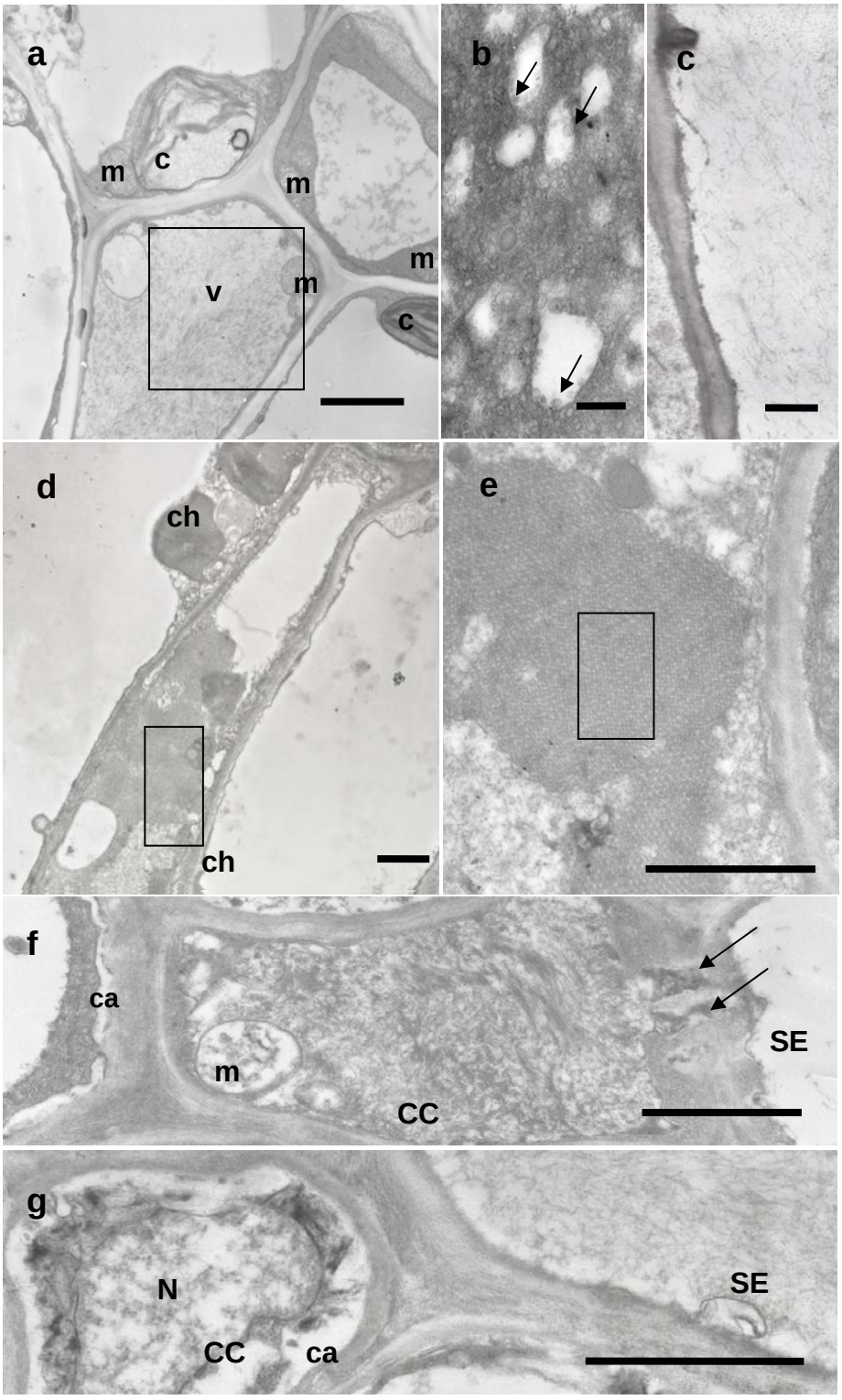


Figure 8.2. Electron micrographs of tomato leaf cells single infected with *Tomato torrado virus* (ToTV). **(a)** Vascular zone showing a defined crystal of isometric virus-like particles (VLPs) of 20-30nm in a phloem parenchyma cell (PhP). A small amount of flexuous particles, probably corresponding to the P-protein, are present in the sieve tubes (SE), Bar= 3.63µm. **(b)** Detail of b. Bar= 1.45 µm. **(c)** Vascular area showing three companion cells (CC) with cytoplasm vesiculation and sieve tubes (SE) containing a small amount of P-protein (arrows). Chloroplasts (ch) do not show alterations. Mitochondria (m) appear hypertrophied. No special cell wall overgrowth is present (N= nucleus; bar = 0.9µm).

Cytopathology of ToTV + ToCV double infection

Leaf tissues single-infected with ToCV revealed flexuous virus-like particles (VLPs) in the sieve tubes and some hypertrophied chloroplasts showing some osmophilic globules (Figure 8.3a) associated with viral infection of other viruses (Francki *et al.,* 1985). The typical cytoplasmic vesiculation (Table 8.2) and the *Beet yellows virus* (BYV)-type membranous inclusion bodies (Fig 8.3b) induced by *Crinivirus* infection were also observed (Medina *et al.,* 2003). Likewise, no plasmalemma deposits were shown like those described for *Lettuce infectious yellows virus* (LIYV) (Medina *et al*., 1998). Under an optical microscope, the leaf tissues infected with ToCV only showed a few necrotic cells of the parenchyma mesophyll (Figure 8.1c).

The observation of a double infection of ToTV and ToCV reveals that the cytopathic effect of ToCV was more evident in these samples (Table 8.2; Figure 8.3c-g). Two types of VLPs were distinguishable: large crystalline masses of isometric VLPs, contained in the companion cells of the phloem (Figure 8.3d, e) and masses of flexuous VLPs inside the sieve tubes (Figure 8.3f, g), together with p-Protein fibers (Figure 8.3c). Chloroplasts reveal only slight alterations (Figure 8.3d), although some mitochondria appeared to be hypertrophied (Figure 8.3f, Table 8.2). Moreover, stronger cell wall growth and callose deposition were observed in the companion cells (Figure 8.3f, g; Table 8.2), as common alterations characteristic of *Criniviruses* infection (Medina *et al.,* 2003). A noticeable increase in size of the plasmodesmata connected with a phloem tube was shown (Figure 8.3f), which was a reported mechanism of cell-to-cell movement of other viruses (Wolf *et al.,* 1989; McLean *et al.,* 1993). Cytoplasm disruption was stronger and viral accumulation in the phloem was clear in the ToTV and ToCV mixed infected samples, probably because cell-wall growth and callose deposition hindered the possible cell-to-cell viral spreading, as reported for plant virus infections in general (Francki *et al*., 1985).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◄ **Figure 8.3.** Electron micrographs of tomato leaf cells single-infected with *Tomato chlorosis virus* (ToCV) **(a,b)** and double-infected with Tomato torrado virus (ToTV) and *Tomato chlorosis virus* (ToCV) **(c,d,e,f,g)**. **(a)** Vascular area showing hypertrophied chloroplast (ch) and flexuous virus-like particles (VLPs) in a sieve tube in ToCV single-infected tissues. Bar= 1.5 µm. **(b)** Membranous inclusion body showing vesicles (arrows) in lacunes in ToCV single-infected tissues. Bar= 0.91 µm. **(c)** Sieve tube showing flexuous VLPs together with fibers of the P-protein in ToTV and ToCV co-infected tissues. Bar= 1.45 µm. **(d)** Companion cell of ToTV and ToCV co-infected leaf tissues, showing a large crystalline mass of isometric virus-like particles (VLPs). Chloroplasts (ch) show slight alterations. Bar= 2.9µm. **(e)** Companion cell of ToTV and ToCV co-infected leaf tissues showing a large crystalline mass of isometric virus-like particles (VLPs). Bar= 1.45µm. **(f)** Companion cell (CC) of ToTV and ToCV co-infected leaf tissues crowded by masses of flexuous VLPs and with hypertrophied mitochondria (m). Note the considerable increased size of the plasmodesmata (arrows) connecting with a phloem tube, the general callose (ca) deposition in plamalemma and the cell wall overgrowth. Bar= 1.45 µm. **(g)** Vascular detail showing a sieve tube of ToCV and ToTV co-infected leaf tissues full of flexuous VLPs and an enormous callose deposition in a companion cell. Bar= 1.45 µm (N= nucleus).

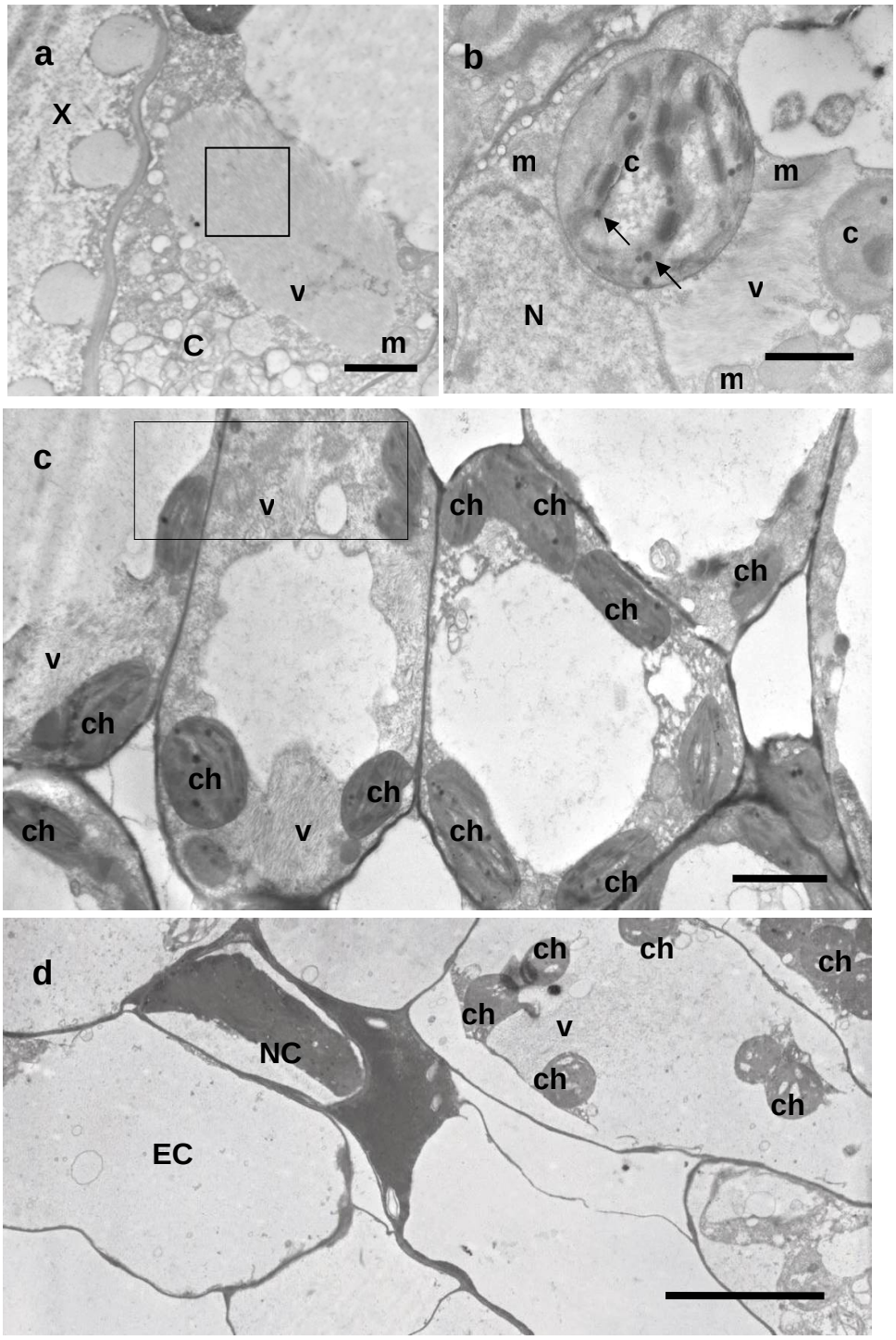
Cytopathology of ToTV + PepMV double infection

The analysis of PepMV single-infected tissue concludes that a very small amount of necrotic cells were present (Figure 8.1d). Mitochondria were not hypertrophied, although their number increased because of infection (Figure 8.4a, b), which also occurred in *Nicotiana glutinosa* infected-leaves with *Tobacco mosaic virus* (TMV), or in other virus-infected apple tissues (Šutić and Sinclair, 1991). All in all, some cells presented a slight hypertrophy of chloroplasts, which were visible under EM, showing osmiophilic globules (Figure 8.4b). Details of the alteration of the tissues observed in PepMV single-infected samples are provided in Table 8.2. Large masses of virions (viroplasms) were present in the different cells which induced alterations and a disruption of contents in extensive areas of the epidermal cells (Figure 8.4a, b). These aggregates containing arrays of filamentous virus-like particles were previously observed in ultrathin sections of *N. glutinosa* leaves infected with the first described isolate of PepMV detected on pepino in Peru (Jones *et al.*, 1980).

There was no evident difference in the cytopathic effect between the two genotypes of PepMV (CH2 and EU) studied in single- or multiple-infected tissues.

The combined infection of ToTV and PepMV led to some necrotic cells of the parenchyma mesophyll (Figure 8.1e) and induced considerable cytopathic effects for tomato leaf cells. Cytoplasm vesiculation was observed in the vascular tissues (Figure 8.4c), as in *Beet necrotic yellow vein virus* infections (BNYVV) (Šutić and Sinclair, 1991). Chloroplasts and mitochondria appeared to be hypertrophied (Figure 8.4c), as in Chinese cabbage infected by *Turnip yellow mosaic virus* (TuYMV), which induced chloroplast hypertrophy with a round shape and appeared aggregated toward the cell walls, or in cucumber cells infected with *Cucumber green mottle mosaic virus* (CGMMV) which showed abnormal mitochondria (Šutić and Sinclair, 1991). However no cell wall overgrowth was determined in the affected samples (Table 8.2). The tissues affected by this double infection sometimes resembled badly embedded samples, which also occurred in the triple infection of ToTV/PepMV/TSWV.

Figure 8.4. Electron micrograph of tomato leaf cells single-infected with the CH2 genotype of *Pepino mosaic virus* (PepMV) **(a, b)** and double-infected with PepMV-CH2 and Tomato torrado virus (ToTV) (**c, d**). **(a)** Phloem cell of single-infected leaf with PepMV showing strong cytoplasmic vesiculation and a mass of flexuous VLPs (viroplasm, v) close to a xylem element (X). Bar=1.81µm. **(b)** Mesophyll cells of single-infected leaf with PepMV showing masses of flexuous VLPs, hypertrophied chloroplasts (ch) with osmiophilic bodies (arrows) and cytoplasmic vesiculation. Bar=1.81µm. **(c)** Mesophyll cells of double-infected leaf with PepMV and ToTV, close to epidermis (upper part) showing a general infection by PepMV-like particles (v). The flexuous virus-like particles (VLPs) seem to be scattered inside the vacuoles. Bar= 3.63µm. **(d)** Some epidermal cells (EC) and lacunar mesophyll cells of double-infected tissues with PepMV and ToTV appear necrosed and/or disrupted (NC). Bar = 7.3 µm.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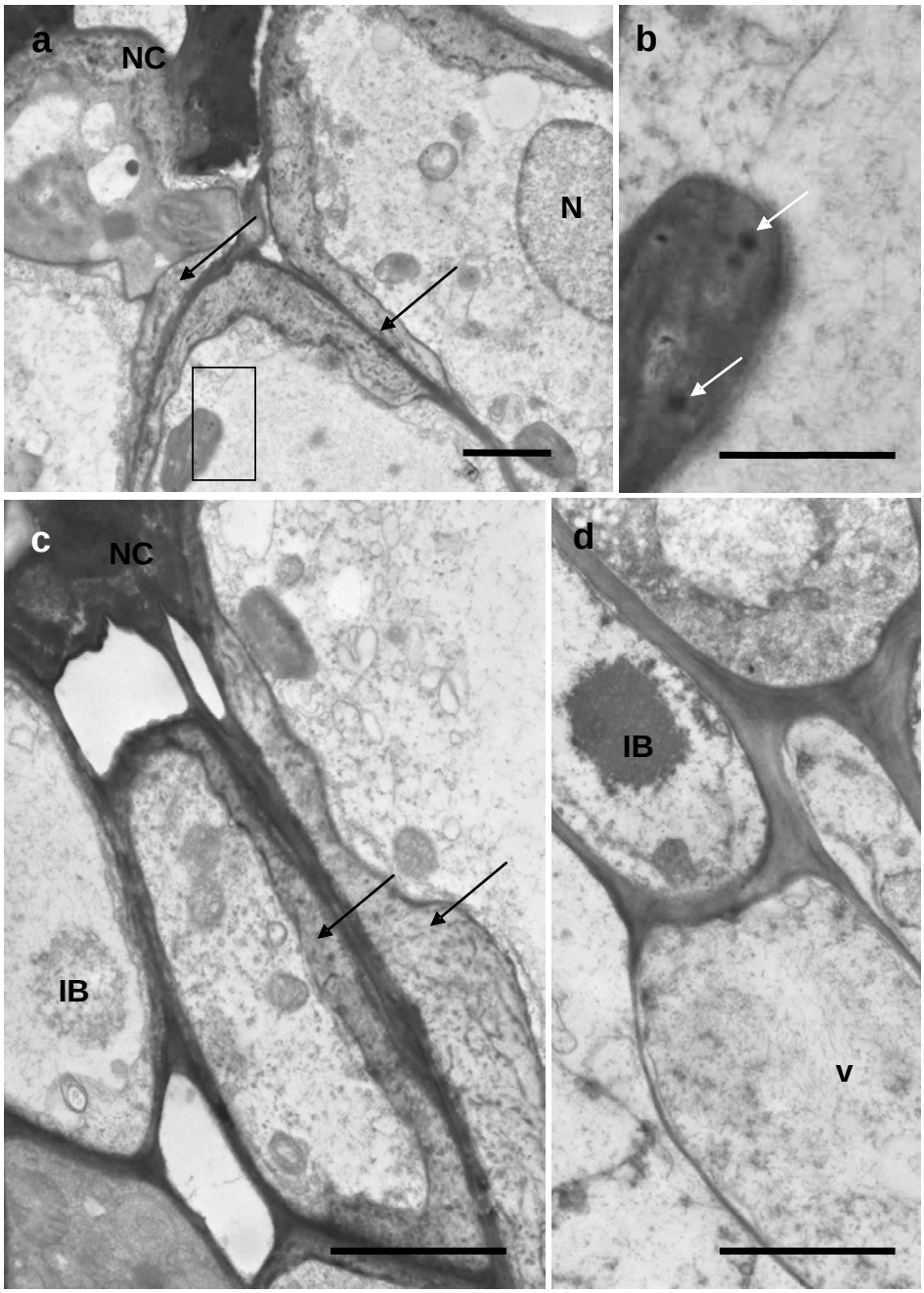
Aggregates of virions were observed in mesophyll cells, and some scattered VLPs in the vacuoles were also present due to the disruption of tonoplasts (Figure 8.4c). Occasionally, some necrotic cells were seen in leaf tissues in a larger amount than in other double or multiple infections (Figure 8.4d, 8.1e).

Cytopathology of ToTV in triple and multiple infections

Cells with a disrupted cytoplasm and necrotic cells were present in a larger amount in triple-infected samples with ToTV, PepMV-CH2 and TSWV (Figs. 8.2f). In such samples, mesophyll cells presented large cell wall overgrowth and, as in the double infection of ToTV/ToCV, scattered flexuous VLPs in the vacuoles (Figure 8.5a) and necrotic cells were observed (Figure 8.5c). Osmophilic bodies were seen in the chloroplasts (Figure 8.5b), while granular inclusion bodies were detected in the mesophyll cells (Figure 8.5c, d). Other triple infections of ToTV with PepMV/ToCV, ToCV/TYLCV also produced evident cell wall overgrowth of the companion cells and the phloem parenchyma cells. However, this cytological change in the bundle sheath cells was only observed in tomato samples infected with ToTV, PepMV and TSWV (Table 8.2). On the other hand, the presence of necrotic cells in the plesionecrotic areas under study was less evident in those samples affected with viruses which were confined to the phloem, i.e., ToTV, ToCV or TYLCV, where the alteration was limited to the vascular tissue (data not shown).

Figure 8.5. Electron micrograph of tomato leaf tissues triple-infected with *Tomato torrado virus* (ToTV), *Pepino mosaic virus* (PepMV) and *Tomato spotted wilt virus* (TSWV). **(a)** Mesophyll cells around a necrotic cell (NC) showing spectacular cell wall overgrowth (arrows) and scattered flexuous virus-like particles (VLPs) in their vacuoles. Bar= 2.9 µm. **(b)** Detail of a. Chloroplasts showing osmiophilic bodies (white arrows). Bar = 1.45µm. **(c)** Palysade mesophyll cells close to a necrosed epidermal cell showing strong cell wall overgrowth and disrupted cytoplasms. One of them shows a granular inclusion body (IB). Bar = 2.3 µm. **(d)** Cells close to a vascular area showing disruption, flexuous VLPs and dense granular IBs. Bar = 2.3 µm. (N= nucleus).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The reaction induced by the infection of ToTV/ToCV produced a general obstruction of the phloem tubes, as observed before (Figure 8.3c), which might avoid PepMV or other viruses spreading (Francki *et al.,* 1985), and consequently, the damage caused by this virus may not occur in these cases.

Furthermore, the results detailed in Table 8.2 show that hypertrophied chloroplasts were more visible in the samples infected with PepMV in any of the combinations studied. Cell wall overgrowth appeared to be a typical feature of ToCV infection. ToCV did not induce observable symptoms in cells outside the phloem, at least in the plesionecrotic tissues studied. As for the multiple infections with ToTV, cell necrosis was more evident, and embedding for EM was harder due to the cell wall overgrowth of cells caused by infection, which made the penetration of fixatives and resins difficult.

CONCLUSIONS

Synergism was observed at the ultrastructural level between different viruses that co-infect samples affected by the 'Torrado' disease. ToTV in single infections led to alterations in cells mainly at the phloem level. However, the co-infection of this virus with other viruses in affected plants produced more severe reactions in either the phloem or other plant tissues, such as the double-infection of ToTV with PepMV or ToCV, and the multiple infections of ToTV, TSWV and PepMV.

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Capítulo 9

Molecular variability of Spanish isolates of Tomato torrado virus

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ABSTRACT

The population structure and genetic variation of Tomato torrado virus (ToTV) were estimated from 19 Spanish isolates collected from 2001 to 2009 in different tomato production areas by analyses of the partial nucleotide sequences of five regions of the virus genome: the protease-cofactor (Pro-Co) and the RNA-dependent RNA-polymerase (RdRp) in the RNA1, and the movement protein (MP) and two subunits of the coat protein (Vp35 and Vp23) in the RNA2. In the analysis, three Hungarian isolates of the virus were also included. All the ToTV isolates clustered together in the phylogenetic analysis of the nucleotide sequences of the different regions. A high similarity was observed among all the isolates and the two published ToTV isolates: ToTV-type isolate and the Polish isolate Wal03. However, some genetic diversity was observed in case of subunits of the CP studied among the Gran Canary isolates and the rest of analysed isolates of ToTV that grouped together. The most variable regions studied were the three encoding regions on the RNA2. Studying the genetic distances between pairs of sequences, the ratio between nonsynonymous and synonymous substitutions was low, indicating a strong negative selective pressure in the studied regions. Nine negatively selected sites (distributed in Pro-Co, MP, Vp23 and Vp35) and just one site (in the Pro-Co) under positive selection for all the genome regions studied were found.

INTRODUTION

Tomato torrado virus (ToTV) is a newly characterized Picorna-like virus, proposed to be a member type of a new genus, Torradovirus (Verbeek *et al.,* 2007a), which also includes two other recently characterized tomato infecting viruses, Tomato apex necrosis virus (ToANV) and Tomato marchitez virus (ToMarV) (Turina *et al.,* 2007; Verbeek *et al.,* 2007b). Virions of ToTV consist of isometric particles with a diameter of 28 nm and their genome is composed of two single-stranded positive sense RNA molecules. RNA1 has approximately 7800 nt and contains one open reading frame (ORF) encoding the characteristic functional domains of the protease cofactor (Pro-Co), helicase, protease and the RNA-dependent RNA polymerase (RdRp). RNA2 has 5389 nt and contains two ORFs. The ORF2, which partly overlaps ORF1, encodes three different subunits of the coat protein (CP), named according to their approximate molecular weight (Vp35, Vp26 and Vp23), and a putative movement protein (MP) since a motif LRVPML, similar to the characteristic region of MP found among other viruses, was determined. The ORF1 showed no homology to other viral proteins and the analysis of functional sites did not identify any specific domains (Verbeek *et al.,* 2007a; Budziszewska *et al.,* 2008). This genome structure resembles those of the members of the genera *Sequivirus*, *Waikavirus, Sadwavirus* and *Cheravirus*. However, a phylogenetic analysis revealed important differences between ToTV and the viruses of these genera (Verbeek *et al.,* 2007a). Recently, a new family, Secoviridae, which would include all those genera as well as *Comovirus*, *Nepovirus*, *Fabavirus* and Torradovirus, has been proposed (Sanfaçon *et al.,* 2009).

ToTV causes a tomato disease which, in Spain, is locally named 'torrado' due to the necrotic burn-like symptoms on leaves and fruits that affected plants show. The virus was reported, apart from mainland Spain (Verbeek *et al.,*

2007a), on tomatoes in Poland (Pospieszny *et al.,* 2007), Australia (IPPC, 2008), Panama (Herrera-Vásquez *et al.,* 2009a) and Hungary (Alfaro-Fernández *et al.,* 2009a), and has been recently included in the EPPO alert list (EPPO, 2009). This virus is efficiently transmitted by whitefly species *Trialeurodes vaporariorum* (Pospieszny *et al.,* 2007) and *Bemisia tabaci* (Amari *et al.,* 2008), and naturally infects several weed species associated with tomato crops (Alfaro-Fernández *et al.,* 2008b).

A comparative sequence analyses of the complete genome of two different isolates: the ToTV-type isolate named PRI-ToTV0301 (Accession numbers DQ388879 and DQ388880) collected from the Murcia Region (Spain) in 2003 and the Polish isolate named Wal03 (Accession numbers EU563948 and EU563947) collected in the Wielkopolska region (Poland) in 2003, revealed a high homology about 98-100% amino acid (aa) identity, although few nucleotide (nt) and amino acid (aa) substitutions were observed in the coding regions (Budziszewska *et al.,* 2008). The study of the three subunits of two Polish isolates (Kra and Ros) collected in 2007, revealed also a high aa identity between them and with the other ToTV sequences described above (Pospieszny *et al.,* 2009). The comparison of some coding regions of other isolates also revealed a high level of nt identity (98-99%) with the ToTV type isolate, such as ToTV-Can (Accession number EF436286) isolated from the Canary Islands (Spain) in 2006 (Alfaro-Fernández *et al.,* 2007a), ToTV-CE (Accession numbers EU476181 and EU476182) trapped on tomato plants in a greenhouse of the Murcia Region (Spain) (Amari *et al.,* 2008), ToTV-Pan1 (Accession numbers FJ357161 and EU934037) collected from the Coclé region (Panama) in 2008 (Herrera-Vásquez *et al.,* 2009a), ToTV-W1 and ToTV-W2 (Accession numbers EU090252 and EU090253) collected from two weed species from the Murcia Region and the Canary islands (Spain) in 2007, and the Hungarian isolates ToTV-H1, ToTV-H2, and ToTV-H3 (Accession Nos. EU835496-FJ616996,

FJ616995-FJ616997, and FJ616994-FJ616998) collected in Szeged, Öcsöd, and Csongrád (Hungary) in 2007 and 2008 (Alfaro-Fernández *et al.,* 2009a). The aim of the present work is to evaluate the molecular variability of the ToTV genome and the phylogenetic relationships among the 19 Spanish isolates collected over 9 different years (2001 to 2009) from diverse geographic origins and three Hungarian isolates, and compare them with the isolates published in the GenBank database.

MATERIALS AND METHODS

Virus isolates, RNA extraction and dot-blot hybridization analysis

Leaf samples of symptomatic plants were collected from different geographical regions of Spain and Hungary in the period 2001-2009. The characteristics of the samples are detailed in Table 9.1. The Spanish isolates collected from tomato were codified by a three letter code indicative of its geographic origin (ALC= Alicante, ALM= Almeria, BCN= Barcelona, GNC= Grand Canary, MUR= Murcia, TEN= Tenerife), followed by the year of collection. Total RNA extraction was performed with 0.1 g of fresh or dried leaf tissue from the infected plants using the silica capture protocol (MacKenzie *et al.,* 1997). One microlitre of the extracted RNA was first denatured with formaldehyde, and then was directly applied to a nylon membrane. The analysis of total nucleic acids by non-isotopic dot-blot hybridization was performed as described previously by Sánchez-Navarro *et al.* (1998) using a dig-RNA probe complementary to a fragment of the polyprotein of RNA2 of ToTV to verify the presence of the virus.

Primer design

ToTV complete genome sequences of the ToTV type isolate PRI-ToTV0301 (RNA1, GenBank Accession number DQ388879 and RNA2, GenBank Accession number DQ388880) and the Polish isolate Wal03 (RNA1, GenBank Accession number EU563948 and RNA2, GenBank Accession number EU563947), which are available in the GenBank database, were aligned using the CLUSTAL X version 1.83 (Thompson *et al.,* 1997). Five different fragments of the virus genome were studied: the partial protease-cofactor (Pro-Co) and the RNA-dependent RNA-polymerase (RdRp) regions in the RNA1, and the partial movement protein (MP) and two subunits of the coat protein in the RNA2 (Vp35 and Vp23). To amplify the corresponding regions, we used different primers which have been previously described, and three ToTV-specific primers (Vp35-D, CP-R and ToTCoF-R) designed using the OLIGO version 4.0 (National Bioscience Inc.; Table 9.2).

RT-PCR amplification, sequencing and sequence analysis

The RT-PCR reactions of the particular zones of the ToTV genome were performed using the SuperScript III one step RT-PCR system with Platinum Taq DNA polymerase kit (Invitrogen Life Technologies) with the specific pair of primers for each region (Table 9.2) at a final concentration of 0.5 μM for each primer. The PCR program consisted of an initial incubation at 50ºC for 30 min followed by 2 min at 94ºC and 40 cycles of 94ºC for 15s, 55ºC for 30s and 68ºC for 1 min, except for the primer combination ToTVB-D/ToTVB-R whose appropriate annealing temperature was 50ºC. A final incubation at 68ºC for 10 min was introduced to finish the incomplete PCR fragments. The amplified PCR products were analysed on 1.2% agarose gel in 1x TAE buffer, stained with ethidium bromide and visualised under UV light.

Table 9.1. Characteristics of the ToTV isolates used in the assay.

Isolate code[a]	Collection date	Original host	Area of origin	GenBank Accession Nos.[b]				
				Pro-Co	RdRp	MP	Vp23	Vp35
MUR-01	2001	Tomato	Murcia, Spain	GQ397365	GQ397383(v)	GQ397433(n)	GQ397399(s)	GQ397415
MUR-02	2002	Tomato	Murcia, Spain	GQ397366	GQ397384	(n)	GQ397400	GQ397416
TEN-03	2003	Tomato	Tenerife, Spain	GQ397368	GQ397385	GQ397434	GQ397401	GQ397417(q)
MUR-03	2003	Tomato	Murcia, Spain	GQ397367(z)	(v)	GQ397435	(r)	GQ397418
W3-04	2004	*S. nigrum*	Murcia, Spain	GQ397374	GQ397386(u)	GQ397436	GQ397402(r)	GQ397419
ALM-04	2004	Tomato	Almería, Spain	GQ397381(w)	GQ397387	GQ397437	GQ397403	GQ397420
GNC-04	2004	Tomato	Grand Canary, Spain	GQ397369(y)	GQ397388	GQ397438	GQ397404	GQ397421
MUR-05	2005	Tomato	Murcia, Spain	(z)	(v)	GQ397439	(r)	GQ397422(p)
GNC-05	2005	Tomato	Grand Canary, Spain	GQ397380	GQ397389	GQ397440	GQ397405	GQ397423
GNC-06 (ToTV-CAN)	2006	Tomato	Grand Canary, Spain	(y)	GQ397390	EF436286[c]	GQ397406	GQ397424
MUR-06	2006	Tomato	Murcia, Spain	GQ397377	GQ397391	GQ397441	GQ397407	GQ397425
ALC-07	2007	Tomato	Alicante, Spain	GQ397372	GQ397392	GQ397442	GQ397408	GQ397426(o)
W4-07	2007	*S. nigrum*	Tenerife, Spain	GQ397371	GQ397393	GQ397445	GQ397409	GQ397427
TEN-07	2007	Tomato	Tenerife, Spain	GQ397378	GQ397394	GQ397444	GQ397410	(q)
MUR-07	2007	Tomato	Murcia, Spain	(w)	GQ397395	GQ397443	(s)	(o)
ToTV-H1	2007	Tomato	Szeged, Hungary	GQ397376	(t)	EU835496[d]	FJ616996[d]	GQ397430
GNC-08	2008	Tomato	Grand Canary, Spain	GQ397370	GQ397396	GQ397446	GQ397411	GQ397428
MUR-08	2008	Tomato	Murcia, Spain	GQ397379	(v)	GQ397447	GQ397412	(o)
BCN-08	2008	Tomato	Barcelona, Spain	GQ397375	(u)	GQ397448	GQ397413	GQ397429
ToTV-H2	2008	Tomato	Öcsöd, Hungary	GQ397373(x)	GQ397398(t)	FJ616995[d]	FJ616997[d]	GQ397431
ToTV-H3	2008	Tomato	Csongrád, Hungary	(x)	(t)	FJ616994[d]	FJ616998[d]	GQ397432
MUR-09	2009	Tomato	Murcia, Spain	GQ397382	GQ397397	GQ397449	GQ397414	(p)

[a]A three letter code indicative of its geographic origin (ALC= Alicante, ALM= Almería, BCN= Barcelona, GNC= Grand Canary, MUR= Murcia, TEN= Tenerife) followed by the year of collection.
[b]Sequences showing the same letter (in parentheses) are 100% nt identical.
[c]Sequence published in the GenBank database and reported by Alfaro-Fernández *et al.* (2007a).
[d]Sequences published in the GenBank database and reported by Alfaro-Fernández *et al.* (2009a).

Table 9.2. Sequences of the ToTV-specific primers used in the assay.

Genome zone	Primer name	Nucleotide sequence (5´-3´)	Location in the sequence (nt)[a]	Expected fragment (bp)	Reference[b]
Pro-Co (RNA1)	ToTGSP2-D[c,d]	GAAGCCTTCGGTTACAGATGCTG	277-299	847	Budziszewska *et al.*, 2008
	ToTCoF-R[e]	AGCCTGCTCCCTTAGATGTTGG	1102-1124		designed
RdRp (RNA1)	TR1F[d]	CAATGTGCCAAAGATGAGCG	4007-4026	1066	Pospieszny *et al.*, 2007
	ToT4b-R[c,e]	AGTTCCTTGATGAGCCCAATG	5053-5073		Budziszewska *et al.*, 2008
MP (RNA2)	ToTVB-R[d]	TTCCAGTAATGATCCAACCAAT	1055-1076	576	van den Heuvel *et al.*, 2006
	ToTVB-D[e]	CCCATCATCACCCTCCTCTTCGTA	1608-1631		van den Heuvel *et al.*, 2006
Vp35 (RNA2)	Vp35-D[d]	ACTGGAAAGGTACGGCATTGTGAA	1880-1903	694	designed
	ToTVCP-R[e]	GACCCGAAATTGCACCCATGCCGG	2551-2574		designed
Vp23 (RNA2)	TR2F[d]	GAAGGACGAAGAGCGACTG	3683-3701	573	Pospieszny *et al.*, 2007
	TR2R[e]	AAGGTAGGTATGCGTTTGC	4238-4256		Pospieszny *et al.*, 2007

[a]Nucleotide location refers to the sequences of the Wal03 isolate available in the GenBank database, Accession numbers EU563947 and EU563947.
[b]Reference where the primer was designed.
[c]Primer used in the opposite direction to which it was designed.
[d]Specific forward primers.
[e]Specific reverse primers.

Fragment sizes were determined by comparison with a 100bp DNA Ladder Plus (MBI Fermentas). The amplified fragments were purified using the High Pure PCR Product Purification Kit (Roche Diagnostics), and were sequenced and deposited in the GenBank database (accession numbers are detailed in Table 9.1. The similarity/identity of the obtained nucleotide and the deduced amino acid sequences was calculated with MatGAT version 2.01 (Matrix Global Alignment Tool; Campanella *et al.,* 2003).

For each genomic region, the nucleotide substitution model that better explained the observed pattern of variability was inferred by a maximum likelihood approach using the FINDMODEL server (http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html).
Similarly, the amino acid substitution model that maximized the likelihood of the observation was inferred using the PROTTEST server (http://darwin.uvigo.es/software/prottest_server.html). A phylogenetic analysis based on the nucleotide and amino acid sequences was performed with the MEGA version 3.1 (Kumar *et al.,* 2004), using neighbour-joining algorithm. The statistical reliability of the constructed tree was assessed by the bootstrap method based on 10,000 pseudoreplicates. The comparative analyses were carried out using the sequences available in GenBank). The genetic distances between pairs of sequences and pairwise synonymous (d_S) and nonsynonymous (d_{NS}) substitutions were calculated according to the Kimura 2-parameter (Kimura, 1980) and to the method Pamilo, Bianchi and Li (Pamilo and Bianchi, 1993; Li, 1993), respectively, using MEGA version 3.1 (Kumar *et al.,* 2004). Confidence estimates for nonsynonymous (amino acid-replacing) and synonymous (silent mutational) nucleotide substitutions were calculated by using the bootstrap method with 1,000 replicates. To identify the specific amino acids under selective constraints, the differences between the nonsynonymous (d_{NS}) and synonymous (d_S) substitution rates were estimated for each position in the

alignment using the fixed-effects likelihood (FEL) and internal fixed-effect likehood (IFEL) methods (Kosakovsky-Pond and Frost, 2005; Kosakovsky-Pond *et al.,* 2006), as implemented in the HYPHY server http://www.datamonkey.org). A d_N - d_S values >0 is taken as evidence for a positive selection, whereas values <0 are a sign of a negative or purify selection.

RESULTS

The genetic diversity of the Spanish isolates of ToTV was analysed by comparing the nucleotide and amino acid sequences of five selected genome fragments; Pro-Co, RdRp (RNA1), and MP, Vp23 and Vp35 (RNA2). In addition, three Hungarian isolates were also included in the comparative analyses. These genomic regions were selected because they were representative of the two RNAs and encoded aa fragments with distinct functions. Fragments of the RdRp, Vp35 and Vp23 were reported as the ToTV genomic regions with more aa substitutions (Budziszewska *et al.,* 2008). All the sequences used in this paper that differed from the complete ToTV sequences included in the GenBank nucleotide sequence database, were introduced into the database (Table 9.1).

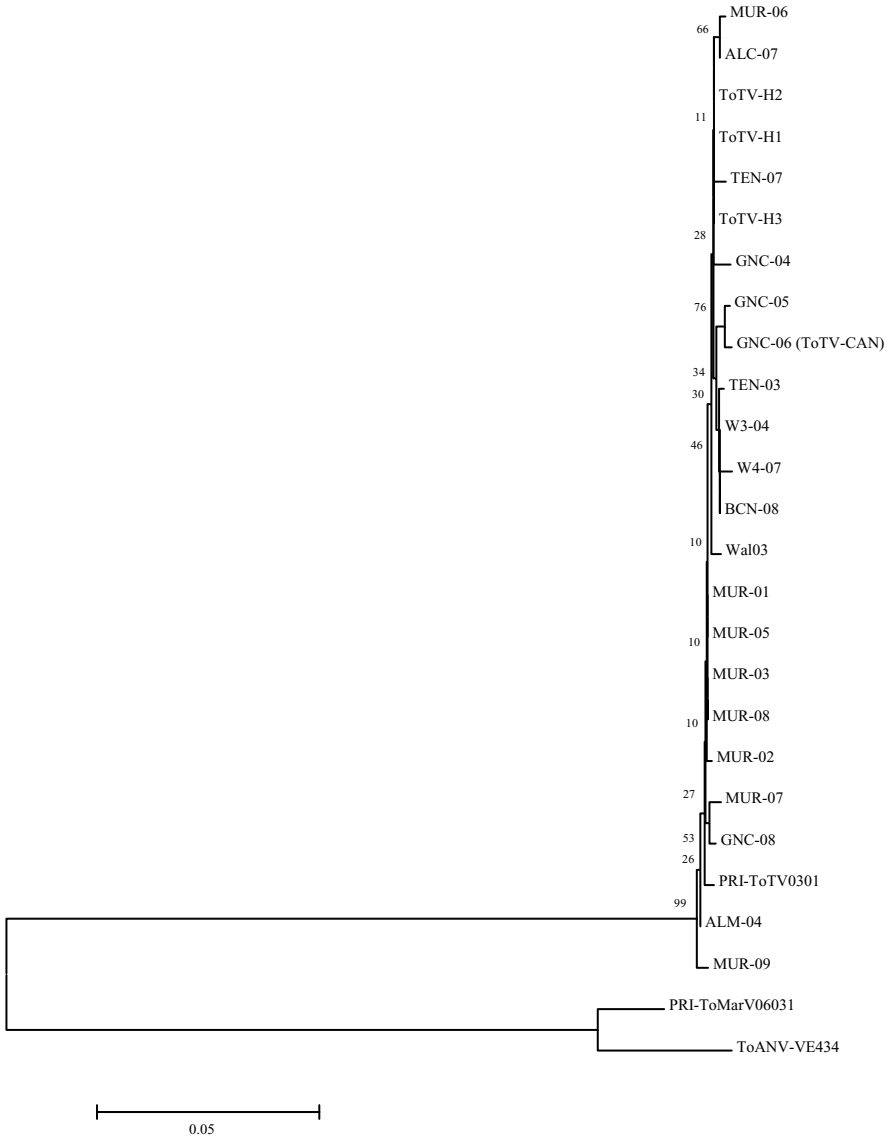
Phylogenetic analyses among the nt and aa sequences of the 22 ToTV isolates studied were inferred for the five analysed genomic regions using the neighbour joining method. It is clearly observed in the five regions studied that all the ToTV isolates clustered separately from ToMarV and ToANV, these being the two other viruses belonging to the newly proposed genus, Torradovirus (Sanfaçon *et al.,* 2009). The isolates studied were very similar and belonged to a single phylogenetic cluster as representatively shows Figure 9.1a. No significant grouping was observed according to the geographic origin or the collection year, except for the Grand Canary isolates using the subunits of the

CP, Vp35 (bootstrap 76%) and Vp23 (bootstrap 100%) regions (Fig 9.1b and 9.1c).

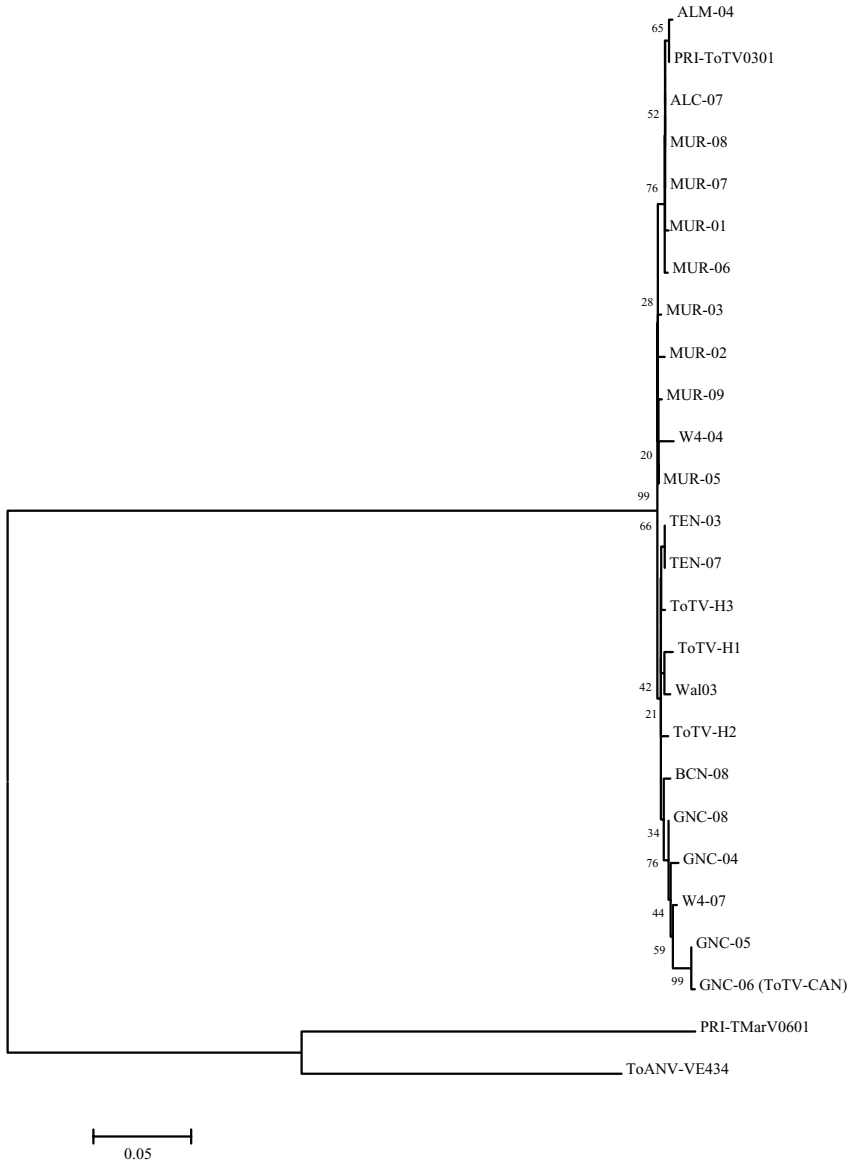
Interesting, the grouping included the ToTV variant isolates from both tomato and weed species (*S. nigrum*, isolate W4-07). In addition the Hungarian isolates did not group separately from the Spanish isolates in any of the study regions. In general, there was no evidence of population subdivision, indicating a close genetic distance among the isolates. The phylogenetic analysis shows that the sequences of the different studied regions were much conserved, and that when the analysis was performed with the predicted aa sequences, all the ToTV isolates were grouped together, and no differences were observed even in the Vp23 and Vp35 sequences (Figure 9.2). Only representative phylogenetic tree among nt and aa have been included, the rest of the trees did not add any information.

Figure 9.1. Representative phylogenetic analysis showing the relationship of the obtained nucleotide sequences of the 19 Spanish and 3 Hungarian ToTV isolates with other isolates, and related viruses in the RdRp **(a)**, the coat proteins subunits Vp35 **(b)** and Vp23 **(c)**. The neighbour-joining phylogenetic trees were obtained using MEGA version 3.1. (Kumar *et al.,* 2004). The statistical reliability of the constructed trees was assessed by the bootstrap method based on 10,000 pseudoreplicates. The number above the nodes indicates the percentage of bootstrap replicates which supported branching. The scale bar represents a genetic distance of 0.05. The accession numbers of the sequences used in the analysis are PRI-ToTV0301 (GenBank Accession numbers DQ388879 and DQ388880), Wal03 (GenBank Accession numbers EU563948 and EU563947), ToTV-CAN (Accession number EF436286), ToTV-Pan1 (Accession numbers FJ357161 and EU934037), ToTV-W1 (Accession number EU090252), ToTV-W2 (Accession number EU090253), ToTV-H1 (Accession numbers EU835496 and FJ616996), ToTV-H2 (Accession numbers FJ616995 and FJ616997), ToTV-H3 (Accession numbers FJ616994 and FJ616998), PRI-ToMarV0603 (GenBank Accession numbers NC010987 and NC010988) and ToANV-VE434 (GenBank Accession numbers EF063641 and EF063642).

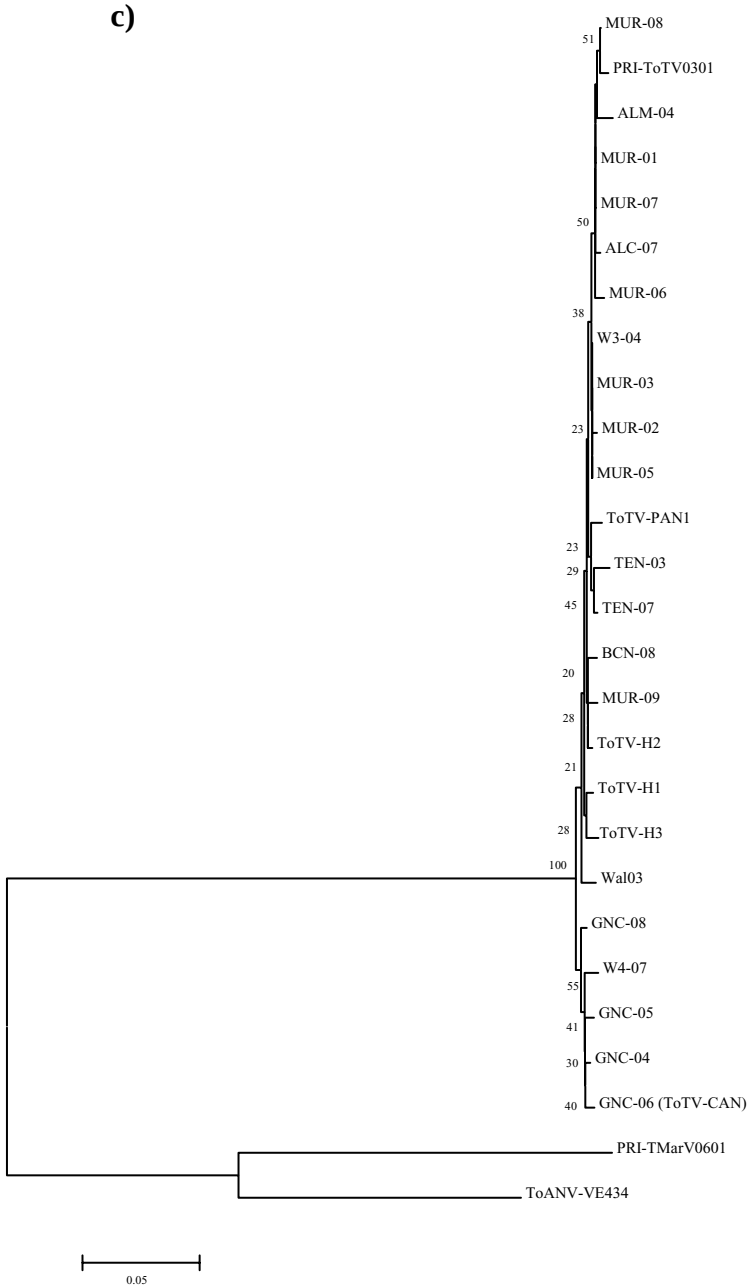
a)


MUR-06
66
ALC-07
ToTV-H2
11
ToTV-H1
TEN-07
ToTV-H3
28
GNC-04
76
GNC-05
GNC-06 (ToTV-CAN)
34
TEN-03
30
W3-04
46
W4-07
BCN-08
10
Wal03
MUR-01
10
MUR-05
MUR-03
10
MUR-08
MUR-02
27
MUR-07
53
GNC-08
26
PRI-ToTV0301
99
ALM-04
MUR-09
PRI-ToMarV06031
ToANV-VE434
0.05

b)


ALM-04
65
PRI-ToTV0301
ALC-07
52
MUR-08
MUR-07
76
MUR-01
MUR-06
28
MUR-03
MUR-02
MUR-09
W4-04
20
MUR-05
99
TEN-03
66
TEN-07
ToTV-H3
ToTV-H1
42
Wal03
21
ToTV-H2
BCN-08
GNC-08
34
76
GNC-04
W4-07
44
GNC-05
59
99
GNC-06 (ToTV-CAN)
PRI-TMarV0601
ToANV-VE434
0.05

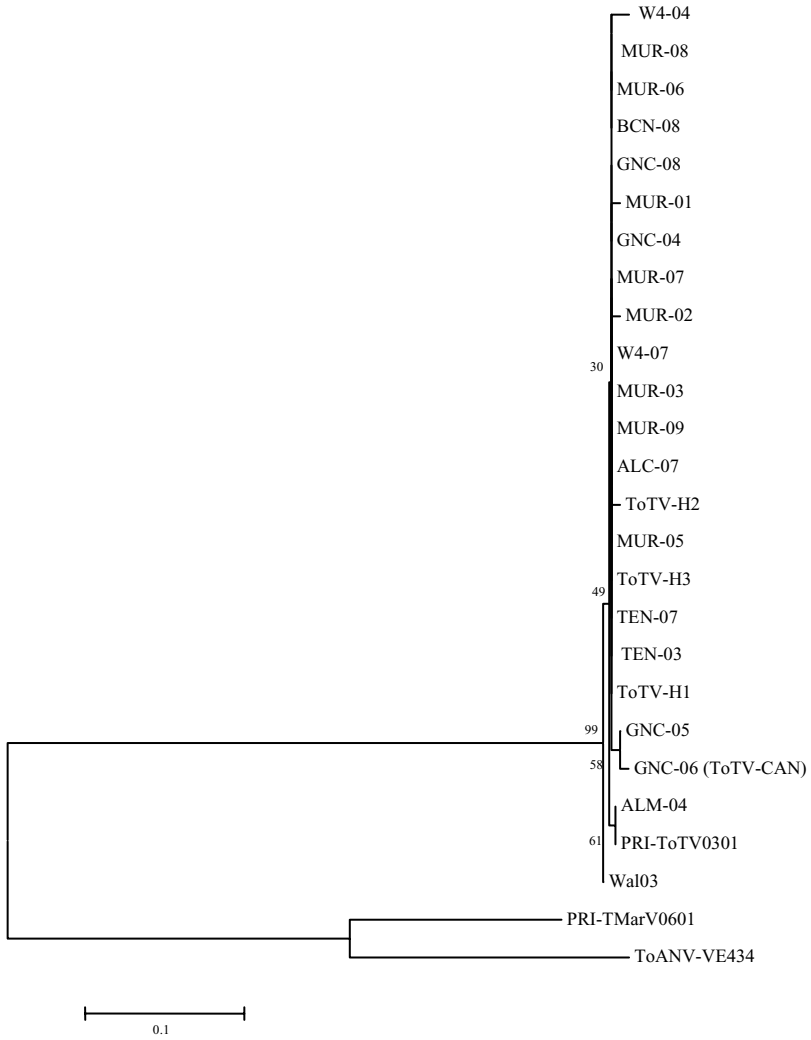
c)


MUR-08
51
PRI-ToTV0301
ALM-04
MUR-01
MUR-07
50
ALC-07
MUR-06
38
W3-04
MUR-03
23
MUR-02
MUR-05
ToTV-PAN1
23
29
TEN-03
45
TEN-07
BCN-08
20
MUR-09
28
ToTV-H2
21
ToTV-H1
28
ToTV-H3
100
Wal03
GNC-08
W4-07
55
GNC-05
41
30
GNC-04
40
GNC-06 (ToTV-CAN)
PRI-TMarV0601
ToANV-VE434
0.05

The percentages of similarity/identity of the predicted amino acid sequences showed that the studied isolates were very similar between each other, and also with the published ToTV-type (PRI-ToTV0301) and the Polish Wal03 isolates (Table 9.3). The identity/similarity among the aa predicted sequences ranged from 97.9% to 100%. The most similar genomic studied coding region was the RdRp and Pro-Co, and the highest differences were found in the RNA2 studied regions, mainly in the Vp23. Isolate W4-07, collected in 2007 from weed species, presented a lower identity/similarity in the MP compared with the published ToTV isolates, ToTV-type and Wal03 (Table 9.3).

Genetic distances for each pair of isolates were estimated for each genomic region analysed by Kimura´s two-parameter method. The mean genetic distance ranged from 0.0044 to 0.117. Although all the studied regions of the genome for all the isolates presented similar genetic distances, the mean value found in RdRp was half the mean value of the other regions studied. The highest genetic distance was shown in Vp23, although the difference among the RNA2 studied regions was minor (Table 9.4).

Figure 9.2. Phylogenetic analysis of the predicted aa sequences of the 19 Spanish and 3 Hungarian ToTV isolates with other isolates, and related viruses in the subunit Vp35 of the CP studied region. The neighbour-joining phylogenetic tree was obtained using MEGA version 3.1. (Kumar *et al.,*2004). The statistical reliability of the constructed trees was assessed by the bootstrap method based on 10,000 pseudoreplicates. The number above the nodes indicates the percentage of bootstrap replicates which supported the branching. The accession numbers of the sequences used in the analysis are PRI-ToTV0301 (GenBank Accession numbers DQ388879 and DQ388880), Wal03 (GenBank Accession numbers EU563948 and EU563947), ToTV-CAN (Accession number EF436286), ToTV-Pan1 (Accession numbers FJ357161 and EU934037), ToTV-W1 (Accession number EU090252), ToTV-W2 (Accession number EU090253), ToTV-H1 (Accession numbers EU835496 and FJ616996), ToTV-H2 (Accession numbers FJ616995 and FJ616997), ToTV-H3 (Accession numbers FJ616994 and FJ616998), PRI-ToMarV0603 (GenBank Accession numbers NC010987 and NC010988) and ToANV-VE434 (GenBank Accession numbers EF063641 and EF063642).


W4-04
MUR-08
MUR-06
BCN-08
GNC-08
MUR-01
GNC-04
MUR-07
MUR-02
W4-07
30
MUR-03
MUR-09
ALC-07
ToTV-H2
MUR-05
ToTV-H3
49
TEN-07
TEN-03
ToTV-H1
99
GNC-05
58
GNC-06 (ToTV-CAN)
ALM-04
61
PRI-ToTV0301
Wal03
PRI-TMarV0601
ToANV-VE434
0.1

Pair-wise genetic differences at the synonymous (d_S) and nonsynonymous (d_{NS}) nucleotide positions were estimated according to the method of Pamilo, Bianchi and Li (Table 9.4). The number of synonymous substitutions per synonymous site (d_S) was higher in Vp23, whereas RdRp presented the smallest values of d_S. The nonsynonymous substitutions per nonsynonymous site (d_{NS}) were smaller than the d_S in all the studied regions. The ratio between nucleotide diversity values in nonsynonymous and synonymous positions (d_{NS}/d_S) provides an estimation of the degree and direction of the selective constraints operating in a coding region. Overall, the obtained values of the d_{NS}/d_S ratio for the five studied areas were markedly low (0.0000-0.2296; Table 9.4).

The highest d_{NS}/d_S ratio was found in the MP region, indicating that this region was under tighter functional constraints than the others studied. In the RdRp region, no nonsynonymous amino acid change was observed; only some silent changes (synonymous) were present in the Spanish isolates (Table 9.4). In addition, we observed nine negatively selected sites and just one site under positive selection for all the proteins analyzed (p-value<0.05) by using the FEL analyses at the HYPHY server. In the case of RdRp, no selected site was observed, however for the Pro-Co region, three negatively selected sites were obtained (S74, S212 and T301). For the MP, three sites were also detected under the negative selection (I148, G156 and G244). For the two coat proteins, we observed three negatively selected sites, one for the Vp23 (E1151) and two for the Vp35 (R469 and A581). Using the IFEL analyses to characterise the selected sites in the internal branches of the tree, we detected the only positive selection case for the Pro-Co proteins (V-I 291). The position of the negative and positive selection sites is referred to the aa complete sequence of the polyprotein in each RNA.

Table 9.3. Percentages of identity/similarity of the amino acid sequences among the studied isolates, ToTV-type PRI-ToTV0301 and Polish isolate Wal03.

		RNA1								RNA2											
		Pro-Co				RdRp				MP				Vp23				Vp35			
		PRI-ToTV0301[a]		Wal03[b]		PRI-ToTV0301[a]		Wal03[b]		PRI-ToTV0301[c]		Wal03[d]		PRI-ToTV0301[c]		Wal03[d]		PRI-ToTV0301[c]		Wal03[d]	
		iden.[e]	simil.[e]	iden.	simil.	iden.	simil.	iden.	simil.	iden.	simil.	iden.	simil.	iden.	simil.	iden.	simil.	iden.	simil.	iden.	simil.
1.	MUR-01	99.6	100.0	99.1	99.6	100.0	100.0	99.6	100.0	99.3	99.3	98.6	99.3	98.8	99.4	99.4	100.0	98.9	100.0	98.9	100.0
2.	MUR-02	99.6	99.6	99.1	99.6	100.0	100.0	99.6	100.0	99.3	99.3	98.6	99.3	98.8	99.4	99.4	100.0	98.9	99.5	98.9	99.5
3.	MUR-03	100.0	100.0	99.6	99.6	100.0	100.0	99.6	100.0	100.0	100.0	99.3	100.0	98.8	99.4	99.4	100.0	99.5	100.0	99.5	100.0
4.	TEN-03	99.6	100.0	99.6	99.6	100.0	100.0	99.6	100.0	99.3	100.0	98.6	100.0	98.2	99.4	99.4	100.0	99.5	100.0	99.5	100.0
5.	ALM-04	100.0	100.0	99.6	99.6	100.0	100.0	99.6	100.0	100.0	100.0	99.3	100.0	99.4	99.4	99.4	100.0	100.0	100.0	98.9	100.0
6.	GNC-04	100.0	100.0	99.6	99.6	100.0	100.0	99.6	100.0	99.3	99.3	98.6	99.3	98.2	98.8	98.8	99.4	99.5	100.0	99.5	100.0
7.	W3-04	100.0	100.0	99.6	99.6	100.0	100.0	99.6	100.0	100.0	100.0	99.3	100.0	98.8	99.4	99.4	100.0	98.4	99.5	98.4	99.5
8.	GNC-05	100.0	100.0	99.6	99.6	100.0	100.0	99.6	100.0	100.0	100.0	99.3	100.0	97.6	98.2	98.2	98.8	98.9	100.0	98.9	100.0
9.	MUR-05	100.0	100.0	99.6	99.6	100.0	100.0	99.6	100.0	100.0	100.0	99.3	100.0	98.8	98.8	99.4	100.0	98.9	99.5	98.9	99.5
10.	GNC-06	100.0	100.0	99.6	99.6	100.0	100.0	99.6	100.0	100.0	100.0	99.3	100.0	98.2	98.8	98.8	99.4	98.4	100.0	98.4	100.0
11.	MUR-06	100.0	100.0	99.6	99.6	100.0	100.0	99.6	100.0	100.0	100.0	99.3	100.0	98.8	99.4	99.4	100.0	99.5	100.0	99.5	100.0
12.	ALC-07	100.0	100.0	99.6	99.6	100.0	100.0	99.6	100.0	100.0	100.0	99.3	100.0	98.8	99.4	99.4	100.0	99.5	100.0	99.5	100.0
13.	MUR-07	100.0	100.0	99.6	99.6	100.0	100.0	99.6	100.0	100.0	100.0	99.3	100.0	98.8	99.4	99.4	100.0	99.5	100.0	99.5	100.0
14.	TEN-07	100.0	100.0	99.6	99.6	100.0	100.0	99.6	100.0	99.3	99.3	98.6	99.3	98.2	99.4	99.4	100.0	99.5	100.0	99.5	100.0
15.	W4-07	100.0	100.0	99.6	99.6	100.0	100.0	99.6	100.0	97.9	98.6	97.2	98.6	98.2	98.8	98.8	99.4	99.5	100.0	99.5	100.0
16.	BCN-08	100.0	100.0	99.6	99.6	100.0	100.0	99.6	100.0	100.0	100.0	99.3	100.0	98.8	99.4	99.4	100.0	99.5	100.0	99.5	100.0
17.	GNC-08	100.0	100.0	99.6	99.6	100.0	100.0	99.6	100.0	99.3	99.3	98.6	99.3	98.2	98.8	98.8	99.4	99.5	100.0	99.5	100.0
18.	MUR-08	99.6	99.6	99.1	99.6	100.0	100.0	99.6	100.0	100.0	100.0	99.3	100.0	98.8	99.4	99.4	100.0	99.5	100.0	99.5	100.0
19.	MUR-09	99.6	99.6	99.1	99.1	100.0	100.0	99.6	100.0	99.3	100.0	98.6	100.0	98.2	98.8	98.8	99.4	99.5	100.0	99.5	100.0
20.	ToTV-H1	99.6	100.0	99.1	99.6	100.0	100.0	99.6	100.0	100.0	100.0	99.3	100.0	98.8	99.4	99.4	100.0	99.5	100.0	99.5	100.0
21.	ToTV-H2	99.6	100.0	99.6	99.6	100.0	100.0	99.6	100.0	100.0	100.0	99.3	100.0	98.8	99.4	99.4	100.0	98.9	99.5	98.9	99.5
22.	ToTV-H3	100.0	100.0	99.6	99.6	100.0	100.0	99.6	100.0	100.0	100.0	99.3	100.0	97.6	99.4	99.4	100.0	99.5	100.0	99.5	100.0

[a] ToTV-type isolate PRI-0301 GenBank accession number DQ388879.
[b] Polish isolate Wal03 GenBank accession number EU563948.
[c] ToTV-type isolate PRI-0301 GenBank accession number DQ388880.
[d] Polish isolate Wal03 GenBank accession number EU563947.
[e] Identity and similarity percentages of the deduced amino acid sequences calculated with MatGAT (Matrix Global Alignment Tool) version 2.01 (Campanella *et al.*, 2003).

Table 9.4. Average number of nucleotide substitutions among the 19 Spanish, the 3 Hungarian isolates, the ToTV-type isolate PRI-0301 (GenBank accession numbers DQ388879 and DQ388880) and the Polish isolate Wal03 (GenBank Accession number EU563948 and EU563947) for the five genomic regions of the ToTV studied.

Genomic region	d[a]	d_{NS}[a]	d_S[a]	d_{NS}/d_S[a]
Pro-Co (RNA1)	0.0080 ± 0.0013	0.0017 ± 0.0008	0.0219 ± 0.0040	0.0776
RdRp (RNA1)	0.0044 ± 0.0011	0.000 ± 0.0000	0.0115 ± 0.0028	0.0000
MP (RNA2)	0.0102 ± 0.0017	0.0048 ± 0.0016	0.0209 ± 0.0043	0.2296
Vp23 (RNA 2)	0.0117 ± 0.0022	0.0033 ± 0.0014	0.0314 ± 0.0075	0.1051
Vp35 (RNA2)	0.0105 ± 0.0021	0.0024 ± 0.0008	0.0281 ± 0.0060	0.0854

[a]d= nucleotide diversity according to Kimuras´s two-parameter method; d_{NS}= average number of nonsynonymous substitutions per nonsynonymous site; d_S= average number of synonymous substitutions per synonymous site (Pamilo and Bianchi, 1993); d_{NS}/d_S= average ratio between the nonsynonymous and the synonymous substitutions for each pair of comparisons.

DISCUSSION

Plant RNA viruses are believed to have a great potential for genetic variation because their replication process is error-prone since no proofreading correction mechanism has been associated with their RNA-dependent RNA polymerase (Holland *et al.,* 1982). Nonetheless, the large mutability need not result in high genetic variability, and other factors such as natural selection or bottleneck facts can reduce the virus genetic variation (Roosnick, 1997). Studies of the variability and changes in the genetic structure of plant virus populations is an important aspect of plant pathology, and may even be highly relevant for the development of strategies to control virus-induced diseases (García-Arenal *et al.,* 2001).

In this work, we analysed the genetic diversity of five different regions of the genome with different functions of 19 ToTV Spanish isolates collected in the main tomato-growing areas over a 9-year period on a local scale. We also compared those isolates with 3 Hungarian isolates collected over a 2-year period and the available sequences of ToTV isolates published in the GenBank database. A phylogenetic analysis of the five genome areas studied reveals that all the ToTV isolates studied clustered together and separately from ToMarV and ToANV, the two other members of the new genus Torradovirus, as observed in previous studies (Verbeek *et al.,* 2007b; Budziszewska *et al.,* 2008; Pospieszny *et al.,* 2009). Remarkably, ToANV has been recently reported as an strain or isolate within the ToMarV species (Sanfaçon *et al.,* 2009). In this sense, the identity/similarity percentages among all the analysed ToTV (including the ToTV variants from Spain, Poland and Hungary) ranged between 97-100% for all the analysed proteins, but lower than 85% when compared with ToMarV (Budziszewska *et al.* 2008). These viruses have been proposed to constitute a

new genus, Torradovirus, which has also been proposed to belong to the new family Secoviridae, which will also include other genera such as *Sequivirus*, *Waikavirus*, *Comovirus*, *Fabavirus*, *Nepovirus*, *Cheravirus*, *Sadwavirus*, and other tentative viruses of this last genus. All these genera present common properties, such as particle structure (icosahedra with pseudo T=3 symmetry), positive-strand RNA genome that has a polyprotein expression strategy, a common replication block including type III helicase, 3C-like cysteine proteinase, type I polymerase, and they clustered together as a single branch in the Pro-Pol dendogram when compared to other picornavirus members (Sanfaçon *et al.,* 2009).

The genetic diversity within the ToTV isolates was very low (ranging from 0.0044 to 0.0117, Table 9.4), but similar to those reported for other viruses (Janssen *et al.,* 2007; Rubio *et al.,* 2001). There was no evidence of any temporal differentiation on a local scale of the Spanish population as occurs in *Tomato yellow leaf curl Sardinia virus* (TYLCSV, Sánchez-Campos *et al.,* 2002), or *Cucurbit yellow stunting disorder virus* (CYSDV, Marco and Aranda, 2005), or of any geographical differentiation in the studied isolates, in general, as in the Spanish isolates of CYSDV (Rubio *et al.,* 2001), *Tobacco mild green mosaic virus* (TMGMV, Fraile *et al.,* 1996) or *Watermelon mosaic virus* (WMV, Moreno *et al.,* 2004). Unlike the rest of the protein sequences analysed, the two subunits of the CP grouped all the Grand Canary isolates in the same cluster, including one isolate characterised in the weed species *S. nigrum* from Tenerife, an island close to Grand Canary. Although the Grand Canary variants (GNC-04, GNC-05, GNC-06 and GNC-08) and the weed variant from Tenerife (W4-07) grouped together which suggests a common origin, the other two Tenerife isolates from tomato clustered separately with the rest of ToTV isolates studied. The geographic grouping correlated to variation in the CP gene has also been reported for other viruses, such as the WMV Iranian isolates (Sharifi *et at.,* 2008)

and the *Zucchini yellow mosaic virus* (ZYMV) Tunisian isolates (Yakoubi *et al.,* 2008).

All the ToTV genome sequences analysed was the high genetic stability that could be attributed to the negative purify selection addressed to maintain the functional integrity of the viral genome (García-Arenal *et al.,* 2001). The degree of negative selection was estimated by the ratio between the nucleotide diversities in the nonsynonymous and synonymous positions (d_{NS}/d_S), which indicate the amount of variation in the nucleic acid that results in the variation of the encoded protein. For the ToTV population studied herein, this ratio was below the unit for all the coding regions, thus suggesting that all proteins are under high negative selective constraints. Interestingly, the MP genomic region presented a slightly higher value than the two subunits of the CP. However, this variation was not reported to correlate with the function of the encoded protein, and varied largely according to the gene and the virus (García-Arenal *et al.,* 2001).

In addition, the stable ecological conditions for the virus, vector and host plant interaction, such as dominant selection pressure, may contribute to generic stability (García-Arenal *et al.,* 2001), as observed in other whitefly-transmitted viruses such as CVYV(Janssen *et al.,* 2007) or CYSDV (Rubio *et al.,* 2001). The overall variability observed suggests a genetic stability that could be explained, at least in part, by the ecological bottleneck in which host plants and vectors are continuously maintained (Fraile *et al.,* 1996; Sanchez-Campos *et al.,* 2002). On the other hand, a viral population, which shows higher genetic variability, is normally considered to be older, although this may or may not always be consistent (Wei *et al.,* 2009). In this sense, the adaptation of a plant virus to a new resistant host or species implies specific molecular changes that confer some advantages to the new host (Jenner et al. 2002; Wallis *et al.,* 2007). A

recent example of fast host-specific adaptation is the convergent evolution of *Pelargonium flower break virus* (PFBV) populations adapted to *Chenopodium quinoa* (Rico *et al.,* 2006). In this example, PFBV incorporated five specific non contiguous amino acids in the CP when it was maintained in the *C. quinoa* host. ToTV has been recently detected in tomato crops and some associated weed species (Alfaro-Fernández *et al.,* 2008b), and has not spread widely in many countries. Remarkably, this virus seems to be adapted mainly to the *Solanaceae* species (Pospieszny et al., 2009), although naturally infects some weed species of different families (Alfaro-Fernández *et al.,* 2008b). The high sequence stability observed in the coding regions could be interpreted as ToTV having already adapted to the tomato host. However, we observed a significant grouping of the Grand Canary isolates together with a *Solanaceae* weed variant, in which some residues are specific for all the group components, such as A1051 in the Vp23, or others like G302 (W4-07 and GNC-08) and Y303 (W4-07, GNC-08, GNC-04) in the MP or the positive selected site V-I 291 in the Pro-Co that contains a specific I amino acid for the W4-07 and GNC-08 isolates. This observation raises two different possibilities: i) that a putative ToTV from a weed host could evolve into the new tomato host, or vice versa, and ii) if this were the case, then we can speculate a putative origin of the recent new ToTV in tomato crops. We are currently checking this possibility by analysing the stability of the ToTV progeny in the different weed and/or tomato hosts. In addition, it would be very interesting to study the new emerging populations of this virus which could become a problem for tomato production worldwide.

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Capítulo 10

Discusión general

DISCUSIÓN GENERAL

La gran complejidad fitosanitaria de un cultivo como el tomate, tan importante desde el punto de vista económico, plantea la necesidad de estudiar los agentes patógenos que le afectan, para poder establecer medidas de control eficaces que minimicen las pérdidas ocasionadas. El presente trabajo se ha centrado principalmente en el estudio de diversos aspectos de las enfermedades causadas por el virus del mosaico del pepino dulce (PepMV) y el virus del torrado de tomate (ToTV), dos agentes que afectan reiteradamente a este cultivo en España desde su identificación. La elección de estos dos virus en el presente estudio se debe a la importancia relativa que presentan en los cultivos protegidos españoles. El PepMV está ampliamente distribuido por nuestro país y presenta una gran variabilidad tanto sintomatológica como genética, apareciendo continuamente nuevos aislados que difieren entre sí por alguna de estas características. Asimismo su eficaz transmisión mecánica y por semilla plantea un riesgo elevado tanto de introducción en nuevas áreas como de una rápida extensión del virus. Por otra parte, el ToTV, agente causal de la enfermedad conocida como "torrao" del tomate es un virus de reciente caracterización, aunque sus síntomas se vienen observando desde 2001. En un primer momento, y antes de la identificación de su agente causal, la enfermedad se asoció a la presencia de PepMV debido a la continua vinculación de los síntomas con la detección de este virus en las plantas afectadas. La detección de este nuevo virus de tipo "picorna" planteó la necesidad de ampliar el conocimiento de su epidemiología, diversidad genética e interacción con otras entidades virales que comúnmente afectan al tomate en España.

En primer lugar, como ya se ha indicado, el PepMV presenta una gran variabilidad molecular, habiéndose diferenciado cinco genotipos muy diferentes entre sí (Ling, 2007), los cuales pueden infectar simultáneamente a una misma planta, apareciendo en ocasiones fenotipos mucho más agresivos (Hanssen *et al.,* 2009b). A tal efecto, disponer de un método de diagnóstico rápido y eficaz para la identificación del genotipo presente en una planta infectada con PepMV sería un aspecto prioritario para realizar estudios de distribución del virus, correlación de genotipos con fenotipos concretos, o incluso, para el diagnóstico rutinario de muestras de campo. Por tanto, en primer lugar se desarrolló en este trabajo un método de diagnóstico capaz de detectar simultáneamente en una muestra infectada los cinco genotipos de PepMV. El método consistía en la realización de una multiplex RT-PCR con una mezcla de seis parejas de cebadores que amplificaron un fragmento del gen de la RNA polimerasa RNA dependiente, así como un control interno, lo cual permitió la identificación de tres grupos de genotipos de PepMV: EU/PE, CH1/US1, CH2/US2. Con el posterior análisis de restricción con la endonucleasa *SacI* realizado a los productos de PCR obtenidos de los distintos grupos se consiguió la separación de cinco genotipos de PepMV: EU, PE, CH1/US1, CH2 y US2.

El desarrollo de este nuevo método supone un avance importante en el diagnóstico del PepMV, ya que hasta el momento únicamente existían dos métodos para la identificación concreta de genotipos del virus sin necesidad de recurrir a la secuenciación del fragmento amplificado. Estos métodos previos se basan en la realización de una RT-PCR seguida del análisis mediante RFLP del fragmento obtenido. Martínez-Culebras *et al.* (2002) discriminaron entre tres tipos de aislados (P1, P2, P3) al realizar una RT-PCR con cebadores específicos del gen de la RdRp y su posterior digestión con *Sau*3A. Sin embargo, la aparición de nuevos genotipos del virus desbancó esta técnica, que era incapaz de detectarlos. Hanssen *et al.* (2008) consiguieron diferenciar los cinco

genotipos de PepMV mediante la amplificación de fragmentos de dos regiones diferentes del genoma del virus (RdRp y CP), realizando dos reacciones de RT-PCR con cebadores específicos de cada zona del genoma. Posteriormente, los fragmentos amplificados se digerían con seis enzimas de restricción diferentes. A la vista de estos estudios previos, el método desarrollado en este trabajo presenta una clara ventaja, ya que reduce significativamente el proceso de análisis, permitiendo además generar un resultado de diferenciación previo sólo con la reacción de RT-PCR multiplex, ya que discrimina entre los grupos EU/PE, CH1/US1 y CH2/US2. Durante este ensayo, al comparar el nuevo método diseñado y el método de RT-PCR-RFLP descrito por Hanssen *et al.* (2008), el primero presentó no sólo una mayor sensibilidad en la identificación del genotipo CH1/US1, sino también una mayor especificidad en la caracterización de algunos aislados del genotipo CH2, los cuales no pudieron ser clasificados mediante el método de RT-PCR-RFLP de Hanssen *et al.* (2008), debido a la variación de un nucleótido en el sitio de restricción diferenciador del genotipo en el gen de la CP.

El método resultó ser altamente específico incluso en infecciones múltiples, presentando a su vez un límite de detección 3125 veces mayor que el ELISA o la hibridación molecular, valor que se encuentra dentro del rango descrito para estas técnicas (Saade *et al.,* 2000; Sánchez-Navarro *et al.,* 1998; Sánchez-Navarro *et al.,* 2006). El análisis de muestras de campo reveló la presencia de un gran porcentaje de muestras con infecciones mixtas de aislados pertenecientes a los genotipos EU con CH2 o CH1, representativos de dos regiones geográficas diferentes (Murcia y las Islas Canarias, respectivamente). En este estudio, se presentó una distribución mayoritaria de los genotipos EU y CH2, al igual que en otros países europeos, donde se ha experimentado un incremento en la distribución del genotipo CH2, en detrimento del EU establecido hasta el momento (Hanssen *et al.,* 2008; 2009b). La presencia del

genotipo CH1/US1 en las Islas Canarias, recientemente determinada (Alfaro-Fernández *et al.,* 2008a), fue confirmada al encontrarse éste en un 25% de las muestras de campo analizadas recogidas en esta región. El método descrito supone un gran avance por el ahorro de costes y tiempo, evitando el uso de digestiones múltiples o la secuenciación. Además facilita la caracterización del estado fitosanitario del tomate determinando la presencia de infecciones simples o múltiples, y la introducción de nuevos genotipos en diferentes áreas.

En segundo lugar, dado que no se ha determinado aún qué región o regiones del genoma del PepMV son las responsables de la expresión de los síntomas (Hanssen *et al.,* 2008). La producción de clones infecciosos de virus vegetales permite el análisis detallado de la estructura-función del virus en la planta. En el presente trabajo, la secuencia genómica completa de un aislado del genotipo CH2 se ha clonado bajo el control del promotor de la RNA polimerasa T7, denominándose la construcción pUS2-CSL. La estrategia empleada fue similar a la descrita por Hasiow-Jarowzeswska *et al.* (2009a) para la obtención de un clon infeccioso de un aislado necrótico de PepMV perteneciente al mismo genotipo. Los RNA transcritos obtenidos *in vitro* a partir de dicho clon se inocularon a plantas de *N. occidentalis* 37b, reproduciéndose la misma sintomatología que el aislado original. La construcción de dicho clon de PepMV se estableció como punto de partida para la introducción del gen de la proteína de fluorescencia verde sintética adaptada a plantas (SGFP). El clon de PepMV mutado con GFP (pUS2-CSL:GFP) se construyó gracias a la inserción de este gen en la región intergénica existente en el genoma del virus entre las regiones codificantes del TGB y la CP. En la inserción del gen ajeno, se empleó la PCR para fusionar fragmentos independientes que poseían 15 pb de solape entre ellos, generando finalmente un único amplicón que contenía la unión de tres fragmentos. Este método que modifica ligeramente el descrito hasta el momento (Hobert, 2002; Charlier *et al.,* 2003), resultó efectivo para la fusión de

fragmentos de PCR suponiendo un ahorro de tiempo en los análisis y permitiendo la inserción de fragmentos independientemente de la existencia de sitios de restricción (Boulin *et al.,* 2006). Esta técnica podría ser aplicada en un futuro para la introducción de otros genes ajenos en el genoma de PepMV, así como en estudios de recombinación con distintos genotipos del virus. Los transcriptos generados *in vitro* a partir del clon de PepMV mutado con GFP (pUS2-CSL:GFP) están siendo estudiados en este momento, ya que en un ensayo previo no resultaron infectivos tras ser inoculados en *N. occidentalis* 37b, al igual que ocurrió con los transcritos generados del clon de PepMV pUS2-CSL, empleado como control. El éxito de este tipo de clones marcados con proteínas fluorescentes, supondría una herramienta eficaz para visualizar aislados de distintos genotipos del virus marcados diferencialmente.

En otro orden de cosas, dentro de la gran variabilidad sintomatológica que se produce en plantas de tomate infectadas con PepMV, se ha asociado la manifestación del síndrome conocido como "colapso" del tomate con la presencia de este virus y el hongo vector *O. brassicae* sl (Córdoba *et al.,* 2004b). A pesar de haberse demostrado dicha asociación, no se había estudiado hasta el momento la posible transmisión del virus mediante dicho hongo vector, responsable de la transmisión de otros virus que causan enfermedades importantes en otras especies hortícolas (Rochon *et al.,* 2004; Sasaya y Koganezawa, 2006). Por tanto, otro de los aspectos abordados en el presente trabajo fue comprobar la capacidad del hongo vector *O. brassicae* sl (concretamente *O. virulentus*) de transmitir PepMV. Como se ha descrito anteriormente se ha utilizado la nueva demarcación de especies del género *Olpidium*, en la cual se determina que la especie tradicionalmente denominada *O. brassicae* sl estaría actualmente constituida por dos especies diferenciadas: *O. brassicae* que, correspondería a la cepa "crucífera" cuyo rango de hospedantes se restringe a dicha familia botánica y se caracteriza por necesitar el

apareamiento entre zoosporas para el desarrollo de esporas de resistencia, y *O. virulentus* que constituiría la cepa "no crucífera" y no requeriría de la reproducción sexual para continuar su ciclo (Koganezawa *et al.,* 2005; Sasaya y Koganezawa, 2006). En dos replicaciones distintas, se demostró la capacidad de *O. virulentus*, originario de un cultivo de tomate, para transmitir PepMV a plantas de tomate sanas regadas con el agua de drenaje recogida de plantas infectadas con ambos agentes. La tasa de transmisión obtenida en el ensayo fue del 8% en ambas repeticiones que, a pesar de resultar baja comparada con la de otros virus transmitidos por las diversas especies de este hongo vector (Lot *et al.,* 2002; Campbell y Lot, 1996; Tomlinson y Thomas, 1986), en este caso puede ser suficiente para una rápida expansión del virus en el cultivo debida a la gran eficacia de éste en la transmisión mecánica. No obstante, el virus nunca se transmitió a plantas sanas regadas con el percolado obtenido del riego de plantas únicamente infectadas con PepMV, lo que pone de manifiesto que el virus no se transmite con el agua de riego sin la presencia del vector, contrariamente a los resultados obtenidos en otros estudios que determinan la distribución del virus en sistemas hidropónicos (Fakhro *et al.,* 2005; Schwarz *et al.,* 2007). Asimismo, *O. virulentus* se detectó mediante multiplex-PCR (Herrera-Vásquez *et al.,* 2009b) en el agua de drenaje obtenida tras el riego de plantas infectadas con dicho agente, mientras que PepMV estaba presente únicamente en las muestras de agua de drenaje procedente de plantas infectadas con el virus y el hongo vector. Este resultado confirma de nuevo la necesidad de ambos agentes para que se produzca la transmisión del virus en estas condiciones. Al producirse la transmisión únicamente con el cultivo del hongo procedente de tomate se reafirmó la alta especificidad que se presenta en las especies de *Olpidium* spp. entre el aislado fúngico, el hospedante y el virus transmitido (Campbell y Sim, 1994; Campbell *et al.,* 1995; Koganezawa *et al.,* 2005; Temmink *et al.,* 1970). La contaminación de *O. virulentus* en sistemas de irrigación es posible al

haberse descrito la presencia de otra especie (*O. bornovanus*), vector eficiente de MNSV en balsas de riego en Almería (Gómez y Velasco, 1991). Todos estos resultados ponen de manifiesto la necesidad de controlar la presencia de *Olpidium* spp. en agua de riego o en el suelo, ya que puede favorecer la aparición del síndrome de "colapso" en campos infectados con PepMV, y presentar además un riesgo potencial de transmisión del virus en el cultivo.

Otro problema planteado fue profundizar en el conocimiento del recientemente caracterizado virus del torrado del tomate (ToTV) como agente causal de la enfermedad conocida como "torrao". En este sentido, se estudiaron su sintomatología concreta, la expansión del virus en España, la co-infección de las plantas infectadas con otros virus que afectan al tomate, entre ellos el PepMV con el cuál se había indicado anteriormente su posible asociación (Alfaro-Fernández *et al.,* 2007b), el posible sinergismo entre estos agentes virales, las técnicas para su diagnóstico y su variabilidad molecular.

Primeramente se realizaron muestreos en las principales áreas productoras de tomate desde 2001 a 2008, para determinar la incidencia y distribución de la enfermedad del "torrao", así como la presencia de ToTV en dichas zonas. En ellos, se evaluó la sintomatología manifestada por las plantas y a su vez se analizaron frente a ToTV y PepMV las muestras recogidas, determinando en este último el genotipo concreto presente en la muestra según el método de multiplex RT-PCR comentado anteriormente (Alfaro-Fernández *et al.,* 2009b). Los resultados determinaron la presencia del nuevo virus en plantas de tomate recogidas en Alicante, Almería, Barcelona, Gran Canaria, Mallorca, Murcia y Tenerife. La incidencia fue variable según los años, pero al estudiarla dentro de un mismo invernadero afectado, el porcentaje de plantas podría representar hasta un 37%. La observación de síntomas determinó que, aunque la gran mayoría de plantas que manifestaba los síntomas típicos de la enfermedad (94,4%) eran positivas al ToTV, existía un porcentaje importante de plantas

infectadas que presentaban otros síntomas necróticos (53,3%) e incluso no manifestaban necrosis (40,5%). Por tanto, no existe una correlación completa entre la sintomatología y la infección con ToTV, presentándose además un elevado número de plantas que aún no han manifestado los síntomas de la enfermedad. Generalmente la severidad y manifestación de síntomas ocasionados por infecciones virales depende de múltiples factores como son el aislado, el cultivar, el estado de la planta y las condiciones ambientales (EPPO, 2004). En general, la determinación de los síntomas en campo puede proporcionar una primera información sobre la etiología concreta de la infección, sin embargo no es lo suficientemente específica, ya que diversos virus pueden producir una sintomatología similar como claramente ocurre en el caso de las necrosis (Córdoba-Sellés *et al.,* 2007a).

En este estudio, se identificaron diferentes virus (TSWV, PVY, PMoV, CMV, ToCV, TYLCV) en plantas con necrosis diversas en la parte aérea. Asimismo, se detectó la presencia de infecciones mixtas en campo, encontrándose la asociación de PepMV y ToTV en el 46,7% de las plantas analizadas. De las plantas infectadas con ToTV más de la mitad presentaban también PepMV (60,5%). Se encontraron asimismo plantas coinfectadas con ToTV y otros virus como ToCV, TSWV, ToMV y TYLCV, aunque en una proporción mucho menor. Sin embargo, los análisis realizados a estos otros virus iban dirigidos en función del síntoma observado, es decir, únicamente se analizaron aquellas plantas sospechosas de estar infectadas; de esta forma puede que la proporción fuera mayor que la observada. No se encontró ningún genotipo concreto de PepMV asociado a la infección con ToTV, al contrario de lo que ocurría en ensayos previos en Murcia donde en las plantas infectadas se encontraban mayoritariamente ToTV y el genotipo CH2 de PepMV (Alfaro-Fernández *et al.,* 2007b). Aunque el estudio estaba dirigido a ampliar la información sobre la enfermedad del "torrao", los muestreos realizados sirvieron

para confirmar de nuevo el un incremento de la presencia del genotipo CH2 de PepMV en detrimento del EU, mayoritariamente extendido hasta entonces (Pagán *et al.,* 2006), asociándose a una posible ventaja biológica del primero de ellos (Hanssen *et al.,* 2008). A pesar de la reciente descripción de virus relacionados con el ToTV en México (ToMarV y ToANV), los análisis realizados a muestras que presentaban sintomatologías similares a las descritas para estas nuevas virosis (Turina *et al.,* 2007), resultaron negativos en todos los casos. Sin embargo, no se descarta la futura introducción de estos nuevos patógenos procedentes de su país de origen, así como la del nuevo virus Tomato chocolate spot virus (ToCsV) descrito en Guatemala (Kuo *et al.,* 2009). Este hecho pone de manifiesto la necesidad de evaluaciones periódicas de los cultivos españoles de tomate sospechosos, para detectar la entrada de estos nuevos patógenos y poder ampliar medidas de control para evitar su expansión por el país.

Por otro lado, se comprobó la gran eficacia de la transmisión de ToTV por la mosca blanca *T. vaporariorum* a diversas especies de solanáceas, describiéndose por primera vez como hospedante *D. stramonium*, aunque estudios previos habían determinado *D. inoxia*, especie del mismo género, como hospedante del virus (Pospieszny *et al.,* 2009). Asimismo se verificó la dependencia del cultivar de tomate empleado en la transmisión del virus, determinada anteriormente por Budziszewska *et al.* (2008). En este estudio los cvs. Boludo, Cedrico y 1123 fueron susceptibles al virus, y sin embargo el cv. Marmande resultó claramente resistente. La presencia en homocigosis de un alelo recesivo de un gen del tomate confiere resistencia natural a la planta frente a la infección con este virus (Maris *et al.,* 2007).

Por otra parte, se evaluó con éxito la fiabilidad del método de diagnóstico de hibridación molecular con sonda no radiactiva complementaria a un fragmento de la ORF2 del RNA2 del virus que contenía la proteína de

movimiento, elegida por ser una zona conservada del genoma del virus, tanto aplicando sobre la membrana el RNA extraído del tejido infectado, como la impresión directa de peciolos de hoja sobre la membrana. Este método permite la evaluación del estado fitosanitario del tomate y presenta una clara ventaja por el ahorro de tiempo y la simplificación en el manejo y procesado de las muestras de campo (Aparicio *et al.,* 2008; Galipienso *et al.,* 2005). Simultáneamente, se muestrearon y analizaron en 2007, especies de la flora arvense que se encontraban habitualmente en los invernaderos de tomate de Murcia, Gran Canaria y Tenerife, para determinar posibles reservorios de ToTV, determinándose su presencia en muestras pertenecientes a especies y géneros de diversas familias botánicas (*Amaranthaceae*, *Caryophyllaceae*, *Chenopodiaceae*, *Cruciferae*, *Malvaceae*, *Polygonaceae* y *Solanaceae*). Se establece por tanto, la necesidad de tener en cuenta el manejo de las poblaciones de malas hierbas en los invernaderos de tomate y zonas colindantes dentro del diseño de estrategias de control de esta enfermedad.

En este mismo orden de cosas se procedió a la evaluación el efecto citopatológico de la infección de ToTV en plantas afectadas por "torrao", así como a la comparación del efecto ultraestructural de las infecciones simples de este virus respecto a las mixtas con otros virus que comúnmente afectan al cultivo del tomate, encontradas en plantas afectadas por dicha enfermedad. La infección simple con ToTV indujo alteraciones celulares a nivel de los tejidos del floema, observándose en las células parenquimáticas de estos tejidos vasculares las típicas inclusiones cristalinas formadas por partículas virales de unos 20 a 30 nm, características de este virus (Pospieszny *et al.,* 2009). Sólo algunas muestras presentaron mitocondrias hipertrofiadas y una desorganización citoplasmática manifiesta, aunque en general las mitocondrias y cloroplastos no manifestaban ninguna alteración evidente. Sin embargo, las co-infecciones de ToTV con otros virus presentaron efectos citopáticos más acusados, como en el

caso de ToTV y ToCV, donde además de observarse las masas de agregados tanto de partículas isométricas como flexuosas, típicas de cada uno de estos virus, las células revelaban mitocondrias hipertrofiadas, engrosamiento de la pared celular y deposiciones de calosa en las células de compañía, alteraciones típicas de otros *Crinivirus* (Medina *et al.,* 2003). Estas dos últimas alteraciones celulares hacían que la acumulación de estos dos virus en el floema fuera mayor, restringiendo tanto su movimiento célula a célula (Francki *et al.,* 1985), así como alterando la posible distribución de otros virus como se observó en infecciones triples con PepMV, donde se redujeron los efectos patogénicos de la infección de éste en otros tejidos. En cambio, en el caso de coinfección entre PepMV y ToTV, no se observó engrosamiento alguno de la pared celular, pero los tejidos presentaron vesiculación citoplasmática e hipertrofia de mitocondrias y cloroplastos, típicas de infecciones causadas por virus (Šutić y Sinclair, 1991). Sin embargo, los tejidos que presentaron efectos más perjudiciales por la infección fueron aquellos con infecciones triples de PepMV, ToTV y TSWV, donde la alteración más evidente fue el engrosamiento de la pared celular tanto en las células de compañía, como en las células parenquimáticas del floema y en las células de la vaina. En este estudio se observó claramente un efecto sinérgico a nivel ultraestructural del ToTV con otras infecciones virales. El tomate es un cultivo habitualmente susceptible a la presencia de infecciones virales múltiples y en él se han determinado importantes sinergismos como es el caso del ToCV y el TSWV, que puede llevar incluso a la ruptura de la resistencia de la planta a este segundo agente (García-Cano *et al.,* 2006).

Por último, en este trabajo se abordó el estudio de la variabilidad molecular de una población de diecinueve aislados españoles de ToTV recogidos durante nueve años distintos en diversas zonas productoras españolas así como tres aislados húngaros del virus. Los aislados de ToTV tanto españoles como húngaros se agruparon conjuntamente en un grupo claramente

diferenciado del constituido por ToMarV y ToANV, al igual que se observó en otros estudios con otros aislados de este virus (Verbeek *et al.,* 2007a; Budziszeswska *et al.,* 2008; Porpieszny *et al.,* 2009). La diversidad genética de los aislados de ToTV fue muy baja, como ocurre en otros virus (Janssen *et al.,* 2007; Rubio *et al.,* 2001). A pesar de no manifestarse ninguna diferenciación en función del año de recolección del aislado, sí se observó un agrupamiento de aislados debido a su origen geográfico en el caso de los procedentes de Gran Canaria, que se englobaban en un subgrupo en las dos subunidades de la CP estudiadas. Este fenómeno de agrupación por procedencia geográfica debido a la variación de la CP ha sido observado en WMV y ZYMV (Sharifi *et al.,* 2008; Yakoubi *et al.,* 2008). En este subgrupo de aislados de Gran Canarias se incluyó también un aislado procedente de *S. nigrum* recogido en Tenerife en 2007, que comparte junto con algunos de los aislados de este grupo, residuos específicos como A1051 en la subunidad de la CP Vp23, G302 e Y303 en la MP, o incluso el único sitio determinado que presenta presión de selección positiva en la zona Pro-Co del RNA1 (V-I 291). Este resultado podría deberse entre otras posibilidades a la evolución de un aislado de ToTV adaptado a esta mala hierba para infectar a tomate, o viceversa, fenómeno ya observado en aislados de *Pelargonium flower break virus* (PFBV) que con cinco aa incorporados a su CP, presentaban una adaptación estable a *C. quinoa* (Rico *et al.,* 2006).

La estabilidad genética observada en las poblaciones de este virus se encuentra favorecida por las condiciones ecológicas estables tanto del virus, como de su vector y la interacción con su hospedante (García-Arenal *et al.,* 2001). Sin embargo, a pesar de que se considera que los virus tienen un gran potencial para generar variabilidad genética debido a su gran facilidad de mutación, la selección natural o los fenómenos de cuello de botella reducen las variaciones genéticas (Holland *et al.,* 1992; Roosnick, 1997).

En definitiva todos los resultados recogidos en el presente trabajo han permitido avanzar y profundizar en el conocimiento y detección del virus del mosaico del pepino dulce (PepMV), ampliamente extendido tanto en España como en otros países de Europa y América del Norte. Además, ha sido posible esclarecer diversos aspectos del recientemente caracterizado virus del torrado del tomate (ToTV), agente responsable de la enfermedad del "torrao" en España, que pueden contribuir al desarrollo de estrategias de diagnóstico y control de dicha enfermedad.

Capítulo 11

Conclusiones

Conclusions

CONCLUSIONES

A lo largo de los distintos capítulos de este trabajo se han obtenido una serie de conclusiones que se enumeran de forma resumida a continuación:

1.- El método de detección simultánea de aislados de PepMV mediante multiplex RT-PCR con seis parejas de cebadores que amplifican un fragmento del gen de la RNA polimerasa RNA dependiente así como un control interno, permite la identificación de tres grupos de genotipos de PepMV: EU/PE, CH1/US1 y CH2/US2. Con el posterior análisis de restricción con la endonucleasa *SacI*, realizado a los productos de PCR obtenidos de los distintos grupos, se consigue la separación de cinco genotipos de PepMV: EU, PE, CH1/US1, CH2 y US2.

2.- El método de multiplex RT-PCR presenta un límite de detección igual al obtenido por el análisis mediante RT-PCR con una única pareja de cebadores específicos y, al compararlo con el de las técnicas DAS-ELISA e hibridación molecular es hasta 3125 veces más sensible, permitiendo así el diagnóstico de un mayor número de positivos en muestras de campo.

3.- El método de diagnóstico de multiplex RT-PCR seguido del análisis de restricción con el enzima *SacI* presenta una mayor sencillez, especificidad y sensibilidad que el método de RT-PCR-RFLP descrito por Hanssen *et al.*, (2008), sobre todo en la detección del genotipo CH1/US1.

4.- El análisis de las muestras de campo con el nuevo método de diagnóstico mediante multiplex RT-PCR seguido de la restricción con *SacI* revela la

presencia habitual de infecciones mixtas en las muestras infectadas con PepMV, encontrándose mayoritariamente los genotipos EU y CH2.

5.- Se ha generado un clon de cDNA de un aislado del genotipo CH2 de PepMV, a partir del cual se sintetizan trascritos *in vitro* que resultan infecciosos tras su inoculación mecánica a plantas de *N. occidentalis* 37b.

6.- El clon de cDNA generado se ha empleado para la construcción de un clon de cDNA mutado con el gen de la proteína de fluorescencia verde (GFP) insertada en la segunda región intergénica del genoma del virus, entre los genes del TGB y la CP. El método de fusión mediante PCR, que consiste en generar un amplicón único a partir de la unión de fragmentos individuales que contienen 15 pb de solape con el adyacente, se presenta como una herramienta sencilla y eficaz durante la construcción del clon mutado. Los transcritos *in vitro* generados a partir de esta construcción están siendo estudiados en este momento para comprobar su infectividad.

7.- La transmisión de PepMV mediante el hongo vector *O. virulentus* procedente de un cultivo de tomate es posible bajo condiciones controladas de cámara de cultivo, consiguiéndose una tasa de transmisión del 8% tras el riego de plantas sanas con el percolado de lotes de plantas infectadas con PepMV cuyas raíces contenían *O. virulentus* procedente de tomate.

8.- *O. virulentus* y PepMV se detecta al procesar y analizar el agua de drenaje obtenida tras el riego de plantas infectadas, aunque solamente resultan positivas al virus aquellas muestras de agua de drenaje recogidas de plantas infectadas con ambos agentes, y nunca en agua de drenaje procedente de plantas infectadas solo con PepMV.

9.- El ToTV ha sido detectado en muestras recogidas en Alicante, Almería, Barcelona, Gran Canaria, Mallorca, Murcia y Tenerife, presentándose una incidencia variable según los años. La mayor parte de las plantas que presenta los síntomas típicamente asociados a la enfermedad del "torrao" del tomate son positivas a ToTV, sin embargo existe un porcentaje de plantas con síntomas necróticos o asintomáticas que también están infectadas por el virus.

10.- El ToTV se encuentra habitualmente en infección mixta con PepMV, y en ocasiones con otros virus habitualmente encontrados en invernaderos de tomate como, por ejemplo, ToCV o TSWV.

11.- El ToTV se transmite de manera eficaz a diversas especies de solanáceas, entre ellas, *D. stramonium* que se describe por primera vez como hospedante del virus. El tomate presenta una respuesta variable a la infección en función del cultivar ensayado, resultando los cv. Boludo, Cedrico y 1123 susceptibles al virus, mientras que el cv. Marmande presenta resistencia a la infección. Los análisis realizados a flora arvense encontrada en invernaderos de tomate revela la existencia de reservorios del virus entre las especies y géneros *Amaranthus* sp., *Spergularia* sp., *Atriplex* sp., *C. ambrosioides*, *Chenopodium* sp., *H. sativus*, *S. didyma, Malva* sp., *Polygonum* sp., *N. glauca* y *S. nigrum*.

12.- La hibridación molecular con sonda no radiactiva complementaria a un fragmento de la ORF2 del RNA2 del virus que contiene la proteína de movimiento resulta un método eficaz y fiable para el diagnóstico de ToTV en muestras de campo tanto al analizar el RNA extraído de los tejidos vegetales, como al realizar improntas directas de peciolos de hoja en la membrana.

13.- La infección de los tejidos foliares de tomate con ToTV presenta alteraciones celulares fundamentalmente a nivel del floema, observándose en las

células parenquimáticas de estos tejidos vasculares las típicas inclusiones cristalinas formadas por sus partículas virales icosaédricas de 20 a 30 nm.

14.- Se produce un efecto sinérgico a nivel ultraestructural en las infecciones mixtas de ToTV con otros virus, como es el caso de las infecciones dobles de este virus con ToCV o PepMV, y las infecciones triples de PepMV, ToTV y TSWV. En estos casos, las alteraciones más evidentes son engrosamientos de la pared celular en las infecciones mixtas de ToTV y ToCV, así como de ToTV, PepMV y TSWV, acompañadas en el primero de los casos con deposiciones de calosa. En cambio, la infecciones dobles de PepMV con ToTV no producen este efecto, aunque sí se manifiestan vesiculaciones citoplasmáticas e hipertrofia de mitocondrias y cloroplastos.

15.- El estudio de cinco regiones del genoma (Pro-Co, RdRp, MP, subunidades Vp23 y Vp35 de la CP) de dos poblaciones, española y húngara, de aislados de ToTV determina que todos los aislados del virus se agrupan de manera conjunta en los análisis filogenéticos de todas las zonas, no observándose correlación entre el año de recolección y la diversidad genética. Sin embargo, existe una cierta relación entre la diversidad genética y el origen geográfico en las dos subunidades de la CP estudiadas, donde se presenta un subgrupo que engloba los aislados muestreados durante distintos años en Gran Canaria, así como un aislado procedente de Tenerife recogido en 2007 de planta de *S. nigrum*.

16.- Las zonas más variables del genoma de ToTV se presentan en las tres regiones del RNA2 estudiadas: MP, Vp23 y Vp35. No obstante, todas las zonas estudiadas del genoma presentan una presión de selección negativa, encontrándose nueve puntos con presión de selección negativa distribuidos en Pro-Co (S74, S212 y T301), MP (I148, G156 y G244), Vp23 (E1151) y Vp35 (R469 y A581), y únicamente uno con presión de selección positiva en la zona Pro-Co (V-I 291).

CONCLUSIONS

Some general conclusions have been obtained in the different chapters of the present Thesis and are briefly listed as follows:

1. The multiplex RT-PCR method with a mix of six primers which amplified a fragment of the RNA-dependent RNA polymerase gene of PepMV genome, plus an internal control, allowed the detection of three groups of PepMV genotypes: EU/PE, CH1/US1 y CH2/US2. Restriction analysis with the *SacI* endonuclease to the PCR obtained products permited the identification of the five described PepMV genotypes: EU, PE, CH1/US1, CH2 y US2.

2. No differences in terms of detection limit were observed when a serially-diluted double-infected sample was analysed by either the multiplex (containing a cocktail of six primers) or the single (containing two primers) RT-PCR. Comparison between the analyses of the serially-diluted samples by multiplex RT-PCR and ELISA or dot-blot hybridization revealed a detection limit of 3125 times higher for the multiplex reaction, allowing the detection of a large number of positives in field samples.

3. Multiplex RT-PCR detection method followed by the restriction analysis with *SacI* is easier and more sensitive that the RT-PCR-RFLP method described by Hanssen *et al.* (2008), overall in the detection of the CH1/US1 genotype.
4. Field samples collected in different Spanish greenhouses analysed by the new multiplex RT-PCR-*SacI* approach revealed the usual presence of mixed infections of different PepMV genotypes, mostly EU and CH2.

5. A full-length cDNA clone of PepMV was constructed and the derived RNA transcripts resulted infectious when mechanically inoculated to *N. occidentalis* 37b.

6. This infectious full-length clone of PepMV was used for the construction of a mutant cDNA clone of PepMV with the green fluorescent protein encoding-gene (GFP) inserted in the intergenic region between the TGB and CP genes. Fusion PCR method, which consists in generating a single amplicon from the joint of different individual fragments that contain 15-bp overlaps in both prime ends, resulted an easy and effective tool used for the construction of the GFP mutant clone. In vitro RNA transcripts generated from this construction is now under study.

7. Transmission of PepMV by the fungal vector *O. virulentus* isolated from soil of tomato crops was possible under controlled conditions, with a transmission rate of 8% after irrigating healthy plants with the drainage water collected from PepMV-infected plants whose roots contained the fungal culture from tomato.

8. Both the virus and the fungus were detected in water samples collected from the drainage water obtained after watering infected plants, however the virus was only detected in those water samples that present infection of both agents, and never in drainage water of plants just infected with PepMV.

9. ToTV was present in samples collected in Alicante, Almería, Barcelona, Gran Canaria, Mallorca, Murcia y Tenerife, although the incidence was variable depending on the year. The majority of the positive samples to ToTV showed typical symptoms of the 'torrado' disease, however some plants with different necrosis symptoms and even asymptomatic plants resulted infected with the virus.

10. Co-infection of ToTV and PepMV occured in a large number of samples, and several samples were infected with other tomato-infecting-viruses, as ToCV o TSWV.

11. ToTV was efficiently transmitted by whitefly *T. vaporariorum* to different *Solanaceae* species, being *D. stramonium* reported as host for the first time. Tomato infection in variety-dependent, whereas cv. Boludo, Cedrico and 1123 were susceptible, cv. Marmande results resistant to the virus. Weed species belonging to *Amaranthus* sp., *Spergularia* sp., *Atriplex* sp., *Chenopodium* sp., *C. ambrosioides*, *H. sativus*, *S. didyma, Malva* sp., *Polygonum* sp., *N. glauca* and *S. nigrum* collected in tomato greenhouses were ToTV hosts.

12. Dot-blot and tissue printing hybridization with a complementary probe to a fragment of the ORF2-RNA2 of ToTV was demonstrated to be a reliable technique which could facilitate the routine diagnosis of ToTV in field samples.

13. Leaves samples single-infected with ToTV presented a cellular alteration more at the phloem level than in other kinds of tomato leaf tissues. Crystalline arrays of isometric virus-like particles of 20-30 nm in the inclusion bodies were observed in phloem parenchyma cells.

14. An ultrastructural synergism effect was observed in mixed infections of ToTV and other viruses. The cytological effects of the infection were more evident in tissues double-infected with ToTV and ToCV or triple-infected with ToTV, PepMV and TSWV, where a cell overgrowth observed. However, double-infections of PepMV and ToTV presented vesiculations in the cytoplasm and mitocondria and chloroplast hypertrophy.

15. The phylogenetic analysis of five regions of the virus genome (Pro-Co, RdRp, MP, subunidades Vp23 y Vp35 de la CP) of Spanish and Hungarian populations determined that all studied ToTV isolates cluster together, but separately of other virus species as ToMarV. However, some genetic diversity was observed in case of the two subunits of the CP studied among the Gran Canary isolates and the rest of ToTV isolates analysed that grouped together. In this sub-group an isolate from *S. nigrum* collected in Tenerife in 2007 was also grouped in the phylogenetic analyses.

16. The most variable regions of the ToTV genome were the three coding regions on the RNA2: MP, Vp23 and VP35. All the studied zones present a negative selected pressure. Specifically, nine negatively selected sites distributed in Pro-Co, MP, Vp23 and Vp35 and just one site in the Pro-Co under positive selection for all the genome regions studied were found.

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