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Emerging viruses infecting solanaceous crops: genetic characterization of the pepper vein yellows virus complex and generation of tomato chlorosis virus infectious clones

Elisa Navas Hermosilla

RIBOZYME PLASMIDS TRIALEURODES VAPORARIORUM
RNA INFECTIOUS LUTEOVIRUS **CRINIVIRUSES** TRANSMISSION
PLANT PATHOGENS **POLEROVIRUS** EMERGENCE
VIRUS CHLOROSIS **PEPPER** MUTATION WHITEFLY **YELLOWING**
BEMISIA TABACI CLONES NICOTIANA TAXONOMY
CLOSTEROVIRUS

TESIS DOCTORAL

Directores

Jesús Navas Castillo y Elvira Fiallo Olivé

Programa de Doctorado en Biología Avanzada
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AUTORA: Elisa Navas Hermosilla

 <https://orcid.org/0000-0003-2804-5326>

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PROGRAMA DE DOCTORADO EN BIOTECNOLOGÍA AVANZADA

**EMERGING VIRUSES INFECTING SOLANACEOUS CROPS:
GENETIC CHARACTERIZATION OF THE PEPPER VEIN
YELLOW VIRUS COMPLEX AND GENERATION OF
TOMATO CHLOROSIS VIRUS INFECTIOUS CLONES**

Memoria de Tesis Doctoral presentada por la Licenciada en Ciencias Biológicas

Elisa Navas Hermosilla

para optar al grado de

Doctora en Biología por la Universidad de Málaga

Directores

Dr. Jesús Navas Castillo

Dra. Elvira Fiallo Olivé



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Parte de los resultados de esta Tesis Doctoral se incluyen en los siguientes artículos publicados en revistas científicas recogidas en el SCI:

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Dedicado a Fon

Lograste que 600 km no parecieran tantos

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ABSTRACT

Emerging viral diseases are not exclusive to humans or other animals; they also seriously affect plants, including major crops, in the past 30 years. The emergence of plant virus diseases is increasingly associated with the international transport of goods and the rise in global temperatures, which facilitates the establishment of vectors in previously unaffected areas. Plant viruses cause significant economic losses, and unlike fungi and other pathogens, there are no direct antiviral agents available for their control. The multitude of viruses infecting plants is a critical factor that limits agriculture worldwide, especially in less-developed countries. Insect vectors play a crucial role in plant virus epidemics by introducing new viruses into the environment. Among these vectors, aphids (Aphididae), leafhoppers (Cicadellidae), and whiteflies (Aleyrodidae) are the major transmitters of more than 70% of known insect-borne plant viruses, comprising over 500 species. In recent years, the appearance of vein yellowing and leaf roll symptoms in pepper crops associated with the presence of poleroviruses (genus *Polerovirus*, family *Solemoviridae*) has been observed in many countries worldwide. Spain was the first European country where pepper yellowing disease was reported. Similarly, tomato chlorosis virus (ToCV) is an emerging plant pathogen that causes yellow leaf disorder in tomato and other solanaceous crops. ToCV belongs to the genus *Crininivirus* (family *Closteroviridae*) and has a positive-sense, single-stranded (ss)RNA bipartite genome with long and flexuous virions. It is phloem-limited, transmitted by whiteflies, and causes interveinal chlorosis, bronzing, and necrosis in the lower leaves of tomato plants, resulting in reduced vigor and fruit yield.

In this study, we sequenced a polerovirus isolate from Spain that infects peppers and demonstrated its transmission by the aphid *Aphis gossypii*. We compared the Spanish isolate's genome with isolates from Japan, Israel, China, and Australia. The Spanish isolate exhibited the typical organization of poleroviruses, comprising seven open reading frames (ORF0 to ORF5 and ORF3a), putatively encoding proteins P0 to P5 and P3a. Comparing the Spanish sequence with the four complete sequences available for poleroviruses infecting peppers revealed a closer relationship to the Israeli isolate, suggesting the existence of a complex with at least five polerovirus species. Considering the similarity of symptoms caused by all described pepper poleroviruses, we propose naming them "pepper vein yellows virus 1" to "pepper vein yellows virus 5" (PeVYV-1 to PeVYV-5), with PeVYV-5 corresponding to the polerovirus from Spain described in this study. Our results, along with previously published findings,

highlight the high complexity of emergent poleroviruses threatening pepper crops worldwide due to recombination events.

Having infectious virus clones available is invaluable for reverse genetic studies, but the size and complexity of closterovirid genomes have hindered this approach. Therefore, we attempted to enhance the infectivity of the existing agroinfectious cDNA clones of ToCV (isolate AT80/99-IC from Spain) by incorporating the hepatitis delta virus (HDV) ribozyme fused to the 3' ends of both RNA1 and RNA2 components. The inclusion of the ribozyme resulted in viral progeny with RNA1 3' ends more similar to those present in the clone used for agroinoculation. However, similar to the original clones, the modified clones were unable to directly infect tomato plants through agroinoculation. Nonetheless, the infectivity of the clones carrying the HDV ribozyme increased by an average of two-fold in *Nicotiana benthamiana* plants compared to the previously available clones.

ToCV RNA molecules are individually encapsidated in flexuous particles with a distinctive structure characterized by a long "body" and a short "tail." The virion body consists of the major coat protein, while the tail contains the minor coat protein (CPm). The gene encoding CPm originated from the duplication and divergence of the CP gene. The CPm of criniviruses is believed to be crucial for transmission by whitefly vectors. Based on available information, we hypothesize that the determinant of dual transmission of criniviruses by members of the whitefly genera *Bemisia* and *Trialeurodes* is located within the CPm. To investigate this, we performed an *in silico* analysis comparing the CPm of characterized criniviruses to date, aiming to identify the putative regions responsible for ToCV transmission by *Bemisia tabaci* and *Trialeurodes vaporariorum*. Our analysis revealed a central region of the CPm exclusive to criniviruses transmitted by *T. vaporariorum*. Subsequently, using a system of ToCV agroinfectious clones, we generated a set of deletion mutants targeting the central region and its sub-regions of the CPm. Four out of five deletion mutants were infectious when agroinoculated into *N. benthamiana* plants. These engineered RNA2 infectious clones lay the foundation for future investigations that will elucidate the role of specific regions of the CPm in transmission by different whitefly species.

RESUMEN EXTENDIDO

RESUMEN EXTENDIDO

Los virus son las entidades biológicas más abundantes en la Tierra, infectan a miembros de los tres superreinos de la vida (Bacterias, Arqueas y Eukarya) y sus pequeños genomas los obligan a ser parásitos intracelulares. Durante las últimas décadas, la humanidad ha enfrentado numerosos brotes de enfermedades infecciosas emergentes y reemergentes, causadas principalmente por virus, con importantes amenazas para la seguridad sanitaria, la economía global e incluso la biodefensa. La reciente pandemia causada por el coronavirus severe acute respiratory syndrome coronavirus 2 es un ejemplo paradigmático de este fenómeno. Las enfermedades víricas emergentes no son exclusivas de los seres humanos u otros animales. Así, las plantas, incluidos los principales cultivos, han sido seriamente afectadas por enfermedades víricas emergentes en los últimos 30 años. La aparición de enfermedades víricas en las plantas está cada vez más asociada al transporte internacional de mercancías y al aumento de la temperatura global, lo que provoca el establecimiento de vectores donde antes no existían. Los virus de plantas son responsables de grandes pérdidas económicas y, a diferencia de los hongos y otros patógenos, no existen agentes antivirales directos disponibles para su control. El gran número de virus que infectan a las plantas constituye uno de los factores críticos que limitan la agricultura en todo el mundo, especialmente en los países menos desarrollados. Más del 70% de los numerosos virus de plantas transmitidos por insectos son transmitidos por miembros del orden Hemiptera, entre los que se encuentran los pulgones (Aphididae), los cicadélidos (Cicadellidae) y las moscas blancas (Aleyrodidae), vectores de virus pertenecientes a más de 500 especies. Estos insectos se distribuyen en regiones tropicales, subtropicales y templadas y algunos de ellos se están estableciendo en nuevas regiones debido al cambio climático global. Además, es importante destacar la amplia gama de huéspedes de algunos vectores hemípteros, como el pulgón *Aphis gossypii* o las moscas blancas del complejo *Bemisia tabaci*, lo que favorece la propagación de los virus. Los virus de plantas transmitidos por insectos se transmiten en modo no persistente, semipersistente o persistente, dependiendo del tiempo mínimo requerido para la adquisición y la duración máxima de retención de los viriones. El período mínimo de adquisición varía desde segundos a minutos para los virus no persistentes, desde minutos a horas para los semipersistentes y horas para los virus persistentes. Los virus no persistentes y semipersistentes son típicamente transmitidos por el estilete y el tracto digestivo anterior, respectivamente. Los virus persistentes se desplazan desde el tracto digestivo anterior hacia el medio y el intestino posterior, desde donde se transportan a la hemolinfa y

luego a las glándulas salivales para ser liberados en los tejidos de las plantas. Los virus persistentes pueden replicarse (propagativos) o no (no propagativos) en el cuerpo del vector.

Actualmente, los virus de las plantas se incluyen en más de 100 géneros agrupados en 24 familias. La mayoría de las familias de virus de plantas están integradas por miembros con genomas de ARN de cadena simple de polaridad positiva. Una característica importante de los virus de ARN en general es su alta capacidad para cambiar en su secuencia genómica y así poder adaptarse a condiciones ambientales cambiantes. Estas variantes se crean mediante mutación, recombinación, duplicación y reordenamiento génico. La diversidad genética de un virus se deriva de interacciones complejas que involucran la aptitud de las variantes seleccionadas, la interacción con el vector, el rango de huéspedes, la interacción con otras especies virales, etc. Estas interacciones pueden tener consecuencias epidemiológicas graves, como cambios en la virulencia, la aparición de aislados capaces de superar la resistencia genética de las plantas o el aumento del rango de huéspedes.

Con más de 15,000 hectáreas de invernaderos cada uno, el tomate y el pimiento son los cultivos protegidos más importantes en España. Los tomates silvestres (*Solanum* sección *Lycopersicon*, familia Solanaceae) son nativos del oeste de América del Sur (Ecuador, Perú y Chile), aunque su domesticación comenzó principalmente en México, Colombia y Bolivia, que se consideran como centros secundarios de diversificación. *Solanum lycopersicum*, el tomate cultivado, es una planta perenne que se comporta como anual cuando se cultiva. El tomate es uno de los frutos más versátiles en la cocina, siendo un elemento esencial en la cocina mediterránea que se utiliza crudo o cocinado y es la base de salsas como el ketchup. El tomate es el sexto cultivo en importancia económica en el mundo; en 2021, se estimó que se produjeron 189,133,955 toneladas en una superficie de 5,167,388 hectáreas, con un valor bruto de producción de más de 100 mil millones de dólares. España es el octavo productor de tomate en el mundo y el segundo en Europa después de Italia, con más de cuatro millones de toneladas producidas en 2021. La producción de tomate en España se encuentra principalmente en las provincias de Badajoz, Sevilla y Cáceres en campo abierto y Almería, Granada y Murcia en cultivo protegido. Un porcentaje importante de los tomates frescos producidos en España se destina a la exportación a Europa. Aunque los principales virus en los cultivos protegidos solían considerarse transmitidos mecánicamente, muchos virus emergentes en las últimas décadas en este tipo de agrosistemas son transmitidos por insectos, principalmente pulgones (Hemiptera:

Aphididae), moscas blancas (Hemiptera: Aleyrodidae) y trips (Thysanoptera: Thripidae). Además de disminuir la producción directamente a través de la alimentación, se ha descrito que los insectos son vectores de más de 130 virus en los cultivos de tomate, causando enfermedades y grandes pérdidas económicas. En España, se han detectado virus transmitidos por insectos pertenecientes a varios géneros en los cultivos comerciales de tomate, incluyendo virus transmitidos por pulgones (cucumber mosaic virus y potato virus Y), virus transmitidos por moscas blancas (tomato chlorosis virus, tomato infectious chlorosis virus, tomato yellow leaf curl virus y virus relacionados, tomato torrado virus y tomato leaf curl New Delhi virus) y virus transmitidos por trips (tomato spotted wilt virus). Además, se encuentran presentes varios virus transmitidos por contacto, como *Parietaria mottle virus*, pepino mosaic virus, tomato mosaic virus y tomato brown rugose fruit virus.

El pimiento (*Capsicum annuum*) es una planta perenne perteneciente a la familia Solanaceae que generalmente se cultiva como planta anual. Las especies silvestres del género *Capsicum* son nativas de Ecuador, Perú y Bolivia, y han sido domesticadas en América Central y del Sur. El pimiento y algunas otras especies de *Capsicum* son un elemento fundamental en la cocina mexicana y mediterránea, en parte debido a sus características picantes. En 2021, se produjeron 41,125,953 toneladas en 3,675,234 hectáreas en todo el mundo. España es el séptimo productor mundial y el primero en Europa, con 1,511,560 toneladas producidas en 22,240 ha en 2021. Las principales provincias productoras en España son Almería, Murcia y Granada, siendo Almería responsable de la mitad de la producción del país, principalmente en invernadero. Las enfermedades virales son probablemente el principal factor limitante para el cultivo de pimiento. Se han identificado más de 70 virus que infectan el pimiento y al menos 20 de ellos, pertenecientes a 15 familias, causan daños graves en los cultivos. En España se han detectado miembros de varios géneros transmitidos por insectos, que incluyen los transmitidos por pulgones (potato virus Y, *Amaranthus leaf mottle virus*, alfalfa mosaic virus, cucumber mosaic virus, broad bean wilt virus 1 y pepper vein yellows virus), los transmitidos por moscas blancas (tomato yellow leaf curl virus y virus relacionados, tomato chlorosis virus y tomato torrado virus) y los transmitidos por trips (tomato spotted wilt virus). Además, se han identificado varios virus transmitidos por contacto que infectan al pimiento en España: tobacco mosaic virus, pepper mild mottle virus y *Parietaria mottle virus*. Se han descrito brotes de una enfermedad transmitida por pulgones que afecta a los cultivos de pimientos en varios países desde la década de 1980. La enfermedad fue detectada por primera vez en la Prefectura de

Okinawa, Japón, en 1981 y los síntomas característicos incluían amarilleo de las venas y enrollamiento de las hojas en plantas cultivadas en invernadero. El agente causal fue identificado como pepper vein yellows virus (PeVYV), un nuevo polerovirus (género *Polerovirus*, anteriormente incluido en la familia *Luteoviridae*, pero actualmente clasificado en la familia *Solemoviridae*) transmitido por los pulgones *Aphis gossypii* y *Myzus persicae* de manera persistente y no transmitido mecánicamente ni por semillas. Una enfermedad similar fue descrita en el Valle de Arava, Israel, en 1998. Los síntomas descritos en este caso incluían acortamiento de los entrenudos del tallo, amarilleo intervenal y enrollamiento de las hojas y decoloración y reducción del tamaño de los frutos. El agente causal de esta enfermedad, también transmitido por *A. gossypii* y *M. persicae* y no transmitido mecánicamente, se denominó pepper yellow leaf curl virus (PYLCV). Síntomas similares a los observados en Japón e Israel también se han observado en cultivos de pimiento en muchos otros países del mundo asociados a la presencia de polerovirus, incluyendo Turquía, Túnez, España, India, Indonesia, Malí, Filipinas, Taiwán, Tailandia, Sudán, Estados Unidos, Costa de Marfil, China, Italia y Australia. Desde noviembre de 2012, se han observado brotes graves de una enfermedad novedosa para los agricultores en cultivos comerciales de pimiento en invernaderos en Almería, en el sureste de España. La prevalencia fue muy alta, alcanzando el 100% en muchos invernaderos. Los síntomas incluían amarilleo intervenal, enrollamiento de las hojas hacia arriba, acortamiento de los entrenudos y maduración anormal de los frutos, lo que reducía su comercialización. Estos síntomas recordaban a los causados por los polerovirus PeVYV en Japón y PYLCV en Israel. La secuenciación de un fragmento de ADN de 1,1 kb obtenido con una pareja de cebadores universales de polerovirus confirmó la infección por polerovirus en muestras de pimiento de Almería. La identidad de nucleótidos de los fragmentos de ADN amplificados mostró la mayor identidad (98,5% a 98,7%) con un aislado de PeVYV de Turquía.

El género *Polerovirus* en la familia *Solemoviridae* (Riboviria, Orthornavirae, Pisuviricota, Pisoniviricetes, Sobelivirales) contiene virus icosaédricos con genomas monopartitos de ARN de cadena sencilla. Anteriormente, el género *Polerovirus* estaba incluido en la actualmente abolida familia *Luteoviridae*. Los miembros de la familia *Solemoviridae* se transmiten por pulgones de manera circulativa persistente. Los viriones no envueltos son icosaédricos, con un diámetro de 25 a 30 nm y sus genomas consisten en una sola molécula de ssRNA de sentido positivo lineal que varía de 4 a 6 kb. El ARN genómico tiene una pequeña proteína viral (protein genome-linked, VPg) unida covalentemente al extremo 5' y carece de

una cola poli(A) en el extremo 3'. Los genomas de polerovirus, ejemplificados por potato leafroll virus, la especie tipo del género, contienen diez marcos de lectura abiertos (ORF0 a ORF8 y ORF3a). El ORF0 codifica un supresor de silenciamiento génico implicado en la sintomatología y el rango de huéspedes. Los ORF1 y ORF2 codifican proteínas relacionadas con la replicación, P1 y P2. P1 es una poliproteína que incluye un dominio transmembrana, la VPg y una proteasa de serina. P2 se expresa mediante un mecanismo de desplazamiento ribosomal a partir del ORF1 y codifica una ARN polimerasa dependiente de ARN. El ORF3 codifica la proteína de la cápsida y el ORF5 contiguo codifica un factor de transmisión por pulgones. El ORF3a codifica una proteína implicada en la infección sistémica. El ORF4, que se superpone con el ORF3, codifica una proteína de movimiento necesaria para el movimiento de célula a célula. Los ORF6 y ORF7 codifican proteínas que regulan la transcripción y la replicación del virus y el ORF8 codifica una proteína asociada a la replicación.

En la década de 1980 se observaron síntomas de amarilleo nuevos para los agricultores en plantas de tomate cultivadas en California. Inicialmente, los síntomas se atribuyeron a trastornos fisiológicos o nutricionales e incluso a toxicidad por pesticidas, aunque la presencia de moscas blancas asociadas a plantas sintomáticas sugería una enfermedad viral. El agente causal se denominó tomato infectious chlorosis virus (TICV), demostrándose que era un miembro del género *Crinivirus*, perteneciente a la familia *Closteroviridae*, transmitido por la mosca blanca *Trialeurodes vaporariorum*. En 1981, se informó de una "enfermedad de hoja amarilla" en plantas de tomate cultivadas en Florida, con síntomas similares a los causados por TICV. Sin embargo, el virus que causaba esta enfermedad no presentaba reacción cruzada en hibridaciones con sondas específicas de TICV. Por otra parte, este virus se transmitía además mediante *Bemisia tabaci*, lo que sugería que se trataba de un segundo crinivirus que infectaba el tomate. Tras su caracterización biológica y genómica parcial, este virus se denominó tomato chlorosis virus (ToCV). Los síntomas foliares causados por ToCV también pueden confundirse con trastornos fisiológicos o nutricionales. Sin embargo, la evolución de la enfermedad presenta características específicas. Así, la clorosis intervenales comienza en las hojas más viejas, avanzando hacia la parte superior de la planta. En la etapa inicial de la infección, las áreas cloróticas suelen tener forma poligonal, limitada por las venas principales. En etapas avanzadas, pueden desarrollarse áreas amarillas intervenales con manchas necróticas de color marrón rojizo. Las hojas inferiores aparecen enrolladas, engrosadas y quebradizas al tacto. La pérdida de área fotosintética podría ser la responsable de la reducción del vigor observado en las plantas

infectadas. La infección por ToCV reduce el crecimiento del fruto de tomate y retrasa su maduración, disminuyendo la cantidad de frutos comercializables. Como se mencionó anteriormente, ToCV se transmite mediante miembros de dos géneros de moscas blancas, *Trialeurodes* y *Bemisia*: *T. vaporariorum*, *T. abutilonea* y varias especies del complejo *B. tabaci* (New World-1, Middle East Asia Minor 1 y Mediterranean). En España, síntomas similares a los asociados con TICV/ToCV en Estados Unidos se detectaron inicialmente en cultivos de tomate en las provincias de Almería y Málaga en 1997. Pronto se confirmó que el virus causal era ToCV, siendo la primera identificación del virus fuera de los Estados Unidos. ToCV se encuentra actualmente ampliamente distribuido en España, incluyendo las provincias de Sevilla, Málaga, Granada, Almería, Murcia y Alicante, así como las Islas Baleares y las Islas Canarias. Desde que ToCV fue identificado por primera vez en Estados Unidos y luego en España, el virus ha ampliado enormemente su distribución geográfica, incluyendo actualmente más de 40 países y territorios en las Américas, Europa, África y Asia. Aunque ToCV se identificó por primera vez en tomates, infecta de forma natural más de 120 especies de plantas pertenecientes a 32 familias, incluyendo 30 especies de la familia Solanaceae. Las solanáceas huéspedes de ToCV incluyen cultivos tan importantes como el pimiento, la patata y la berenjena. El género *Crinivirus* en la familia *Closteroviridae* (Riboviria, Orthornavirae, Kitrinoviricota, Alsuviricetes, Martellivirales) contiene virus largos y flexuosos con genomas bipartitos de ARN de cadena sencilla (con la excepción de una especie con genoma tripartito). La familia *Closteroviridae* también contiene los géneros *Ampelovirus*, *Bluvavirus*, *Closterovirus*, *Menthavirus*, *Olivavirus* y *Velarivirus*. Las características generales de los miembros de la familia incluyen la restricción al floema y a las células acompañantes, así como genomas de ARN de cadena sencilla de polaridad positiva de gran tamaño, carecen de una cola de poli (A), siendo los genomas de ARN más grandes entre los virus de las plantas (de 15.5 a 19.5 kb). Este tamaño se traduce en una gran complejidad génica, originada por la adquisición de genes eucarióticos (por ejemplo, el homólogo de la proteína de choque térmico 70) y la duplicación de genes (por ejemplo, la duplicación del gen de la proteína de la cápsida que da lugar a una proteína principal de la cápsida (CP) y una proteína menor de la cápsida (CPm). Estos virus se transmiten de manera semipersistente por insectos pertenecientes al orden Hemiptera. Los miembros de cada género en la familia *Closteroviridae* se transmiten mediante insectos vectores específicos: ampelovirus y velavivirus por cochinillas (*Pseudococcidae*), crinivirus por moscas blancas (*Aleyrodidae*) y closterovirus por pulgones (*Aphididae*). Sus viriones (de 650 a 2200 de longitud y 12 nm de diámetro) poseen una morfología helicoidal

similar a la de muchos otros virus filamentosos de plantas, pero son únicos al tener una morfología asimétrica, con un "cuerpo" constituido por la CP y una "cola" principalmente por la CPM, de ahí el nombre de "serpiente de cascabel" dado a esta peculiar estructura. Las moléculas de ARN de los crinivirus se encapsidan en viriones separados. ToCV posee un genoma bipartito con la organización típica de los miembros del género *Crinivirus*. El ARN1 contiene cuatro ORFs flanqueados por dos regiones no traducidas (UTRs) ubicadas en los extremos 5' y 3' de la molécula. El ORF1a se traduce en una poliproteína que contiene las regiones conservadas de proteasa, metiltransferasa y helicasa presentes en todos los miembros de la familia. El ORF1b codifica la ARN polimerasa dependiente de ARN, que se traduce mediante un desplazamiento de lectura +1 del ribosoma. El ORF2 codifica una proteína de 22 kDa que se ha descrito como un potente supresor de silenciamiento génico. El ORF3 codifica una proteína putativa de 6 kDa con un dominio transmembrana predicho, similar a otras proteínas ubicadas en el extremo 3' de otros crinivirus, con función desconocida. El ARN2 de ToCV codifica nueve ORFs también flanqueados por UTRs ubicadas en ambos extremos de la molécula. El ARN2 incluye el bloque de cinco genes característico de la familia *Closteroviridae*, que estarían involucrados en la encapsidación del genoma, el movimiento viral en la planta y la interacción con el insecto vector. Este bloque de genes está constituido por el ORF4 que codifica la proteína p4, rica en residuos hidrofóbicos; el ORF5 que codifica la proteína Hsp70h (relacionada con la familia de proteínas Hsp70 de eucariotas); el ORF7 que codifica la proteína p59; y el ORF9 y el ORF10 que codifican la proteína CP y CPM, respectivamente. Se ha sugerido que la proteína CPM, por analogía con lo descrito para lettuce infectious yellows virus, estaría involucrada en la transmisión por el insecto vector. Ambas proteínas de la cápsida, CP y CPM, se han identificado como supresores del silenciamiento génico. El ORF6 codificaría una proteína putativa p8 con tamaño, posición genómica y secuencia similares a las proteínas de otros crinivirus. El ORF8 codificaría una putativa proteína p9, que se encuentra solo en miembros del género *Crinivirus* y cuya función es desconocida. El ORF11 codifica la proteína p27, que parece ser exclusiva de los crinivirus. Por último, el ORF12 codificaría una putativa proteína p7 con un dominio transmembrana que no muestra similitud significativa con ninguna proteína codificada por los genomas de otros crinivirus, por lo que parece ser exclusiva de ToCV.

Los clones infectivos son poderosas y versátiles herramientas para por ejemplo identificar sin ambigüedad el virus asociado a una enfermedad, confirmando así los postulados

de Koch. Nos permiten obtener poblaciones virales puras evitando infecciones mixtas, incluso en casos de virus presentes en bajas concentraciones en la planta huésped como ocurre en el caso de los closterovirus. La disponibilidad de dichos clones también es extremadamente útil para determinar el rango de huéspedes de un virus, reproduciendo la enfermedad que causa, así como para identificar fuentes de resistencia genética. Además, los clones infectivos son indispensables para realizar estudios funcionales mediante genética inversa, introduciendo diferentes tipos de mutaciones para obtener información sobre la funcionalidad de las proteínas virales y la biología del virus. Para obtener clones infectivos de virus de plantas de ARN de cadena sencilla de polaridad positiva, se sintetiza el ADNc de longitud completa del genoma viral mediante transcripción inversa, se amplifica por PCR y se clona en un vector adecuado. A veces, los genomas largos se ensamblan mediante la unión de fragmentos superpuestos que comparten sitios de restricción. Para iniciar la transcripción *in vitro* a partir de los clones de longitud completa, se fusiona un promotor de la ARN polimerasa de un fago al extremo 5' del ADNc y se incorpora un sitio de restricción único en el extremo 3' para finalizar la transcripción mediante digestión. En muchos casos, la incorporación de un análogo de caperuza (*cap*) en el extremo 5' de los transcritos *in vitro* aumenta la infectividad. Los transcritos obtenidos *in vitro* se pueden inocular mecánicamente en las plantas huésped. En los casos en que se prefiere la transcripción *in vivo*, el promotor constitutivo 35S de cauliflower mosaic virus es un candidato excelente para iniciar la transcripción, utilizando el terminador de la sintasa de nopalina (*nos*) de *Agrobacterium tumefaciens* para finalizar la transcripción. En estos casos, las construcciones suelen clonarse en un vector binario para mediar la infección utilizando *A. tumefaciens* transformado (agroinoculación). Los clones infectivos deben diseñarse de manera que el primer nucleótido viral se posicione en el primer nucleótido transcrito tras el promotor, evitando también nucleótidos adicionales en el extremo 3'. Se ha demostrado que los nucleótidos no virales adicionales en ambos extremos de los transcritos tienen efectos adversos en la infectividad. En este sentido, cabe mencionar que se han identificado dos señales de poliadenilación que crean diferentes sitios de terminación/poliadenilación en el terminador *nos*. De hecho, el mapeo del sitio de poliadenilación en los transcritos controlados por el terminador *nos* muestra un patrón difuso, con la escisión ocurriendo en varias ubicaciones diferentes después de la señal de poliadenilación. También debe tenerse en cuenta muchos virus de ARN, como por ejemplo los miembros de las familias *Closteroviridae* y *Solemoviridae* no poseen colas poli(A) en sus genomas. Una alternativa para solucionar estas dificultades es incorporar la secuencia del ribozima de hepatitis delta virus (HDV-Rb) en el extremo 3' del genoma viral,

coincidiendo así el sitio de autoescisión con el último nucleótido viral. La secuencia HDV-Rb se puede incluir en un vector de clonación para facilitar la construcción de clones infectivos. Un ejemplo de tales vectores de clonación es el vector pJL89, que se ha utilizado con éxito para acomodar grandes insertos de ADNc para su transcripción sin nucleótidos virales adicionales. El gran tamaño de algunos ARN virales, como en el caso de la familia *Closteroviridae* de virus de plantas y la familia *Coronaviridae* de virus de animales, presenta varios obstáculos al construir un ADNc infectivo o un transcrito *in vitro*: i) las secuencias de ARN largas dificultan la síntesis de una molécula de ADNc precisa, ya que la fidelidad de la transcripción reversa y PCR (RT-PCR) para la amplificación de ADNc disminuye proporcionalmente a la longitud del ARN, ii) las secuencias de ARN largas tienen más probabilidades de codificar proteínas tóxicas, lo que hace que la secuencia de ADNc sea inestable en los plásmidos, y iii) es difícil encontrar un vector adecuado que pueda acomodar grandes insertos de ADNc. Aun así, estas dificultades se han superado en gran medida y se han construido clones infectivos incluso para citrus tristeza virus que posee el mayor genoma de ARN entre los virus de plantas (aproximadamente 20 kb) y para coronavirus. Una vez construidos, los clones infectivos de closterovirus son difíciles de mantener debido a la existencia de secuencias virales que supuestamente codifican productos con toxicidad para *Escherichia coli*. La inestabilidad causada por la toxicidad se ha superado en los closterovirus y otros virus de ARN de cadena simple mediante el uso de cepas especiales de *E. coli* cultivadas a bajas temperaturas, la inserción de intrones de plantas para interrumpir la expresión de secuencias tóxicas, la introducción de mutaciones de cambio del marco de lectura y mediante el uso de vectores con bajo número de copias. Los clones infectivos diseñados para producir transcritos virales *in vitro* presentan una serie de limitaciones: los transcritos largos y frágiles son difíciles de manipular e inocular y la presencia de mutaciones, nucleótidos no virales, ausencia de estructura *cap* y la necesidad de secuencias precisas en los extremos 5' y 3' pueden comprometer la infectividad. Además, generalmente es necesario inocular los transcritos en protoplastos de plantas modelo como *N. benthamiana* o *N. tabacum* para generar una progenie viral. En los casos en los que se obtienen viriones en protoplastos, se necesitan pasajes sucesivos para obtener una cantidad suficiente de virus para inocular plantas completas. Para evitar las dificultades asociadas con la producción e inoculación de transcritos virales obtenidos *in vitro*, la transcripción *in vivo* de clones infectivos de ADNc es una buena alternativa. Estos clones se pueden inocular en plantas mediante bombardeo biolístico o por agroinoculación. En ambos casos, el ADNc viral suele posicionarse entre el promotor constitutivo 35S y el terminador *nos*, con o sin secuencia HDV-Rb. Después de grandes

esfuerzos, se han desarrollado clones infectivos para la transcripción *in vitro* de seis miembros de la familia *Closteroviridae*: beet yellows virus, citrus tristeza virus, lettuce infectious yellows virus, lettuce chlorosis virus, tomato chlorosis virus y cucurbit yellow stunting disorder virus. Por otro lado, se han desarrollado clones agroinfectivos para los siguientes miembros de esta familia: BYV, grapevine leafroll-associated closterovirus 2 (GLRaV-2), CTV, LIYV, LCV y ToCV. Los clones agroinfectivos de los miembros de la familia *Closteroviridae* no pueden, en general, infectar directamente a sus huéspedes naturales. Varias razones pueden explicar este fenómeno, incluida la degradación del ARN desencadenada por las formas replicativas de doble cadena debido a la maquinaria de silenciamiento génico de la planta y la indisponibilidad de cepas de *A. tumefaciens* capaces de infectar huéspedes específicos. Incluso en los casos en que se ha demostrado que los transcritos *in vitro* son infectivos en protoplastos del huésped natural (por ejemplo, CTV en cítricos, CYSDV en pepino y ToCV en tomate), no se ha logrado la infección de plantas completas. Aunque la replicación de GLRaV-2 tiene lugar en plantas jóvenes de vid micropropagadas tras su agroinoculación con un clon infectivo, estos resultados no se pudieron reproducir en plantas maduras. Se han desarrollado varias estrategias para superar las barreras para la infección de plantas de los huéspedes naturales. Así, los viriones de LIYV y LCV purificados de plantas de *N. benthamiana* se han transmitido a plantas de lechuga mediante mosca blanca y un sistema de alimentación artificial a través de membrana. Además, se ha logrado la infección de plantas de cítricos con CTV mediante la inoculación mecánica con viriones parcialmente purificados de hojas de plantas de *Nicotiana benthamiana* agroinfectadas. Asimismo, se infectaron plantas de tomate con ToCV mediante el injerto de tallos de plantas de *N. benthamiana* agroinfectadas. La manipulación de clones infectivos de miembros de la familia *Closteroviridae* ha permitido incrementar nuestro conocimiento sobre el papel de las proteínas virales en la replicación, encapsidación, movimiento a través de la planta y supresión del silenciamiento génico. Además, los mutantes virales generados a partir de clones infectivos de lettuce infectious yellows virus se han utilizado para analizar la transmisión del virus por el insecto vector.

En la investigación en el ámbito de la virología vegetal se siguen varios pasos clave para la caracterización y control de los virus de plantas. En primer lugar, la identificación y caracterización viral implican el aislamiento y secuenciación del genoma viral para determinar su taxonomía. En segundo lugar, es crucial comprender los mecanismos de transmisión de los virus de plantas, incluyendo la identificación de los vectores responsables de propagar los virus

en la naturaleza. Además, dilucidar las interacciones entre el virus y la planta huésped a nivel molecular ayuda a comprender el proceso de infección y los mecanismos de defensa de la planta. Además, estudiar la epidemiología y ecología de los virus de plantas ayuda a determinar sus patrones de propagación y los factores ambientales que influyen en su prevalencia. Por último, desarrollar estrategias efectivas de control, como la selección de cultivares resistentes o la implementación de prácticas culturales, es esencial para mitigar el impacto de las enfermedades virales en la producción de los cultivos. Estas áreas contribuyen en conjunto a avanzar en nuestro conocimiento de los virus de plantas y a diseñar estrategias para su manejo y control. Varios de estos pasos clave se han considerado en este trabajo, utilizando como modelos virus emergentes que afectan los cultivos de solanáceas en España, tomate y pimiento, definiendo los principales objetivos de los tres capítulos:

- i. Caracterizar genéticamente un aislado español de polerovirus que infecta los cultivos protegidos de pimiento, incluyendo la secuenciación completa del genoma y resolviendo su posición taxonómica.
- ii. Generar clones infectivos de tomato chlorosis virus (ToCV) con una mayor eficiencia de agroinoculación mediante la incorporación de la secuencia del ribozima de hepatitis delta virus.
- iii. Desarrollar un conjunto de clones agroinfectivos del RNA2 de ToCV con deleciones parciales que afectan el gen de la proteína menor de la cápsida (CPm), con el objetivo de determinar los determinantes específicos de transmisión por especies de mosca blanca.

Capítulo 1

En este estudio, secuenciamos un aislado de polerovirus de España que infecta pimiento y demostramos su transmisión por el pulgón *Aphis gossypii*. Se llevó a cabo la comparación del genoma del aislado español con aislados de polerovirus que infectan pimiento de Japón, Israel, China y Australia. El aislado español exhibió la organización típica de los polerovirus, compuesta por siete marcos de lectura abiertos (ORF0 a ORF5 y ORF3a), que posiblemente codifican las proteínas P0 a P5 y P3a. Al comparar la secuencia española con las cuatro secuencias completas disponibles de polerovirus que infectan pimiento, encontramos una relación más cercana con el aislado israelí, sugiriéndose también la existencia de un complejo con al menos cinco especies de polerovirus. Considerando la similitud de los síntomas causados por todos los polerovirus descritos que infectan de forma natural al pimiento, proponemos denominarlos "pepper vein yellows virus 1" a "pepper vein yellow virus 5" (PeVYV-1 a

PeVYV-5), siendo PeVYV-5 el correspondiente al polerovirus de España descrito en este estudio. Nuestros resultados, junto con hallazgos previamente publicados, resaltan la alta complejidad de los polerovirus emergentes que amenazan los cultivos de pimientos en todo el mundo, destacando los eventos de recombinación que están en el origen de dicha complejidad y diversidad.

Capítulo 2

La disponibilidad de clones infectivos de virus de plantas es un recurso indispensable y de gran valor para llevar a cabo estudios de genética reversa. En el caso de los miembros de la familia *Closteroviridae*, dado el tamaño y la complejidad de sus genomas, la construcción de dichos clones ha sido dificultosa. En este trabajo hemos intentado incrementar la infectividad de los clones existentes de ADNc agroinfectivos de ToCV (aislado AT80/99-IC de España) mediante la incorporación del ribozima de hepatitis delta virus (HDV) fusionado a los extremos 3' de los componentes genómicos RNA1 y RNA2. La inclusión del ribozima resultó en una progenie viral con extremos 3' del RNA1 más similares a los del clon utilizado para la agroinoculación. Sin embargo, al igual que los clones originales, los clones modificados no pudieron infectar directamente plantas de tomate mediante agroinoculación. No obstante, la infectividad en *N. benthamiana* de los clones que incluían el ribozima de HDV aumentó al doble, en promedio, en comparación con los clones disponibles anteriormente.

Capítulo 3

Los componentes genómicos de ToCV, ARN1 y ARN2, se encapsidan individualmente en partículas flexuosas con una estructura distintiva caracterizada por un "cuerpo" largo y una "cola" corta, de ahí el nombre de "serpiente de cascabel" dado a esta peculiar estructura. El cuerpo del virión consiste en la proteína CP, mientras que la cola contiene la proteína CPm. El gen que codifica la CPm se originó a partir de la duplicación y divergencia del gen de la CP. Se cree que la CPm de los crinivirus es crucial para la transmisión por moscas blancas que actúan de vectores naturales. Basándonos en la información disponible, formulamos la hipótesis de que el o los determinantes de la transmisión dual de los crinivirus por miembros de los géneros de mosca blanca *Bemisia* y *Trialeurodes* se encuentran localizados en la CPm. Para investigar este aspecto de la biología de los crinivirus, realizamos en primer lugar un análisis *in silico* comparando la CPm de las especies caracterizadas hasta la fecha, con el objetivo de identificar las posibles regiones responsables de la transmisión de ToCV por *B. tabaci* y *T. vaporariorum*.

Nuestro análisis reveló una región central de la CPm exclusiva de los crinivirus transmitidos por *T. vaporariorum*. Posteriormente, utilizando un sistema de clones agroinfectivos de ToCV, generamos un conjunto de mutantes de delección dirigidos a la región central de la CPm y sus subregiones. Cuatro de los cinco mutantes de delección fueron infectivos cuando se agroinocularon en plantas de *N. benthamiana*. La disponibilidad de estos clones infectivos de ARN2 de ToCV sientan las bases para futuras investigaciones que podrán dilucidar el papel de regiones específicas de la CPm en la transmisión de este virus por diferentes especies de mosca blanca.

OBJECTIVES

In plant virus research, several key steps are followed to investigate these pathogens. Firstly, viral identification and characterization involve isolating and sequencing the viral genome to determine its taxonomy. Secondly, understanding the transmission mechanisms of plant viruses is crucial, including identifying the vectors responsible for spreading the viruses in nature. Additionally, elucidating the interactions between the virus and the host plant at the molecular level helps comprehend the infection process and the host's defense mechanisms. Moreover, studying the epidemiology and ecology of plant viruses aids in determining their spread patterns and the environmental factors influencing their prevalence. Lastly, developing effective management strategies, such as breeding resistant cultivars or implementing cultural practices, is essential to mitigate the impact of plant viral diseases on crop production. These areas collectively contribute to advancing our knowledge of plant viruses and devising strategies for their control and management.

Several of these key steps have been considered in this work, using emerging viruses affecting solanaceous crops in Spain as models, defining the main objectives of the three chapters:

- i. Genetically characterize a Spanish polerovirus isolate infecting protected pepper crops, including full genome sequencing and clarification of its taxonomic position.
- ii. Engineer infectious clones of the crinivirus tomato chlorosis virus with improved agroinoculation efficiency by incorporating the hepatitis delta virus ribozyme sequence.
- iii. Develop a set of agroinfectious clones of tomato chlorosis virus RNA2 with partial deletions affecting the minor coat protein gene, aiming to determine the specific transmission determinants by whitefly species.

INTRODUCTION

INTRODUCTION

I.1. Emerging plant viruses transmitted by insects

Viruses, from the Latin *virus* meaning poison, are the most abundant biological entities on Earth (Rohwer, 2003). Viruses infect members of all three superkingdoms of life (Bacteria, Archaea and Eukarya) (Koonin *et al.*, 2015) and their small genomes obligate them to be intracellular parasites. During the last decades, mankind has faced numerous outbreaks of emerging and re-emerging infectious diseases, mainly caused by viruses, with important threats to health security, global economy and even biodefense (Bloom *et al.*, 2017; Smith *et al.*, 2019). The recent pandemic caused by severe acute respiratory syndrome coronavirus 2 is a paradigmatic example of this phenomenon (Wu *et al.*, 2020). Emerging virus diseases are not exclusive to the human being or other animals. Thus, plants, including major crops, have been seriously affected by emerging virus diseases in the last 30 years or so (Jones and Naidu, 2019). The emergence of plant virus diseases is increasingly associated with the active international transport of goods and the increase of global temperature provoking establishment of vectors where they previously did not exist (Canto *et al.*, 2009; Hanssen *et al.*, 2010; Navas-Castillo *et al.*, 2011).

Plant viruses are responsible for great economic losses and unlike fungi and other pathogens, there are no direct antiviral agents available for their control. The large number of viruses infecting plants constitute one of the critical factors limiting agriculture worldwide, especially in lesser-developed countries (Oerke and Dehne, 2004). Insect vectors are also an important factor in plant virus epidemics as introducers of new viruses in the environment. More than 70% of known insect-borne plant viruses are transmitted by members of the order Hemiptera, among which aphids (*Aphididae*), leafhoppers (*Cicadellidae*) and whiteflies (*Aleyrodidae*) are the major vectors, transmitting viruses belonging to more than 500 species (Ferreles and Raccach, 2015). These insects are distributed in tropical, sub-tropical and temperate regions, some of them establishing into new regions due to global climate change. For example, several studies have reported the impact of climate change on life history, distribution, population dynamics and efficacy of management strategies of whiteflies, including several species of the *Bemisia tabaci* complex (Aregbesola *et al.*, 2019). Also, it should be stressed the broad host range of some hemipteran vectors, as the aphid *Aphis gossypii* (Xu *et al.*, 2020) or the whiteflies of the *Bemisia tabaci* complex (De Barro, 1995), thus favoring virus spread.

Insect-born plant viruses are transmitted by their insect vectors in either non-persistent, semi-persistent or persistent mode depending on the minimum time period required for acquisition and maximum duration of retention of the virions (Nault 1997; Ng and Falk 2006b) (Table I.1). The minimum acquisition access period varies from seconds to minutes for non-persistent, from minutes to hours for semi-persistent and hours for persistent viruses. Nonpersistent and semipersistent viruses are typically stylet-born and foregut-born, respectively. Persistent viruses move from the foregut to the mid- and hindgut, from where they are transported to the hemolymph and further to the salivary glands to be released into plant tissues. Persistent (circulative) viruses either replicate (propagative) or do not replicate (nonpropagative) in the body of the insect vector.

Table I.1. Modes of transmission of plant viruses by hemipteran vectors.

Transmission characteristic	Mode of transmission			
	Nonpersistent	Semipersistent	Persistent	
			Nonpropagative	Propagative
Acquisition time	sec-min	min-hours	hours	hours
Retention time	min-hours	hours-days	days-life	days-life
Transtadial passage	no	no	yes	yes
Virus in hemolymph	no	no	yes	yes
Latent period	no	no	hours	days-weeks
Replication in the insect	no	no	no	yes
Transovarial transmission	no	no	no	often
Example	potyviruses by aphids	criniviruses by whiteflies	poleroviruses by aphids	phytoreoviruses by leafhoppers

The diversity of viruses is ordered by the International Committee on Taxonomy of Viruses (ICTV). Although classification of viruses is not an easy task, it is possible to organize them into taxonomic categories based on various characteristics such as genome features (DNA or RNA, single or double strain, monopartite or multipartite), presence of envelope, mode of transmission, infectious cycle, and host range. The recently accepted rank hierarchy includes: Realm, Kingdom, Phylum, Class, Order, Family, Genus and Species, plus seven intermediate ranks (Gorbalenya *et al.*, 2020). Currently, plant viruses are classified into more than 100 genera

grouped into 24 families (<https://ictv.global/taxonomy>).

Most families of plant viruses have members with single-stranded RNA genomes of positive polarity. One important characteristic of RNA viruses in general is a high capacity for change in their genome sequence. The quick generation of new RNA viral variants makes it possible the rapid adaptation to changing environmental conditions (Domingo and Perales, 2019). These variants are created by mutation, recombination, gene duplication, and rearrangement (Roossinck, 1997). Mutations are created by errors of the viral RNA-dependent RNA polymerase (RdRp) and lack of correction mechanisms. For RdRps of animal RNA viruses, for example, mutation rates of about 10^{-4} (i.e. one error per 10-kb genome) have been estimated, in contrast with error rates of 10^{-9} for cellular DNA-dependent DNA polymerases (Roossinck, 1997). RNA recombination may have evolutionary consequences as it could repair deleterious mutations from error-prone replication, create new gene combinations with increased fitness or even generate new viral species (Nagy and Simon, 1997). Gene duplication and subsequently diversification could create new viral functions or modify them (Boyko *et al.*, 1992; Fazeli and Rezaian, 2000; Roossinck, 1997). Besides, the exchange of complete genome components in segmented viruses (rearrangement or pseudo-recombination) may create rapidly new genome combinations (Fraile *et al.*, 1997; Roossinck, 1997; Zhao *et al.*, 2018).

The actual viral diversity of a virus derives from complex interactions involving the fitness of selected variants, interaction with the vector, range host, complementation with other viral species, etc. (García-Arenal *et al.*, 2001). These interactions can result in serious epidemiological consequences as changes in virulence, viral outbreaks, the appearance of isolates capable of overcoming genetic resistance or increase of the host range (García-Arenal and McDonald, 2003; Gibbs *et al.*, 2001; Monci *et al.*, 2005).

I.2. Protected tomato and pepper crops in Spain: importance and viruses affecting them

With more than 15,000 hectares of greenhouses each, tomato and pepper are the most important protected crops in Spain.

Wild tomatoes (*Solanum* section *Lycopersicon*, family Solanaceae) are native to western South America (Ecuador, Peru, and Chile) although their domestication began mainly in

Mexico, Colombia and Bolivia, which are considered as secondary centers of diversification (Peralta *et al.*, 2005). *Solanum lycopersicum*, the domesticated tomato, is a perennial herb that when cultivated behaves as annual. Tomato is one of the most versatile fruits in the kitchen, being an essential element in Mediterranean cuisine used fresh, pickled, stewed or stir-fried, and is the base of sauces such as ketchup. Tomato is the sixth crop by economic importance in the world; in 2021, 189,133,955 tons were estimated to be produced from a land area of 5,167,388 hectares, with a gross production value of more than 100 billion dollars (<http://faostat.fao.org>).

Spain is the eighth producer of tomato in the world and the second in Europe after Italy, with more than four million tons produced in 2021 (<http://faostat.fao.org>). Tomato production in Spain is mainly located in the provinces of Badajoz, Sevilla y Cáceres in open field and Almería, Granada and Murcia in protected crops. An important percentage of the fresh tomatoes produced in Spain is intended for export to Europe.

Although the major viruses in protected crops were once considered to be mechanically transmitted (Moury and Verdin, 2012), many viruses emerging in the last few decades in this type of agrosystem are transmitted by insects, mainly aphids (Hemiptera: Aphididae), whiteflies (Hemiptera: Aleyrodidae) and thrips (Thysanoptera: Thripidae) (Kenyon *et al.*, 2014). In addition to decreasing production directly by feeding, insects have been described as vectors of more than 130 viruses in tomato crops causing diseases and great economic losses (Hanssen and Lapidot, 2012). In Spain, insect-borne viruses belonging to several genera have been detected in commercial tomato crops including aphid-transmitted viruses [pepino mosaic virus (Jordá *et al.*, 2001), potato virus Y (Abad and Jordá, 2000) and cucumber mosaic virus (García-Luque *et al.*, 1983)], whitefly-transmitted viruses [tomato chlorosis virus (Navas-Castillo *et al.*, 2000), tomato infectious chlorosis virus (Font *et al.*, 2002), tomato yellow leaf curl virus and related viruses (Díaz-Pendón *et al.*, 2010; Moriones and Navas-Castillo, 2000), tomato torrado virus (Verbeek *et al.*, 2007) and tomato leaf curl New Delhi virus (Ruiz *et al.*, 2015)] and thrips-transmitted viruses [tomato spotted wilt virus (Laviña *et al.*, 1996)]. Also, a number of contact-transmitted viruses are present infecting tomato in the country [Parietaria mottle virus (Aramburu, 2001), tomato mosaic virus (Aramburu and Galipienso, 2005) and tomato brown rugose fruit virus (Alfaro-Fernández *et al.*, 2021)].

Pepper (*Capsicum annuum*) is a perennial plant belonging to the family Solanaceae that

is usually cultivated as an annual plant. Wild species of the genus *Capsicum* are native to Ecuador, Peru and Bolivia, having been domesticated in Central and South America (Ibiza *et al.*, 2012). Pepper and some other *Capsicum* species are a fundamental element in Mexican and Mediterranean cuisine, in part due to their pungent characteristics. In 2021, 41,125,953 tones were produced in 3,675,234 hectares in the world (<http://faostat.fao.org>). Spain is the seventh producer of pepper in the world and the first in Europe with 1,511,560 tons produced in 22,240 ha in 2021 (<http://faostat.fao.org>). The main Spanish producing provinces are Almería, Murcia, and Granada, with Almería producing half of the production of the country, mostly under greenhouse conditions.

Viral diseases are very probably the main limiting factor for pepper cultivation. More than 70 viruses have been reported infecting pepper and at least 20 of them, belonging to 15 families, cause severe damage to crops (Moury and Verdin, 2012). In Spain, members of several genera transmitted by insects have been detected; these include the aphid-transmitted [potato virus Y (Arteaga, 1989), Amaranthus leaf mottle virus (Segundo *et al.*, 2007), alfalfa mosaic virus (Bergua *et al.*, 2010), cucumber mosaic virus (Simón *et al.*, 2016), broad bean wilt virus 1 (Rubio *et al.*, 2002) and pepper vein yellows virus (Villanueva *et al.*, 2013)], whitefly-transmitted [tomato yellow leaf curl virus and related viruses (Reina *et al.*, 1999; Morilla *et al.*, 2005), tomato chlorosis virus (Lozano *et al.*, 2004) and tomato torrado virus (Amari *et al.*, 2008)]; thrips-transmitted [tomato spotted wilt virus (Parrella *et al.*, 2003)]. Also, a number of contact-transmitted viruses have been identified infecting pepper in Spain: tobacco mosaic virus, pepper mild mottle virus (Alonso *et al.*, 1989) and Parietaria mottle virus (Janssen *et al.*, 2005).

1.3. Pepper poleroviruses

Outbreaks of a distinct aphid-transmitted disease affecting pepper crops have been reported in a number of countries since the 1980s. The disease was first detected in Okinawa Prefecture, Japan, in 1981 and the characteristic symptoms included vein yellowing and leaf rolling in bell pepper grown under greenhouse conditions. The causal agent was identified as pepper vein yellows virus (PeVYV), a new polerovirus (genus *Polerovirus*, formerly included in the family *Luteoviridae* but currently classified in the family *Solemoviridae*) transmitted by the aphids *Aphis gossypii* and *Myzus persicae* in a persistent manner and not transmitted mechanically or by seeds (Yonaha *et al.*, 1995). A similar disease was described in Arava

Valley, Israel, in 1998 (Antignus *et al.*, 2001). Symptoms were described in this case as shortening of stem internodes, interveinal yellowing, leaf rolling and discoloration and size reduction of fruits. The causal agent of this disease, also transmitted by *A. gossypii* and *M. persicae* and not mechanically transmitted, was named pepper yellow leaf curl virus (PYLCV) (Dombrovsky *et al.*, 2010). The experimental host range of PYLCV, in addition to pepper, includes *Chenopodium amaranticolor*, *Cucurbita pepo*, *Datura stramonium*, *Gomphrena globosa*, *Nicotiana clevelandii*, *N. benthamiana*, *Petunia hybrida* and *Physalis floridana* (Dombrovsky *et al.*, 2010).

Symptoms similar to those observed in Japan and Israel have been also observed in pepper crops in many other countries worldwide associated with the presence of poleroviruses, including Turkey and Tunisia (Buzkan *et al.*, 2013); Spain (Villanueva *et al.*, 2013); India, Indonesia, Mali, Philippines, Taiwan, and Thailand (Knierim *et al.*, 2013); Sudan (Alfaro-Fernández *et al.*, 2014); United States (Alabi *et al.*, 2015); Ivory Coast (Bolou *et al.*, 2015); China (Zhang *et al.*, 2015; Tan *et al.*, 2015); Italy (Tomassoli *et al.*, 2016) and Australia (Maina *et al.*, 2016).

Since November 2012, severe outbreaks of a disease novel to the farmers in the area were observed in commercial pepper crops grown under greenhouse conditions in Almería, southeastern Spain. Prevalence was very high, reaching 100% in many greenhouses. Symptoms included interveinal yellowing, upward leaf curling, internode shortening and abnormal ripening of fruits thus reducing their marketability (Figure I.1) (Villanueva *et al.*, 2013). These symptoms were reminiscent of those caused by the poleroviruses PeVYV in Japan and PYLCV in Israel (Villanueva *et al.*, 2013). The sequencing of a 1.1 kb DNA fragment obtained with a pair of polerovirus universal primers supported a polerovirus infection in pepper samples from Almería. The nucleotide identity of the amplified DNA fragments showed the highest identity (98.5 to 98.7%) with a PeVYV isolate from Turkey (Villanueva *et al.*, 2013).

Since the detection of the disease in Spain, similar symptoms to those caused by PeVYV/PYLCV have also been observed in pepper plants associated with polerovirus infections in India, Indonesia, Mali, Philippines, Taiwan and Thailand (Knierim *et al.*, 2013), Sudan (Alfaro-Fernández *et al.*, 2014), United States (Alabi *et al.*, 2015), Ivory Coast (Bolou *et al.*, 2015), China, (Tan *et al.*, 2015; Zhang *et al.*, 2015), Italy (Tomassoli *et al.*, 2016),



Figure I.1. Symptoms caused by pepper vein yellows virus on sweet pepper grown under greenhouse conditions in Almeria (southern Spain). A. General view of a greenhouse showing a high level of infection. B. A close view of an infected plant showing interveinal yellowing and upward leaf curling. C. Symptoms on red-ripening (upper photographs) and orange-ripening (lower photographs) fruits. Photographs on the left correspond to fruits from healthy plants. Panel C is reproduced from Villanueva *et al.* (2013).

Australia (Maina *et al.*, 2016), Greece (Lotos *et al.*, 2017), Saudi Arabia (Kamran *et al.*, 2018), Pakistan (Ahmad *et al.*, 2018), Brazil (Pantoja *et al.*, 2019), and Malaysia (Sau *et al.*, 2020).

I.3.1. Genome organization

The genus *Polerovirus* (from the species *Potato leafroll virus*) in the family *Solemoviridae* (from the genus names *Sobemovirus* and *Polemovirus*) (*Riboviria*, *Orthornavirae*, *Pisuviricota*, *Pisoniviricetes*, *Sobelivirales*) contains icosahedral viruses with monopartite ssRNA genomes. The family *Solemoviridae* also includes the genera *Enamovirus*, *Polemovirus* and *Sobemovirus* plus seven unassigned virus species (Sõmera *et al.*, 2021). Until recently, the genus *Polerovirus* was included in the abolished family *Luteoviridae* (Scheets *et al.*, 2020).

Solemovirids are transmitted by aphids in a persistent circulative manner (Brault *et al.*, 2011; Fereres and Raccah, 2015). Virions are non-enveloped and icosahedral in shape, 25 to 30 nm in diameter (Figure I.2), and genomes consist of a single molecule of linear, positive-sense ssRNA ranging from 4 to 6 kb (Sõmera *et al.*, 2021). Genomic RNA has a small protein (virus protein genome-linked, VPg) covalently linked to the 5′ end and lack a 3′-terminal poly (A)-tail.

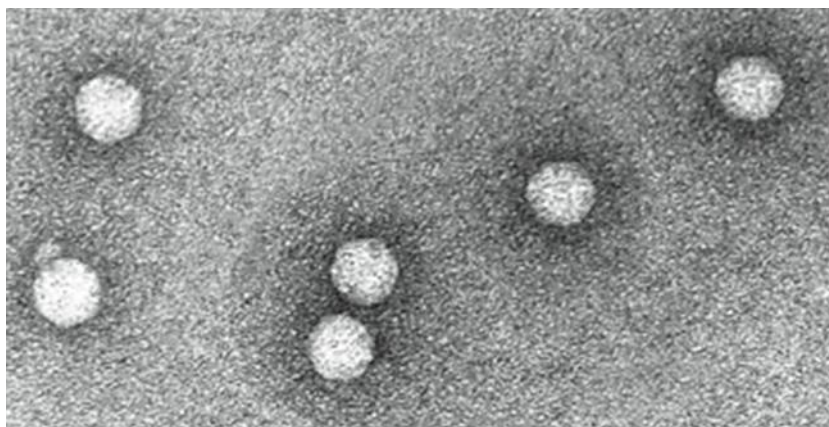


Figure I.2. Icosahedral morphology of a typical solemovirid, pea enation mosaic virus 1, revealed by electron microscopy. Reproduced from Domier (2012).

Polerovirus genomes, exemplified by potato leafroll virus, the type species of the genus, contain ten open reading frames (ORF0 to ORF8 and ORF3a) (Figure I.3) (Sõmera *et al.*, 2021). ORF0 encodes a suppressor of RNA silencing involved in symptomatology and host range. Overlapping ORF1 and ORF2 encode replication-related proteins P1 and P2. P1 is a polyprotein including a transmembrane domain, the VPg, and a serine protease. P2 is expressed by a -1 ribosomal frameshift from ORF1 and encodes a RNA-dependent RNA polymerase. ORF3

encodes the coat protein and the contiguous ORF5 encodes an aphid transmission factor by a readthrough mechanism. ORF3a encodes a protein involved in systemic infection. ORF4, overlapping with ORF3, encodes a movement protein required for cell-to-cell movement. ORFs 6 and 7 encode proteins regulating virus transcription and replication and ORF8 encodes a replication-associated protein. ORFs present in the 3' moiety of the genome are expressed from subgenomic RNAs.

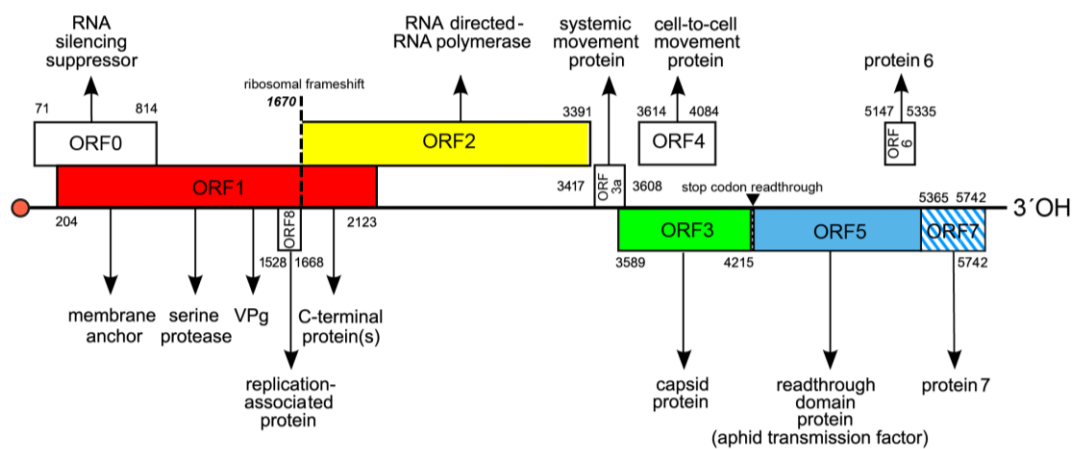


Figure I.3. Genome organization of potato leafroll virus, the member type of genus *Polerovirus*. Red bullet indicates the virus protein genome-linked (VPg) covalently attached at the 5'-end of the RNA. Open reading frames (ORFs) are indicated above and below the genome. ORF1 (red) encodes the polyprotein composed of a transmembrane domain, VPg, a serine protease, and C-terminal protein(s). ORF2 (yellow) encodes the RNA directed-RNA polymerase. ORF 3 (green) encodes the capsid protein. ORF5 (blue) encodes, by a readthrough mechanism, an aphid transmission factor. Other ORFs have no sequence homology at the family level. ORF0 encodes a viral RNA silencing suppressor. Protein encoded by ORF3a is needed for systemic infection. ORF4 encodes a movement protein. ORFs 6 and 7 encode proteins regulating the virus transcription and replication. ORF8 encodes a replication-associated protein. The hatched area indicates an overlap between two ORFs in the same reading frame. The switch to ORF2 is determined by a -1 ribosomal frameshift (dashed line). ORFs 3a to ORF7 are expressed from subgenomic RNAs. Reproduced from Sõmera *et al.* (2021).

I.4. Tomato chlorosis virus

Symptoms of yellowing novel to the farmers were observed in tomato plants grown in California in the 1980s. Initially, the symptoms were attributed to physiological or nutritional

disorders or even to cytotoxicity of pesticides, although the presence of whiteflies associated with symptomatic plants suggested a viral disease. The causal agent was named tomato infectious chlorosis virus (TICV), showing that it was a member of the genus *Crinivirus*, belonging to the family *Closteroviridae* transmitted by the whitefly *Trialeurodes vaporariorum* (Duffus *et al.*, 1996). In 1981, a “yellowing leaf disease” was reported in tomato plants grown in Florida, with similar symptoms to those caused by TICV. However, the virus causing this disease lacked cross-hybridization with TICV-specific probes which along differences in the range of insect vectors, the new one being transmitted in addition to *T. vaporariorum* by *Bemisia tabaci*, suggested the presence of a second crinivirus infecting tomato (Wisler *et al.*, 1998b). After biological and partial genomic characterization, this virus was named tomato chlorosis virus (ToCV).

As with symptoms caused by TICV, leaf symptoms caused by ToCV can be confused with physiological or nutritional disorders. However, the evolution of the disease presents specific patterns (Fiallo-Olivé and Navas-Castillo, 2019; Wisler *et al.*, 1998b). Thus, interveinal chlorosis begins in older leaves, progressing toward the upper part of the plant. In the initial stage of the infection, chlorotic areas are frequently polygonal in shape, which is limited by the main veins. In advanced stages, interveinal yellow areas can develop reddish-brown necrotic flecks. Lower leaves appear to be rolled, thickened and brittle, being crispy to the touch (Figure I.4). Loss of photosynthetic area could be the responsible for the observed reduction of plant vigor. Although floral abortion has been described in ToCV-infected pepper (Fortes *et al.*, 2012), no conspicuous symptoms are observed in infected tomato flowers. However, ToCV infection reduces tomato fruit growth and delays ripening, diminishing marketable fruit yield.

As it was stated above, ToCV is transmitted by members of two whitefly genera, *Trialeurodes* and *Bemisia*, i.e. *T. vaporariorum*, *T. abutilonea* and several species of the *B. tabaci* complex (New World-1, Middle East Asia Minor 1 and Mediterranean) (Navas-Castillo *et al.*, 2000; Wisler *et al.*, 1998b).

In Spain, symptoms similar to those associated with TICV/ToCV in the United States were initially detected in tomato crops in Almería and Málaga provinces in 1997. It was soon confirmed that the causal virus was ToCV, this being the first report of the virus outside the United States (Navas-Castillo *et al.*, 2000). A survey carried out in this area in 1999 showed that infections were already very common in 1999. Subsequent studies revealed a wide

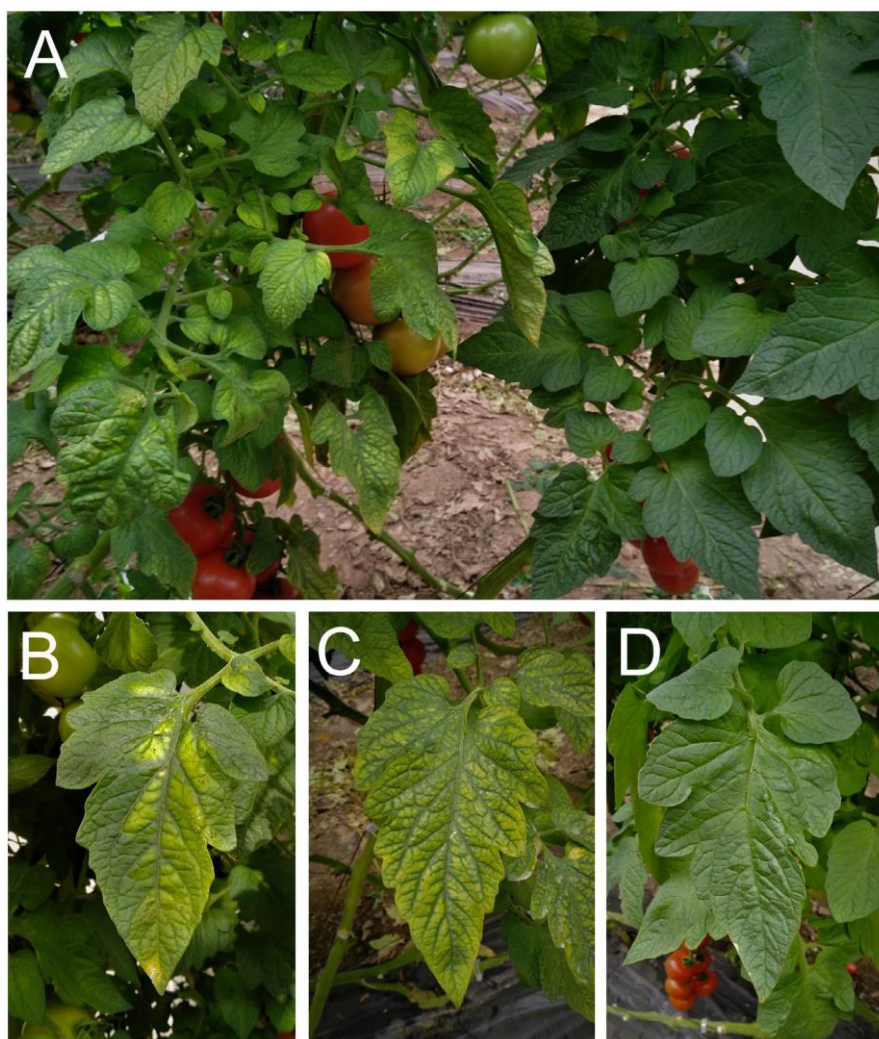


Figure I.4. Symptoms of tomato chlorosis virus infection on naturally infected tomato plants in southern Spain. A. Commercial plant showing generalized yellowing (left) close to a healthy plant (right). Individual leaflets showing polygonal chlorotic areas (B) or interveinal bright yellowing (C) are shown alongside an asymptomatic leaflet (D). Reproduced from Fiallo-Olivé and Navas-Castillo (2019).

distribution of the virus in Spain, including the southern provinces (Almería, Málaga and Seville), the eastern provinces (Alicante and Castellón), the Balearic Islands and the Canary Islands (Font *et al.*, 2003).

Since ToCV was first identified in the United States and then in Spain, the virus has been reported to greatly expand its geographic range. Thus, ToCV has been reported to date from more than 40 countries and territories in the Americas, Europe, Africa and Asia (Figure

I.5, Table I.2). In those cases where national-scale surveys have been carried out, ToCV has generally showed a wide distribution, e.g. in Greece (Dovas *et al.*, 2002; Orfanidou *et al.*, 2014), Indonesia (Suastika *et al.*, 2011), Korea (Lee *et al.*, 2018) and South Africa (Moodley *et al.*, 2019a,b). Access to powerful detection techniques, including increasing availability of next-generation sequencing platforms, will presumably broaden the number of locations where ToCV will be reported.

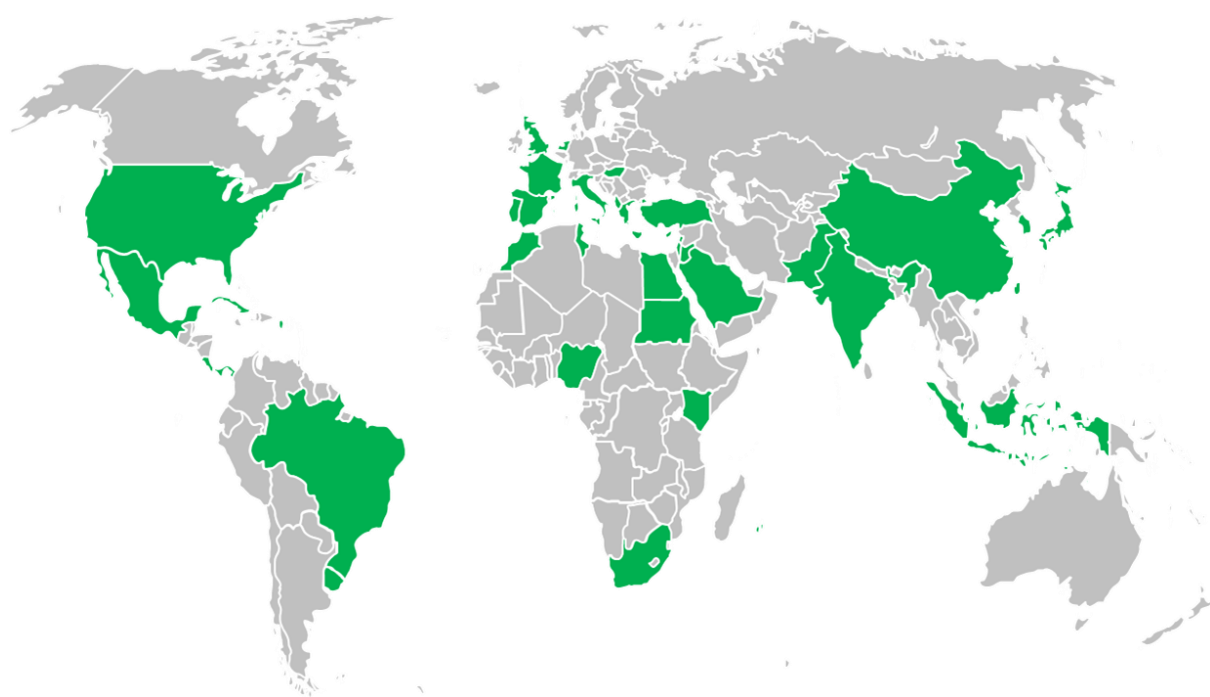


Figure I.5. Geographical distribution of tomato chlorosis virus. Countries and territories where the virus has been found (listed in Table I.2) are in green.

Although ToCV was first identified in tomatoes, it naturally infects more than 30 species belonging to family *Solanaceae*, including important crops as sweet pepper (*Capsicum annuum*) (Lozano *et al.*, 2004), potato (*Solanum tuberosum*) (Fortes and Navas-Castillo, 2008) and eggplant (*Solanum melongena*) (Zhou *et al.*, 2015). In total, natural ToCV infection has been reported in more than 120 dicotyledonous plant species belonging to 32 families (Table I.3). Also, 25 additional species have been reported as experimental hosts (Table I.4).

Table I.2. Dates and references for first reports of tomato chlorosis virus in the countries and territories showed in Figure I.5.

Year	Country/Territory	Reference
1996	United States	Wisler <i>et al.</i> , 1998b
1997	Spain	Navas-Castillo <i>et al.</i> , 2000
1998	Portugal	Louro <i>et al.</i> , 2000
1998	Taiwan	Tsai <i>et al.</i> , 2004
2000	Italy	Accotto <i>et al.</i> , 2001
2000	Morocco	Hanafi, 2002
2000	South Africa	Jones, 2001
2001	Canary Island (ES)	Font <i>et al.</i> , 2003
2001	Greece	Dovas <i>et al.</i> , 2002
2001	Puerto Rico (US)	Wintermantel <i>et al.</i> , 2001
2002	France	Dalmon <i>et al.</i> , 2005
2003	Israel	Segev <i>et al.</i> , 2004
2004	Cyprus	Papayiannis <i>et al.</i> , 2006
2004	Réunion (FR)	Delatte <i>et al.</i> , 2006
2005	Mayotte Island (FR)	Massé <i>et al.</i> , 2008
2005	Mexico	Alvarez-Ruiz <i>et al.</i> , 2007
2006	Brazil	Barbosa <i>et al.</i> , 2008
2006	Cuba	Martínez-Zubiaur <i>et al.</i> , 2008
2006	Lebanon	Abou-Jawdah <i>et al.</i> , 2006
2007	Costa Rica	Castro <i>et al.</i> , 2009
2007	Mauritius	Lett <i>et al.</i> , 2009
2008	Indonesia	Suastika <i>et al.</i> , 2011
2008	Japan	Hirota <i>et al.</i> , 2010
2008	Turkey	Çevik and Erkiş, 2008
2010	Hungary	Bese <i>et al.</i> , 2011
2010	Tunisia	Gharsallah <i>et al.</i> , 2015
2011	Sudan	Fiallo-Olivé <i>et al.</i> , 2011
2012	China	Zhao <i>et al.</i> , 2013
2012	Uruguay	Arruabarrena <i>et al.</i> , 2014
2013	Korea	Lee <i>et al.</i> , 2018
2014	Jordan	Salem <i>et al.</i> , 2015
2014	Saudi Arabia	Al-Saleh <i>et al.</i> , 2014
2016	India	Chaudhary <i>et al.</i> , 2023
2017	Kenya	Harun <i>et al.</i> , 2020
2017	Netherlands	EPPO, 2018a
2017	Nigeria	Mohammed <i>et al.</i> , 2018
2018	United Kingdom	EPPO, 2018b
2018	Panama	Galipienso <i>et al.</i> , 2021
2019	Egypt	Abdel-Salam <i>et al.</i> , 2019
2019	Pakistan	Raza <i>et al.</i> , 2020
2021	Albania	Orfanidou <i>et al.</i> , 2022

Table I.3. Natural plant hosts reported for tomato chlorosis virus.

Family	Plant Species	Reference
Acanthaceae	<i>Daedalacanthus nervosus</i>	Abdel-Salam <i>et al.</i> , 2019
Amaranthaceae	<i>Amaranthus retroflexus</i>	Orfanidou <i>et al.</i> , 2014
	<i>Amaranthus sylvestris</i>	Abdel-Salam <i>et al.</i> , 2019
	<i>Amaranthus viridis</i>	Shakeel <i>et al.</i> , 2017
	<i>Alternanthera philoxeroides</i>	Tang <i>et al.</i> , 2017
	<i>Chenopodium album</i>	Orfanidou <i>et al.</i> , 2014
	<i>Chenopodium amaranticolor</i>	Gharsallah <i>et al.</i> , 2015
	<i>Chenopodium ficifolium</i>	Kil <i>et al.</i> , 2015b
	<i>Chenopodium murale</i>	Font <i>et al.</i> , 2004
	<i>Chenopodium opulifolium</i>	Shakeel <i>et al.</i> , 2017
Apiaceae	<i>Ammi majus</i>	Abdel-Salam <i>et al.</i> , 2019
	<i>Coriandrum sativum</i>	Abdel-Salam <i>et al.</i> , 2019
Apocynaceae	<i>Calotropis procera</i>	Shakeel <i>et al.</i> , 2017
	<i>Metaplexis japonica</i>	Abdel-Salam <i>et al.</i> , 2019
Araceae	<i>Anadendrum affine</i>	Abdel-Salam <i>et al.</i> , 2019
Araliaceae	<i>Aralia nudicaulis</i>	Shakeel <i>et al.</i> , 2017
	<i>Schefflera arboricola</i>	Abdel-Salam <i>et al.</i> , 2019
Asteraceae	<i>Bidens bipinnata</i>	Abdel-Salam <i>et al.</i> , 2019
	<i>Cirsium arvense</i>	Orfanidou <i>et al.</i> , 2014
	<i>Conyza sp.</i>	Orfanidou <i>et al.</i> , 2014
	<i>Conyza canadensis</i>	Kil <i>et al.</i> , 2015b
	<i>Erechtites hieracifolia</i>	Kil <i>et al.</i> , 2015a
	<i>Erigeron annuus</i>	Kil <i>et al.</i> , 2015a
	<i>Lactuca saligna</i>	Shakeel <i>et al.</i> , 2017
	<i>Lactuca serriola</i>	Shakeel <i>et al.</i> , 2017
	<i>Lactuca sativa</i>	Orfanidou <i>et al.</i> , 2014
	<i>Sonchus asper</i>	Kil <i>et al.</i> , 2015b
	<i>Sonchus olereaceus</i>	Shakeel <i>et al.</i> , 2017
	<i>Zinnia elegans</i>	Tsai <i>et al.</i> , 2004
	<i>Youngia japonica</i>	Kil <i>et al.</i> , 2015b
Boraginaceae	<i>Heliotropium lasiocarpum</i>	Shakeel <i>et al.</i> , 2017
	<i>Trigonitis peduncularis</i>	Kil <i>et al.</i> , 2015b
Brassicaceae	<i>Brassica sp.</i>	Solórzano <i>et al.</i> , 2011
	<i>Brassica oleracea</i>	Abdel-Salam <i>et al.</i> , 2019
	<i>Cardamine flexuosa</i>	Kil <i>et al.</i> , 2015b
	<i>Eruca vesicaria</i>	Boiteux <i>et al.</i> , 2016
	<i>Raphanus raphanistrum</i>	Boiteux <i>et al.</i> , 2016
	<i>Sisymbrium irio</i>	Abdel-Salam <i>et al.</i> , 2019
Caryophyllaceae	<i>Cerastium glomeratum</i>	Kil <i>et al.</i> , 2015b
	<i>Stellaria media</i>	Kil <i>et al.</i> , 2015b
Chenopodiaceae	<i>Chenopodium album</i>	Orfanidou <i>et al.</i> , 2014

	<i>Chenopodium murale</i>	Shakeel <i>et al.</i> , 2017
	<i>Chenopodium opulifolium</i>	Shakeel <i>et al.</i> , 2017
Convolvulaceae	<i>Convolvulus arvensis</i>	Orfanidou <i>et al.</i> , 2014
	<i>Ipomoea batatas</i>	Abdel-Salam <i>et al.</i> , 2019
	<i>Ipomoea coccinea</i>	Kil <i>et al.</i> , 2015b
	<i>Ipomoea hederaceae</i>	Kil <i>et al.</i> , 2015b
Cucurbitaceae	<i>Cucumis melo</i>	Abdel-Salam <i>et al.</i> , 2019
	<i>Cucumis sativus</i>	Abdel-Salam <i>et al.</i> , 2019
	<i>Cucurbita moschata</i>	Solórzano <i>et al.</i> , 2011
	<i>Luffa aegyptiaca</i>	Abdel-Salam <i>et al.</i> , 2019
	<i>Momordica charantia</i>	Shakeel <i>et al.</i> , 2017
Euphorbiaceae	<i>Codiaeum variegatum</i>	Abdel-Salam <i>et al.</i> , 2019
	<i>Euphorbia geniculata</i>	Abdel-Salam <i>et al.</i> , 2019
	<i>Jatropha integerrima</i>	Abdel-Salam <i>et al.</i> , 2019
	<i>Ricinus communis</i>	Abdel-Salam <i>et al.</i> , 2019
Fabaceae	<i>Bauhinia variegata</i>	Abdel-Salam <i>et al.</i> , 2019
	<i>Glycine max</i>	Abdel-Salam <i>et al.</i> , 2019
	<i>Leucaena leucocephala</i>	Abdel-Salam <i>et al.</i> , 2019
	<i>Phaseolus vulgaris</i>	Shakeel <i>et al.</i> , 2017
	<i>Vicia angustifolia</i>	Kil <i>et al.</i> , 2015b
	<i>Vicia faba</i>	Abdel-Salam <i>et al.</i> , 2019
	<i>Vigna unguiculata</i>	Wang <i>et al.</i> , 2018
	<i>Vicia tetrasperma</i>	Kil <i>et al.</i> , 2015b
Fumariaceae	<i>Fumaria officinalis</i>	Orfanidou <i>et al.</i> , 2014
Geraniaceae	<i>Pelargonium hirsutum</i>	Abdel-Salam <i>et al.</i> , 2019
Lamiaceae	<i>Tectona grandis</i>	Borges <i>et al.</i> , 2019
Malvaceae	<i>Abelmoschus esculentus</i>	Shakeel <i>et al.</i> , 2017
	<i>Abutilon theophrasti</i>	Orfanidou <i>et al.</i> , 2014
	<i>Alcea rosea</i>	Abdel-Salam <i>et al.</i> , 2019
	<i>Corchorus olitorius</i>	Abdel-Salam <i>et al.</i> , 2019
	<i>Gossypium barbadense</i>	Abdel-Salam <i>et al.</i> , 2019
	<i>Gossypium hirsutum</i>	Abdel-Salam <i>et al.</i> , 2019
	<i>Hibiscus cannabinus</i>	Abdel-Salam <i>et al.</i> , 2019
	<i>Hibiscus rosa-sinensis</i>	Abdel-Salam <i>et al.</i> , 2019
	<i>Malva parviflora</i>	Shakeel <i>et al.</i> , 2017
	<i>Malva sylvestris</i>	Orfanidou <i>et al.</i> , 2014
Mazaceae	<i>Mazus pumilus</i>	Kil <i>et al.</i> , 2015b
Moraceae	<i>Ficus carica</i>	Abdel-Salam <i>et al.</i> , 2019
	<i>Ficus nitida</i>	Abdel-Salam <i>et al.</i> , 2019
	<i>Morus alba</i>	Abdel-Salam <i>et al.</i> , 2019
Oxalidaceae	<i>Oxalis pes-caprae</i>	Orfanidou <i>et al.</i> , 2014
Phytolacaceae	<i>Phytolacca americana</i>	Kil <i>et al.</i> , 2015b
	<i>Phytolacca icosandra</i>	Solórzano <i>et al.</i> , 2011
Plantaginaceae	<i>Plantago major</i>	Solórzano <i>et al.</i> , 2011

Polygonaceae	<i>Rumex sp.</i>	Orfanidou <i>et al.</i> , 2014
Portulacaceae	<i>Portulaca oleracea</i>	Orfanidou <i>et al.</i> , 2014
Primulaceae	<i>Anagalis foemina</i>	Orfanidou <i>et al.</i> , 2014
Rubiaceae	<i>Pentas lanceolata</i>	Abdel-Salam <i>et al.</i> , 2019
Rutaceae	<i>Ruta chalepensis</i>	Solórzano <i>et al.</i> , 2011
Solanaceae	<i>Capsicum annuum</i>	Lozano <i>et al.</i> , 2004
	<i>Datura stramonium</i>	Alvarez-Ruiz <i>et al.</i> , 2007
	<i>Nicandra physaloides</i>	Souza <i>et al.</i> , 2019
	<i>Nicotiana tabacum</i>	Fiallo-Olivé <i>et al.</i> , 2014
	<i>Physalis angulata</i>	Fonseca <i>et al.</i> , 2013
	<i>Physalis ixocarpa</i>	Trenado <i>et al.</i> , 2007
	<i>Physalis peruviana</i>	Trenado <i>et al.</i> , 2007
	<i>Solanum aethiopicum</i>	Fonseca <i>et al.</i> , 2016
	<i>Solanum americanum</i>	Arruabarrena <i>et al.</i> , 2015
	<i>Solanum arcanum</i>	García-Cano <i>et al.</i> , 2010
	<i>Solanum chilense</i>	García-Cano <i>et al.</i> , 2010
	<i>Solanum chmielewskii</i>	García-Cano <i>et al.</i> , 2010
	<i>Solanum corneliomulleri</i>	García-Cano <i>et al.</i> , 2010
	<i>Solanum elaeagnifolium</i>	Gharsallah <i>et al.</i> , 2015
	<i>Solanum galapagense</i>	García-Cano <i>et al.</i> , 2010
	<i>Solanum habrochaites</i>	García-Cano <i>et al.</i> , 2010
	<i>Solanum huaylasense</i>	García-Cano <i>et al.</i> , 2010
	<i>Solanum jamaicense</i>	Boiteux <i>et al.</i> , 2018
	<i>Solanum lycopersicum</i>	Wisler <i>et al.</i> , 1998b
	<i>Solanum mammosum</i>	Boiteux <i>et al.</i> , 2018
	<i>Solanum melongena</i>	Zhou <i>et al.</i> , 2015
	<i>Solanum neorickii</i>	García-Cano <i>et al.</i> , 2010
	<i>Solanum nigrescens</i>	Alvarez-Ruiz <i>et al.</i> , 2007
	<i>Solanum nigrum</i>	Font <i>et al.</i> , 2004
	<i>Solanum paniculatum</i>	Boiteux <i>et al.</i> , 2018
	<i>Solanum pennellii</i>	García-Cano <i>et al.</i> , 2010
	<i>Solanum peruvianum</i>	García-Cano <i>et al.</i> , 2010
	<i>Solanum pimpinellifolium</i>	García-Cano <i>et al.</i> , 2010
	<i>Solanum scuticum</i>	Boiteux <i>et al.</i> , 2018
	<i>Solanum sessiliflorum</i>	Boiteux <i>et al.</i> , 2018
	<i>Solanum sisymbriifolium</i>	Arruabarrena <i>et al.</i> , 2015
	<i>Solanum stramoniiifolium</i>	Boiteux <i>et al.</i> , 2018
	<i>Solanum subinerme</i>	Boiteux <i>et al.</i> , 2018
	<i>Solanum tuberosum</i>	Fortes and Navas-Castillo, 2008
	<i>Solanum velleum</i>	Boiteux <i>et al.</i> , 2018
	<i>Withania somnifera</i>	Abdel-Salam <i>et al.</i> , 2019
Zygophyllaceae	<i>Tribulus terrestris</i>	Shakeel <i>et al.</i> , 2017

Table I.4. Experimental plant hosts reported for tomato chlorosis virus.

Family	Plant species	Reference
Aizoaceae	<i>Tetragonia expansa</i>	Wintermantel and Wisler, 2006
Amaranthaceae	<i>Beta vulgaris</i>	Fariña <i>et al.</i> , 2019
	<i>Beta macrocarpa</i>	Wintermantel and Wisler, 2006
	<i>Chenopodium capitatum</i>	Wintermantel and Wisler, 2006
	<i>Chenopodium quinoa</i>	Fariña <i>et al.</i> , 2019
	<i>Gomphrena globosa</i>	Wintermantel and Wisler, 2006
	<i>Spinacia oleracea</i>	Wintermantel and Wisler, 2006
Apocynaceae	<i>Vinca rosea</i>	Wintermantel and Wisler, 2006
Asteraceae	<i>Calendula officinalis</i>	Wintermantel and Wisler, 2006
	<i>Callistephus chinensis</i>	Wintermantel and Wisler, 2006
Plumbaginaceae	<i>Limonium latifolium</i>	Wintermantel and Wisler, 2006
Solanaceae	<i>Cyphomandra betacea</i>	Wintermantel and Wisler, 2006
	<i>Jaltomata procumbens</i>	Fariña <i>et al.</i> , 2019
	<i>Nicotiana benthamiana</i>	Wintermantel <i>et al.</i> , 2001
	<i>Nicotiana clevelandii</i>	Wisler <i>et al.</i> , 1998b
	<i>Nicotiana edwardsonii</i>	Wintermantel and Wisler, 2006
	<i>Nicotiana glutinosa</i>	Hirota <i>et al.</i> , 2010
	<i>Nicotiana megalosiphon</i>	Wintermantel and Wisler, 2006
	<i>Petunia hybrida</i>	Wintermantel and Wisler, 2006
	<i>Physalis alkengi</i>	Wintermantel and Wisler, 2006
	<i>Physalis pruinosa</i>	Fariña <i>et al.</i> , 2019
	<i>Physalis wrightii</i>	Wintermantel <i>et al.</i> , 2001
	<i>Solanum acaule</i>	Wintermantel and Wisler, 2006
	<i>Solanum viarum</i>	Fariña <i>et al.</i> , 2019
Umbelliferae	<i>Anthriscus cereifolium</i>	Morris <i>et al.</i> , 2006

I. 4.1. Genome organization

The genus *Crinivirus* (from Latin *crinis*, meaning hair, from the appearance of the very long thread-like particles) in the family *Closteroviridae* (from Greek *kloster*, meaning spindle or thread) (*Riboviria*, *Orthornavirae*, *Kitrinoviricota*, *Alsuviricetes*, *Martellivirales*) contains long flexuous viruses with bipartite (with the exception of a tripartite species) ssRNA genomes. The family *Closteroviridae* also includes the genera *Ampelovirus*, *Bluvavirus*, *Closterovirus*, *Menthavirus*, *Olivavirus* and *Velarivirus* (Fuchs *et al.*, 2020; <https://ictv.global/taxonomy>).

General features of closterovirids include restriction to phloem and companion cells and large ssRNA genomes of positive polarity lacking a poly (A)-tail, the largest RNA genomes among plant viruses (15.5 to 19.5 kb). This size translates into a great gene complexity, including the acquisition of eukaryotic genes (e.g. the chaperone heat shock protein 70

homologue) and gene duplication [e.g. duplication of the capsid protein gene resulting in a major (CP) and a minor (CPm) coat protein] (Dolja *et al.*, 2006). Closterovirids are transmitted in a semi-persistent manner by insects belonging to the order Hemiptera. Members of each genus in the family *Closteroviridae* are transmitted by specific insect vectors, i.e. ampeloviruses and velaviruses by mealybugs (Pseudococcidae), criniviruses by whiteflies (Aleyrodidae), and closteroviruses and menthavirus by aphids (Aphididae) (Fuchs *et al.*, 2020; Karasev, 2000; Tzanetakis *et al.*, 2007; Zhang *et al.*, 2022).

Closterovirid virions (650 to 2200 in length and 12 nm of diameter) have a helical morphology similar to many other filamentous plant viruses as members of families *Alphaflexiviridae*, *Betaflexiviridae* or *Poytyviridae*. However, closterovirids are unique in that their virions have an asymmetric morphology, with a “body” and a “tail”, the body constituted by the CP and the tail by the CPm and units of some other viral proteins, hence the name “rattlesnake” for this peculiar structure (Figure I.6) (Agranovsky *et al.*, 1995; Dolja, 2003; Tian *et al.*, 1999). The RNA molecules of segmented closterovirids, i.e. the criniviruses, are encapsidated in separate virions (Liu *et al.*, 2000).

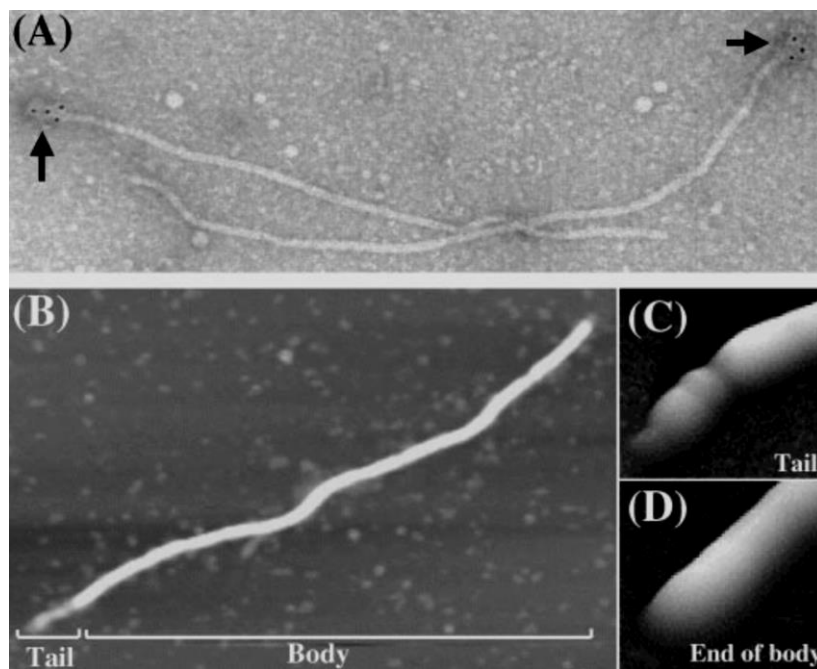


Figure I.6. Morphology of a typical monopartite closterovirid, beet yellows virus (BYV). (A) Electron micrograph of two BYV virions. Virion tails marked by arrows were immunogold labelled using anti-CPm serum. (B) Atomic force microscopy image of a single BYV virion showing the body and the tail. (C, D) 3D reconstruction of the virion ends obtained using atomic force microscopy. Reproduced from Dolja (2003).

ToCV has a bipartite genome with the typical organization of members of the genus *Crinivirus* (Figure I.7). RNA1 contains four ORFs flanked by two untranslated regions (UTRs) located at the 5' and 3' ends of the molecule. ORF1a is translated into a polyprotein containing the protease (PRO), methyltransferase (MET) and helicase (HEL) domains conserved in all closterovirids (Karasev, 2000). ORF1b encodes the RNA-dependent RNA polymerase, which is translated by a ribosomal +1 frameshift. ORF2 encodes a 22 kDa protein which has been described as a potent suppressor of RNA silencing (Cañizares *et al.*, 2008). ORF3 encodes a putative protein of 6 kDa with a predicted transmembrane domain, similar to other proteins located at the 3' end of other criniviruses, with unknown function.

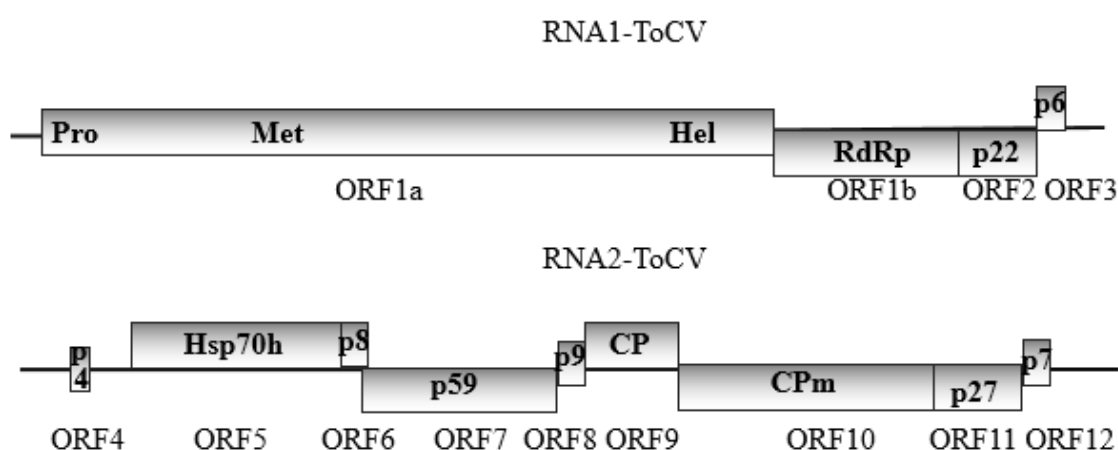


Figure I.7. Genome organization of tomato chlorosis virus RNA1 and RNA2. Putative protein products are indicated inside the open reading frames (ORFs). CP, coat protein; CPm, minor coat protein; Hel, helicase; HSP70h, heat shock protein 70 homologue; Met, methyl transferase; Pro, protease; RdRp, RNA-dependent RNA polymerase.

ToCV RNA2 encodes nine ORFs also flanked by UTRs located at both ends of the molecule. RNA2 includes the block of five genes characteristic of the family Closteroviridae that would be involved in genome encapsidation, viral movement in plant and interaction with the insect vector. This gene block is constituted by ORF4 that encodes protein p4, rich in hydrophobic residues; ORF5 encoding the Hsp70h (related to the heat shock protein 70 homologue (Hsp70) eukaryotic protein family); ORF7 encoding protein p59, and ORF9 and ORF10 encoding the major (CP) and minor (CPm) capsid proteins, respectively. Both CP and CPm have been identified as suppressors of gene silencing (Cañizares *et al.*, 2008). ORF6 encodes a putative p8 protein with size, genomic position and sequence similar to proteins of

other criniviruses (Livieratos *et al.*, 2004; Orflio and Navas-Castillo, 2009). ORF8 encodes a putative p9 protein, found only in members of the genus *Crinivirus* and unknown function. ORF11 encodes the p27 protein, which seems to be unique to criniviruses (Dolja *et al.*, 2006). Finally, ORF12 encodes a putative p7 with a transmembrane domain which does not show significant similarity to any protein encoded by the genomes of other criniviruses, thus seeming to be exclusive of ToCV (Lozano *et al.*, 2006; Wintermantel *et al.*, 2005).

In base of similarity with other closterovirids, the CP protein would form the long virion body and the CPm protein, along with units of at least Hsp70h and p59 proteins, would form the virion tail (Agranovsky *et al.*, 1995). The CPm protein, by homology with what has been described for lettuce infectious yellows virus would be involved in transmission by the insect vector (Chen *et al.*, 2011; Stewart *et al.*, 2010; Tian *et al.*, 1999).

Each of the 5' UTRs of ToCV RNA1 and RNA2 consists of 301-303 and 238 nucleotides, respectively, with a moderate nucleotide identity of 33% between them. The first six nucleotides are identical in RNA1 and RNA2, a characteristic phenomenon of segmented viral genomes. The nucleotide sequences of the 3'-UTRs of RNA1 and RNA2 consist of 176 and 212-218 nt, respectively, having an identity of 81 to 88,6% depending on the ToCV isolate (Lozano *et al.*, 2006, 2007; Wintermantel *et al.*, 2005). The 3'-UTR of the negative chains are putatively involved in viral replication by initiation of positive chains. On the other hand, motifs present in secondary structures of 5'-UTR of citrus tristeza virus and grapevine leafroll-associated virus 3 have been suggested to be involved in virion assembly initiation (Gowda *et al.*, 2003; Jarugula *et al.*, 2018). Also, hairpin structures and pseudoknots have been identified in the 3'-UTR end of positive sense RNAs of ToCV and other closterovirids that would be putatively involved in replication by initiation and regulation of the negative chain synthesis (Kataya *et al.*, 2008; Kreuze *et al.*, 2002; Livieratos *et al.*, 2004; Mongkolsiriwattana *et al.*, 2016; Satyanarayana *et al.*, 2002).

I.5. Infectious clones of members of the family Closteroviridae

Infectious clones are versatile tools for granting the unambiguous identity of plant viruses, thus confirming Kock's postulates (Bos, 1981). They allow us to obtain pure viral populations avoiding mixed infections even in the cases of viruses found in low titres in the plant hosts, such as closterovirids. The availability of such clones is also extremely useful for,

by reproducing the disease caused by a particular virus, determining its host range or identifying sources of genetic resistance. Also, viral infectious clones are indispensable when carrying out functional studies through reverse genetics by introducing different types of mutations, thus providing information about functionality of viral proteins and virus biology (Boyer and Haenni, 1994).

To obtain infectious clones of ssRNA plant viruses, the full-length cDNA of the viral genome uses to be synthesized by reverse transcription, occasionally hampered by secondary structures in RNA template, PCR amplified, and cloned into a suitable vector reviewed by Boyer and Haenni (1994). Long genomes are sometimes assembled by sorting out overlapping fragments that share restriction sites (Dawson *et al.*, 1986), requiring high fidelity polymerases for those longer templates (Lai, 2000). To initiate *in vitro* transcription from the full-length clones, a bacteriophage (T7, SP6 or T3) RNA polymerase promoter can be fused to the 5'-end of the cDNA and a restriction enzyme in the 3' end is used to terminate transcription. Post-transcriptionally, incorporation of a cap analogue (m7GpppN) at the 5' end of the *in vitro* transcripts has been reported to increase the infectivity in many cases (López-Moya and García, 2000). Capped *in vitro* transcripts can be inoculated mechanically in the host plants. In those cases where *in vivo* transcription is preferred, the constitutive 35S promoter of cauliflower mosaic virus is an excellent candidate to initiate transcription, the nopaline synthase terminator (*nos*) of *Agrobacterium tumefaciens* being used to terminate transcription. In these cases, constructs use to be cloned in a binary vector to mediate infection using transformed *Agrobacterium tumefaciens* (agroinoculation).

Infectious clones need to be designed to position the first viral nucleotide in the first transcribed nucleotide behind the promoter, also avoiding extra nucleotides at the 3' end. Extra non-viral nucleotides at both ends of transcripts have been shown to have adverse effects on infectivity (reviewed by Boyer and Haenni, 1994). In this regard, it should be mentioned that two polyadenylation signals creating different termination/polyadenylation sites have been identified in the *nos* terminator (Depicker *et al.*, 1982). In fact, mapping of the polyadenylation site in transcripts controlled by the *nos* terminator shows a diffuse pattern, with cleavage happening in several different locations after the polyadenylation signal. Also, it should be taking in account that a poly (A) tail is not present in many RNA viruses, as it the case for example of members of families *Closteroviridae* and *Solemoviridae*. An alternative to solve

these difficulties is to incorporate the hepatitis delta virus ribozyme sequence (HDV-Rb) at the 3' end of the viral genome, thus matching the auto-scission site with the last viral nucleotide. HDV-Rb sequence can be included within a cloning vector to facilitate the construction of infectious clones. An example of such cloning vectors is the pJL89 vector (Lindbo, 2007) that has been successfully used to accommodate large cDNA inserts to be transcribed without any extra viral nucleotides (e.g. Ferriol *et al.*, 2016; Ruiz-Ramón *et al.*, 2019).

The large size of some viral RNAs, as is seen in the case of plant closterovirids and animal coronavirids, present several obstacles when constructing an infectious cDNA or RNA transcript: i) long RNA sequences make the synthesis of an accurate cDNA molecule difficult, because the fidelity of reverse transcriptase–PCR (RT-PCR) for the amplification of cDNA decreases in proportion to the RNA length, ii) long RNA sequences are more likely to code for toxic proteins, which make the cDNA sequence in plasmids unstable, and iii) it is difficult to find a suitable vector that can accommodate large cDNA inserts (reviewed by Lai, 2000). Even so, these difficulties have been largely overcome and infectious clones have been constructed for the largest RNA genome (ca. 20 kb) of a plant virus, the closterovirus citrus tristeza virus (Satyanarayana *et al.*, 1999), and for coronaviruses (Almazán *et al.*, 2000).

Once constructed, the infectious clones of closterovirids are difficult to maintain due to the existence of viral sequences putatively encoding products with toxicity for *Escherichia coli*. The instability caused by toxicity has been overcome for closterovirids and other ssRNA viruses with long genomes by using special strains of *E. coli* grown at low temperatures (30 °C) with extra nutritious culture media (Sun *et al.*, 2009), by introducing plant intron insertions to interrupt the toxic sequences (Ambrós *et al.*, 2011), by introducing frameshift mutations to avoid translation of toxic proteins (Satyanarayana *et al.*, 2003), and by using low copy number vectors (Almazán *et al.*, 2000).

Infectious clones designed to produce *in vitro* viral transcripts present a number of limitations. Thus, long and fragile *in vitro* transcripts are difficult to manipulate and inoculate (Satyanarayana *et al.*, 2001) and the presence of mutations, extraviral nucleotides, absence of cap structure, and the requirement of precise 5'- and 3'-end sequences can compromise transcript infectivity (Boyer and Haenni, 1994). Also, transcripts usually need to be inoculated into protoplasts of model plants such as *N. benthamiana* or *N. tabacum* to generate a viral progeny. In the cases where virions are obtained in protoplasts, successive passes are needed to

obtain a virus amount sufficient to inoculate plants (Satyanarayana *et al.*, 2001).

To avoid the difficulties associated with the production and inoculation of viral transcripts obtained *in vitro*, *in vivo* transcription of infectious cDNA clones is an alternative. These clones can be inoculated into plants by biolistic bombardment (Gal-On *et al.*, 1995) or by agroinoculation (Grimsley *et al.*, 1986). In both cases, the viral cDNA uses to be positioned between the 35S constitutive promoter and *nos* terminator, with or without HDV-Rb sequence. After great efforts, infectious clones for *in vitro* transcription have been developed for six members of the family *Closteroviridae*: beet yellows virus (BYV) (Peremyslov *et al.*, 1998), citrus tristeza virus (CTV) (Satyanarayana *et al.*, 1999), lettuce infectious yellows virus (LIYV) (Klaassen *et al.*, 1996), lettuce chlorosis virus (LCV) (Mongkolsiriwattana *et al.*, 2011) tomato chlorosis virus (Orílio *et al.*, 2014), and cucurbit yellow stunting disorder virus (CYSDV) (Owen *et al.*, 2016). On the other hand, agroinfectious clones have been developed for the following members of the family *Closteroviridae*: BYV (Prokhnevsky *et al.*, 2002), grapevine leafroll-associated closterovirus 2 (GLRaV-2) (Liu *et al.*, 2009), CTV (Ambrós *et al.*, 2011; Gowda *et al.*, 2005), LIYV (Wang *et al.*, 2009), LCV (Chen *et al.*, 2012), and ToCV (Orílio *et al.*, 2014; Zhao *et al.*, 2016).

Agroinfectious clones of members of family *Closteroviridae* are reluctant to directly infect their natural hosts. Several reasons can explain this phenomenon, including RNA degradation triggered by double-stranded RNA viral replicative forms by plant RNA silencing (Baulcombe, 1999) and unavailability of *A. tumefaciens* strains capable of infecting specific hosts (Pruss *et al.*, 2008). Even in the cases in which *in vitro* transcripts from infectious clones have been proven to be infectious in protoplasts of the natural host [e.g. CTV in citrus (Satyanarayana *et al.*, 2001), CYSDV in cucumber (Owen *et al.*, 2016) and ToCV in tomatoes (Orílio *et al.*, 2014)], infection of whole plants has not been achieved. Although replication of grapevine leafroll-associated virus 2 takes place in young micropropagated grapevine plants after agroinoculation with an infectious clone, these results could not be reproduced in mature plants (Kurth *et al.*, 2012).

Several strategies have been developed to overcome the natural host barriers and achieve plant infection. Thus, LIYV and LCV virions purified from *N. benthamiana* plants have been transmitted to lettuce using whiteflies and an artificial membrane feeding system (Chen *et al.*, 2012; Wang *et al.*, 2009). Also, citrus trees were infected with CTV by slash-cut mechanical

inoculation in the presence of partially purified virions from leaves of agroinfected *N. benthamiana* leaves (Ambrós *et al.*, 2011; Gowda *et al.*, 2005). Also, tomato plants were infected with ToCV by grafting of stems from agroinfected *N. benthamiana* plants (Orílio *et al.*, 2014).

The manipulation of closterovirid infectious clones has allowed to increase our knowledge of the role of viral proteins in replication, encapsidation, movement through the plant and gene silencing suppression (Alzhanova *et al.*, 2000, 2001, 2007; Chiba *et al.*, 2006; Gowda *et al.*, 2003; Klaassen *et al.*, 1996; Napuli *et al.*, 2003; Peremyslov *et al.*, 1998, 1999, 2004a, 2004b; Prokhnevsky *et al.*, 2002, 2005). Also, viral mutants generated from lettuce infectious yellow virus infectious clones have been used to analyze the transmission of the virus by the insect vector (Ng and Falk, 2006a).

CHAPTER 1

EVIDENCE FOR A COMPLEX OF EMERGENT POLEROVIRUSES AFFECTING PEPPER WORLDWIDE



1.1 INTRODUCTION

The genus *Capsicum* (*Solanaceae*) is native to tropical Central and South America and comprises 27 species, though only five of them have been domesticated (Ibiza *et al.*, 2012). Among them *Capsicum annuum* L., including pungent (chili or hot pepper) and non-pungent (sweet pepper) types, is the most widely commercially cultivated, being an increasingly important spice and vegetable crop worldwide (Gniffke *et al.*, 2013). Although there are more than 70 distinct viruses known to infect pepper, only about 20 of them have been reported to cause serious damage to this crop (Moury and Verdin, 2012). Most of the viruses infecting pepper are transmitted in nature by insects, including aphids (Hemiptera: Aphididae), whiteflies (Hemiptera: Aleyrodidae) and thrips (Thysanoptera: Thripidae) (reviewed by Kenyon *et al.*, 2014).

In 1981, symptoms of vein yellowing and leaf roll were observed in pepper plants grown in greenhouses at Kitanakagusuku, Okinawa prefecture, Japan, although the disease was not reported until more than ten years later (Yonaha *et al.*, 1995). A similar disease was observed in greenhouses and open field in the Arava Valley, Israel, during 1998 (Antignus *et al.*, 2001). In addition to the symptoms observed in Japan, the Israeli group reported symptoms in fruit, which were smaller than normal and discolored, resulting in reduced commercial value. In both Japan and Israel, the disease was shown to be transmitted by the aphids *Aphis gossypii* Glover and *Myzus persicae* (Sulzer) in a persistent manner (Yonaha *et al.*, 1995; Dombrovsky *et al.*, 2010). A luteovirus was shown to be the causal agent of the disease in both countries, which was named pepper vein yellows virus in Japan (PeVYV) (Yonaha *et al.*, 1995) and pepper yellow leaf curl virus in Israel (PYLCV) (Dombrovsky *et al.*, 2010).

The family *Luteoviridae* is composed of three genera: *Luteovirus*, *Polerovirus* and *Enamovirus*, plus several species of unassigned viruses (Domier, 2012; ICTV, 2015). Virions of members of the family *Luteoviridae* are 25 to 30 nm in diameter, icosahedral and have no envelope. Poleroviruses are transmitted by aphids in a persistent circulative manner (Brault *et al.*, 2011; Fereres, and Raccach, 2015). Polerovirus genomes consist of a single molecule of linear, positive sense ssRNA ranging from 5.6 kb to 6.2 kb. A small protein (VPg) is covalently linked to the 5' end of the genomic RNA, which does not have a 3'-terminal poly (A) tract.

The typical genome organization of poleroviruses consists of seven open reading frames (ORF0 to ORF5 and ORF3a) which encode proteins P0 to P5 and P3a (Domier, 2012; Smirnova

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et al., 2015). P0 functions as a suppressor of RNA silencing. ORF1 and ORF2 overlap and encode the replication-related proteins (P1 and P2). P1 contains protease motifs and the VPg is processed from its C-terminal region. P2 is an RNA-dependent RNA polymerase (RdRp). P3 and P5 are the major and minor coat proteins, respectively, P5 being expressed as a C-terminal fusion to the core major coat protein. P5 acts as an aphid transmission and virus particle stability factor. P4 is required for long-distance movement of some poleroviruses. P3a, translated from a non-AUG codon, seems to have an essential role in viral movement.

Nucleotide sequencing of the complete genome of two virus isolates causing the yellowing disease of pepper in Japan and Israel confirmed that they belong to the genus *Polerovirus* in the family *Luteoviridae* of plant viruses (PeVYV, Murakami *et al.*, 2011; PYLCV, Dombrovsky, *et al.*, 2013). Recently, two additional isolates assigned to PeVYV, from China and Australia, respectively, have been completely sequenced (Liu *et al.*, 2016; Maina *et al.*, 2016).

In recent years, symptoms similar to those observed in Japan and Israel have been associated with the presence of poleroviruses in many countries worldwide: Turkey and Tunisia (Buzkan *et al.*, 2013); Spain (Villanueva *et al.*, 2013); India, Indonesia, Mali, Philippines, Taiwan, and Thailand (Knierim *et al.*, 2013); Sudan (Alfaro-Fernández *et al.*, 2014); United States (Alabi *et al.*, 2015); Ivory Coast (Bolou *et al.*, 2015); China (Zhang *et al.*, 2015; Tan *et al.*, 2015); Italy (Tomassoli *et al.*, 2016) and Australia (Maina *et al.*, 2016). The detection of a polerovirus associated with a yellowing disease of pepper in Spain (Villanueva *et al.*, 2013) was the first in Europe. This virus was associated with a severe outbreak of the disease in Almería province, a region in southeastern continental Spain, where almost 10,000 ha of sweet pepper are cultivated in greenhouses for fresh consumption. Disease prevalence in many greenhouses in the area reached levels of 100%, resulting in discolored pepper fruits and reduced commercial value.

In this work, the complete nucleotide sequence of a polerovirus associated with the yellowing disease of pepper in Spain was determined. The genome (6125 nt in length) has the typical organization of poleroviruses and was closer (90.7% nt sequence identity) to a pepper polerovirus from Israel. A comparison of the sequence from Spain with the four complete sequences available for PeVYV/PYLCV isolates supports, following the officially accepted sequence demarcation criterion for species of the genus *Polerovirus*, i.e., differences in amino

acid sequence identity of any gene product of greater than 10%, the existence of a complex of at least five polerovirus species whose members threaten pepper crops worldwide. Also, it was shown that recombination plays a key role in the speciation of this group of emergent poleroviruses.

1.2 MATERIALS AND METHODS

1.2.1 Virus isolate and aphid transmission

Adult aphids (*Aphis gossypii* Glover) naturally infesting symptomatic sweet pepper plants showing symptoms of interveinal yellowing and curling in leaves (Figure 1.1A) were collected from a greenhouse in Almería province (southern Spain) in February 2013. The isolate was named Spain-Almería 2-2013 (ES-Alm2-13). Aphids were transferred to three sweet pepper (cv. California Wonder) plants at the four leaf stage (Figure 1.1B), where they were allowed to feed for 48 hours. At the end of this inoculation period, plants were sprayed with insecticide and maintained in an insect- proof greenhouse with temperature control (22–27 °C day/17–20 °C night). Also, grafting was used to transmit the virus from the three sweet pepper plants experimentally infected by aphids to four additional sweet pepper plants (Figure 1.1C).

1.2.2 Virus detection

The presence of polerovirus RNA in leaf samples was determined either by reverse transcription-polymerase chain reaction (RT-PCR) or by tissue blot molecular hybridization. For RT-PCR, the pair of primers MA1754 (5'-CTG GAG CGT TGC GGA ATG-3') / MA1755 (5'-GTG TCA GGC TGT TGG TAA TCA GAC-3') was designed based on the available partial polerovirus sequence from Spain (isolate Almería-1) (Villanueva *et al.*, 2013). These primers amplify a region of 658 nt which includes part of the RdRp gene and the intergenic region. For tissue blot hybridization, freshly cross-sectioned leaf petioles and stems were squash- blotted on positively charged nylon membranes (Roche Diagnostics) and hybridized with a digoxigenin- labeled RNA probe specific to the RdRp/coat protein region of the Almería-1 isolate (Villanueva *et al.*, 2013) cloned in pGEM-T Easy (Promega). After linearization with EcoO109I, the plasmid was used for *in vitro* transcription using SP6 RNA polymerase (Roche Diagnostics) and DIG RNA Labeling Mix (Roche) to generate a RNA probe of negative

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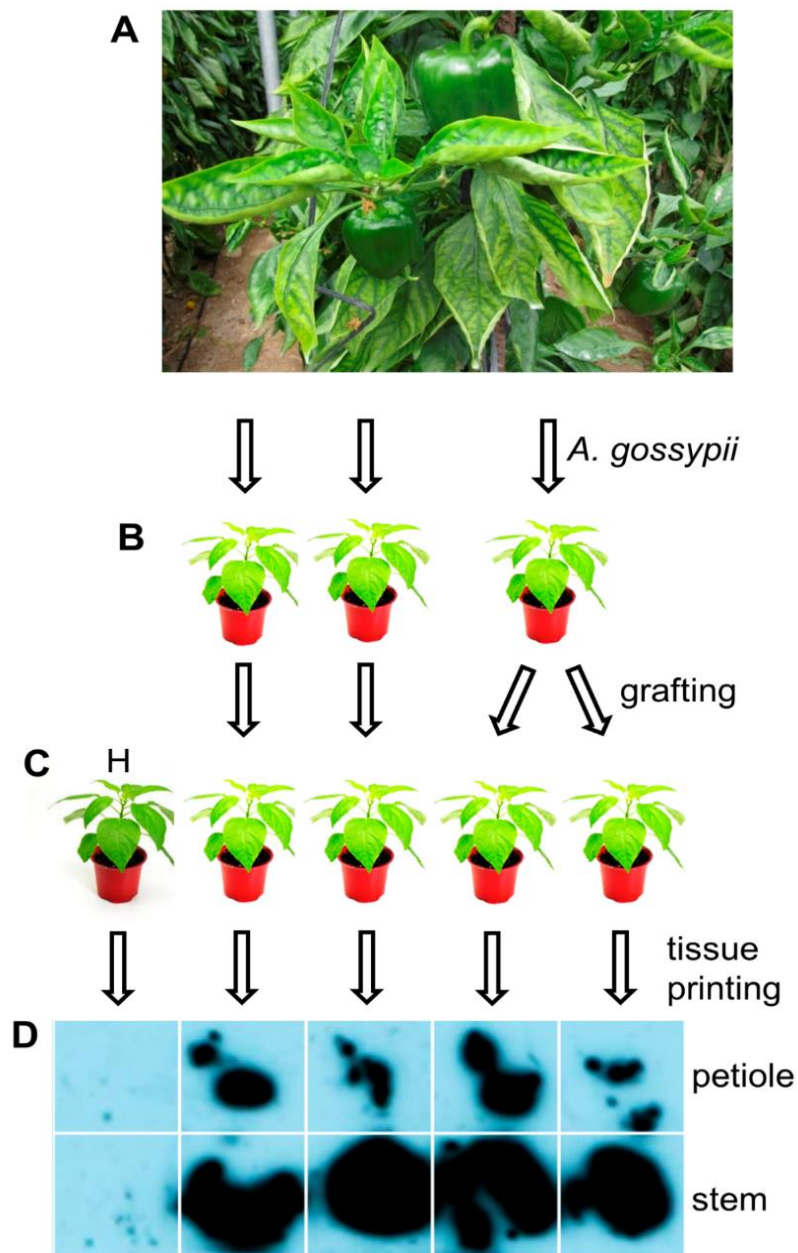


Figure 1.1. Aphid and grafting transmission assays. **A:** Polerovirus-infected pepper plant from a greenhouse located in the Almería region (southern Spain). **B:** Pepper (*Capsicum annuum*) plants (cv. California Wonder) inoculated with viruliferous aphids (*Aphis gossypii*) collected from the infected plants. **C:** Pepper plants (cv. California Wonder) inoculated by grafting. **D:** Tissue printing hybridization of petiole and stem from grafted pepper plants and of a healthy plant (H) included as a negative control.

polarity. Membranes were prehybridized for two hours with pre-hybridization solution (5 x SSC [750 mM NaCl and 75 mM sodium citrate, pH 7.0], 1% *N*-Lauroylsarcosine, 0.02% SDS and 2% blocking agent [Roche Diagnostic]) and 50% formamide. After pre-hybridization, the specific probe was added and incubated at 65°C overnight. Hybridization was carried out under high stringency conditions (washing steps at 65°C in 0.1 x SSC and 0.1% SDS) following standard procedures. Hybridization signals were detected on X-ray film (Mamoray HT, AGFA) after treatment with CDP-Star (Roche Diagnostic).

1.2.3 RNA extraction and RT-PCR amplification

Total RNA was extracted from symptomatic pepper leaf samples using TRIsure Reagent (Bioline) according to the manufacturer's instructions. RT-PCR was carried out with AMV (Promega) and Expand High Fidelity PCR System (Roche Diagnostic) with several pairs of primers (Table 1.1) designed based on the three complete sequences of PeVYV/PYLCV deposited in GenBank when this study was initiated (Japan [AB594828], Israel [HM439608] and China [KP326573]) or on the sequence generated in this work. The primers were designed to obtain overlapping genome fragments. Both ends of the genome were polyadenylated and amplified using the 5'/3' RACE Kit, 2nd Generation (Roche Diagnostic) (Table 1.1). Amplicons of the expected sizes were cloned using the pGEM-T Easy Vector System (Promega) and at least five clones per each genome segment were sequenced at Macrogen Inc. (Seoul, South Korea).

1.2.4 Sequence and phylogenetic analysis

SeqMan and EditSeq (available in MEGALIGN [DNASStar Inc., Madison, WI, USA]) were used for assembling and editing of DNA sequences, respectively. BLAST (<https://www.ncbi.nlm.nih.gov/blast/>) was used for initial sequence similarity searches. Sequences were aligned with MUSCLE (Edgar, 2004) and pairwise identity scores were calculated with SDT (Sequence Demarcation Tool) (Muhire *et al.*, 2014). Phylogenetic analysis of the pepper polerovirus from Spain was performed with the neighbour-joining method (1000 replicates) available in the MEGA6 package (Tamura *et al.*, 2013).

1.2.5 Recombination analysis

To detect putative recombinant fragments in the polerovirus nucleotide sequence

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Table 1.1. List of primers used for the amplification of the pepper polerovirus isolate from Spain

Genome fragment	Number of clones sequenced	Primers	Primer sequence (5'-3')	Position in the cloned genome
5' RACE	6	MA2314 (-)*	GGTCCAGGAGACAATGACCC	596-615
		MA2315 (-)*	CTTGCCATAGCAGATCCAGCGC	421-442
1	6	MA2156 (+) ^{\$}	ACAAAATATACGAAGAGAGAGAGCCC	1-26
		MA2157 (-)*	AACACAGGACACTATTGCAGATG	2960-2982
2	6	Pol-G-F [#]	GAYTGCTCYGGYTTYGACTGGAG	2835-2857
		Pol-G-R [#]	GATYTTATAYTCATGGTAGGCCTTGAG	3935-3959
3	6	MA2186 (+)*	AATCAAGGATAGTTCCTCAGG	3857-3877
		MA2241 (-)*	CCTTCTACCTATTTCCGGTTGTG	4236-4258
4	7	MA2242 (+)*	GGAACAAAGAACGTTGCCGCCG	4182-4203
		MA2325 (-) ^{\$}	CATCRGCTTCAAGATAGACRTGG	4550-4572
5	6	MA2327 (+) ^{\$}	CKATGTAYAAGTGGGAAGATGAG	4432-4454
		MA2243 (-)*	CCGGGTGAGACCACGCCATTTC	4988-5010
6	5	MA2244 (+)*	CATATCCGTCTCTATAAACGAAG	4925-4947
		MA2190 (-) ^{\$}	GCTCGTTATTTTAAAGTCATAG	5906-5927
3' RACE	8	MA2316 (+)*	CCTCCGAACCCTGACCCTTTC	5790-5810
		MA2317 (+)*	GGAGGATTTGCAGTCTAAGCTC	5815-5837

*Designed based on sequences generated in this work.

^{\$}Designed based on the PeVYV/PYLCV sequences AB594828, HM439608 and KP326573.

[#]Knierim *et al.* (2013).

reported here, a search for potential parental viruses in the GenBank database was conducted using SWeBLAST (Fourment *et al.*, 2008). Recombination analysis was conducted using RDP4 (Martin *et al.*, 2015) from the alignment generated with MUSCLE implemented in MEGA6 (Tamura *et al.*, 2013). To illustrate the mosaic nature of the polerovirus genome due to recombination, similarity plots were generated using SimPlot with the Kimura 2-parameter distance model (Lole *et al.*, 1999). Phylogenetic evidence of recombination was also analyzed using the neighbour-net method in the program SplitsTree4 (Huson and Bryant, 2006) and statistically verified with the pairwise homoplasy index (PHI) test (Bruen *et al.*, 2006).

1.3 RESULTS AND DISCUSSION

1.3.1 The pepper polerovirus present in Spain is transmissible by *Aphis gossypii*

The three sweet pepper plants (cv. California Wonder) inoculated with *A. gossypii* adults collected from plants displaying virus-like symptoms showed the typical interveinal yellowing, upward leaf curling and internode shortening associated with PeVYV/PYLCV, identical to the

symptoms observed in the plants from which the aphids were collected and to those previously reported in Spain (Villanueva *et al.*, 2013). The presence of a polerovirus was confirmed by RT-PCR analysis of the three inoculated plants using primers MA1754/MA1755, which rendered the expected DNA fragment of 658 bp and by tissue blot hybridization (not shown). The isolate was named Spain-Almería 2-2013 (ES-Alm2-13). The plants initially inoculated by aphids were propagated by grafting onto healthy sweet pepper plants, the latter confirmed infected by tissue blot hybridization (Figure 1.1D).

PeVYV/PYLCV isolates from Japan and Israel were also shown to be transmitted by *A. gossypii* (Yonaha *et al.*, 1995; Dombrovsky *et al.*, 2010). These isolates were also transmissible by *M. persicae*, but this aphid species was not assayed in our work.

1.3.2 The pepper polerovirus present in Spain has a recombinant origin

Assembling of overlapping genomic PCR fragments from the polerovirus isolate from Spain yielded a consensus contig of 6125 nt (Figure 1.2A). The highest nucleotide identities were with PeVYV/PYLCV from Israel (90.7%), Japan (89.5%), China (89.1%), and Australia (88.4%). The genome of the polerovirus isolate from Spain has the typical structure of members of the genus, containing seven ORFs (ORF0 to ORF5 and ORF3a) (Figure 1.2B) putatively encoding proteins P0 to P5 and P3a. Percent identities of the deduced amino acid sequence of each protein with the equivalent proteins of other pepper polerovirus isolates are shown in Figure 1.3.

Phylogenetic analysis of the complete genome of pepper polerovirus sequences, supported by high bootstrap values, showed that the polerovirus isolate from Spain clusters with the Israeli isolate (Figure 1.4). Isolates from China, Japan, and Australia grouped together in a different clade. When the phylogenetic analysis was carried out separately for the amino acid sequences of the seven proteins putatively coded by the pepper polerovirus genomes, some inconsistencies were observed (not shown). Thus, the isolates from Spain and Israel clustered together only in the case of P0, P1 and P2 proteins. In the case of P3, P4, P5, and P3a, the isolate from Israel branched separately from the cluster formed by the rest of the isolates.

To detect putative recombinant fragments in the novel genome, a search for potential parental viruses in the GenBank database was conducted using SWeBLAST. The sequences with the highest SWeBLAST scores were aligned and analyzed with the RDP4 package. This

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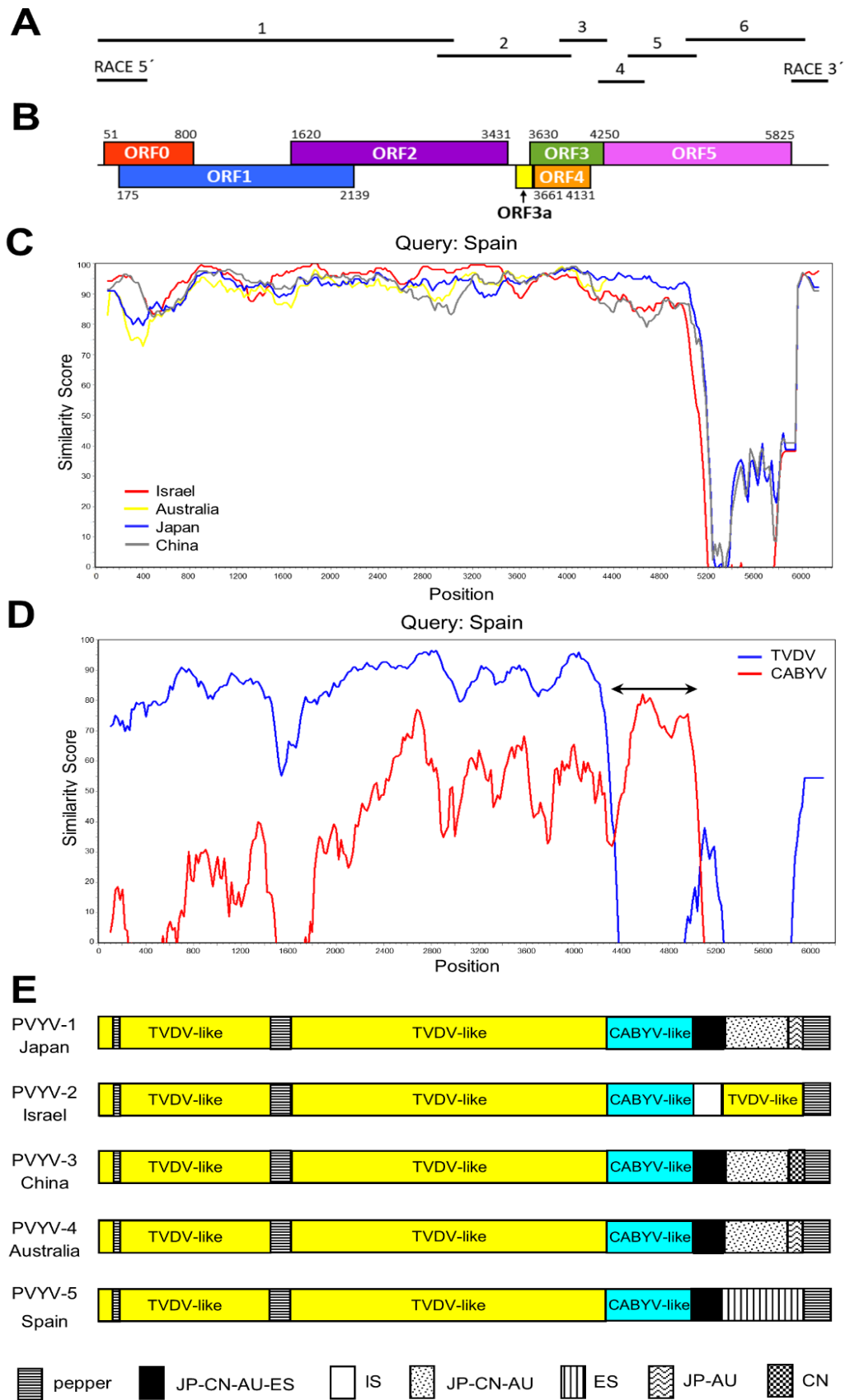


Figure 1.2. Genome organization and recombination analysis of the pepper polerovirus from Spain (pepper vein yellows virus 5, PeVYV-5). **A:** cDNA fragments cloned and sequenced to get the complete genome sequence of PeVYV-5. Table 1.1 for details on the primers used for amplification and number of clones sequenced. **B:** Schematic representation of the genome organization of PeVYV-5. The boxes represent the predicted ORFs and coordinates for them are indicated. **C:** Similarity plot diagram made using the program SimPlot comparing the nucleotide sequence of PeVYV-5 (Spain) with pepper polerovirus isolates from Israel, Australia, Japan, and China. **D:** SimPlot diagram comparing the nucleotide sequence of PeVYV-5 (Spain) with tobacco vein distorting virus (TVDV, EF529624) and cucurbit aphid-borne yellows virus (CABYV, X76931). The main putative recombinant fragment (major parent: TVDV, minor parent: CABYV, $p=1.193 \times 10^{-68}$) is indicated with a double-headed arrow. **E:** Schematic diagram showing putative recombinant fragments for pepper polerovirus isolates. Yellow and blue rectangles indicate sequences related to TVDV and CABYV, respectively. Similar black and white shading indicates sequences shared by different sets of pepper poleroviruses.

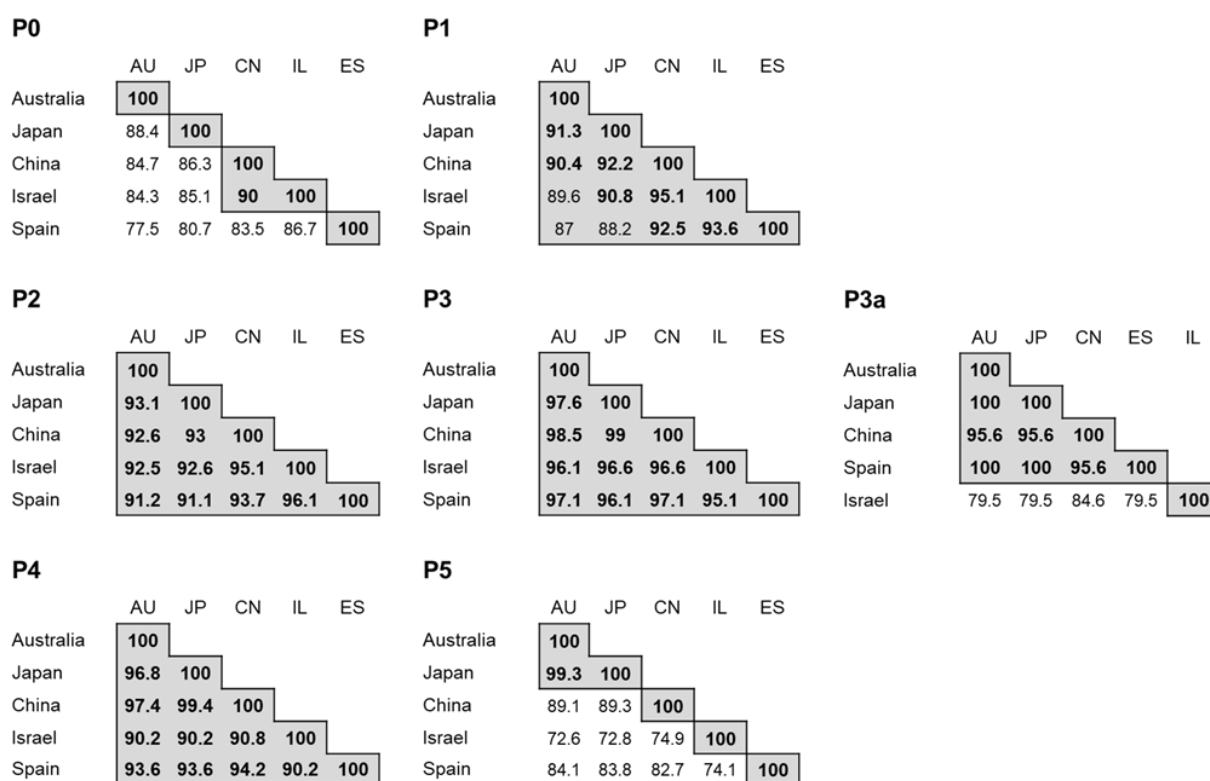


Figure 1.3. Pairwise amino acid sequence identity percentages calculated with Sequence Demarcation Tool (SDT) between the proteins (P0-P5 and P3a) coded by the pepper polerovirus genomes from Australia (AU), Japan (JP), China (CN), Israel (IL) and Spain (ES). Values higher than 90% (criterion used for polerovirus species demarcation) are in bold and the values that define species-level units are grouped by boxes.

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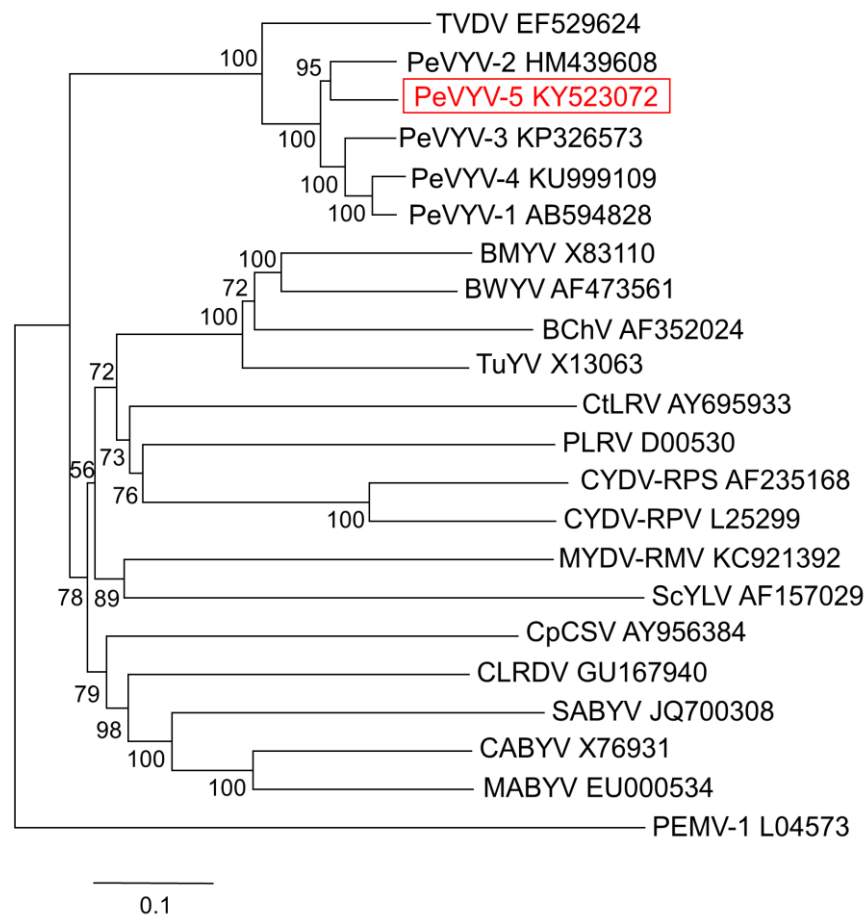


Figure 1.4. Phylogenetic tree illustrating the relationship of the complete genome sequence of the pepper polerovirus from Spain (pepper vein yellows virus 5, PeVYV-5) to isolates of all accepted polerovirus species at the time of analysis. The tree was constructed using the neighbour-joining method available in the MEGA7 package, and bootstrap values (1000 replicates) are shown at the nodes. BChV, beet chlorosis virus; BMV, beet mild yellowing virus; BWYV, beet western yellows virus; CtLRV, carrot red leaf virus; CYDV-RPS, cereal yellow dwarf virus-RPS; CYDV-RPV, cereal yellow dwarf virus-RPV; CpCSV, chickpea chlorotic stunt virus; CLRDV, cotton leafroll dwarf virus; CABYV, cucurbit aphid-borne yellows virus; MYDV-RMV, maize yellow dwarf virus-RMV; MABYV, melon aphid-borne yellows virus; PeVYV-1 to PeVYV-4, pepper vein yellows virus 1 to pepper vein yellows virus 4; PLRV, potato leafroll virus; SABYV, suakwa aphid-borne yellows virus; ScYLV, sugarcane yellow leaf virus; TVDV, tobacco vein distorting virus; TuYV, turnip yellows virus. The enamovirus pea enation mosaic virus 1 (PEMV-1) was used as an outgroup. The bar below the tree indicates 0.1 nucleotide substitutions per site.

analysis showed several putative recombination events between PeVYV/PYLCV genomes known to date as well as the genomes of the poleroviruses tobacco vein distorting virus (TVDV, EF529624) and cucurbit aphid-borne yellows virus (CABYV, X76931), as illustrated in Fig. 1.2C, D, and E. Several small putative recombinant fragments exchanged between pepper

poleroviruses were detected with p values higher than 10^{-10} (not shown). Only one putative recombination event with very low p value (1.193×10^{-68} using the RDP method) was detected in the polerovirus isolate from Spain (see Fig. 1.2D). This recombination event would involve TVDV as major parent and CABYV as minor parent. Evidence of phylogenetic inconsistencies, as shown by reticulate networks after neighbour-net analysis (Fig. 1.5), additionally supported the involvement of recombination in the origin of the pepper poleroviruses ($p = 0.0$, PHI test).

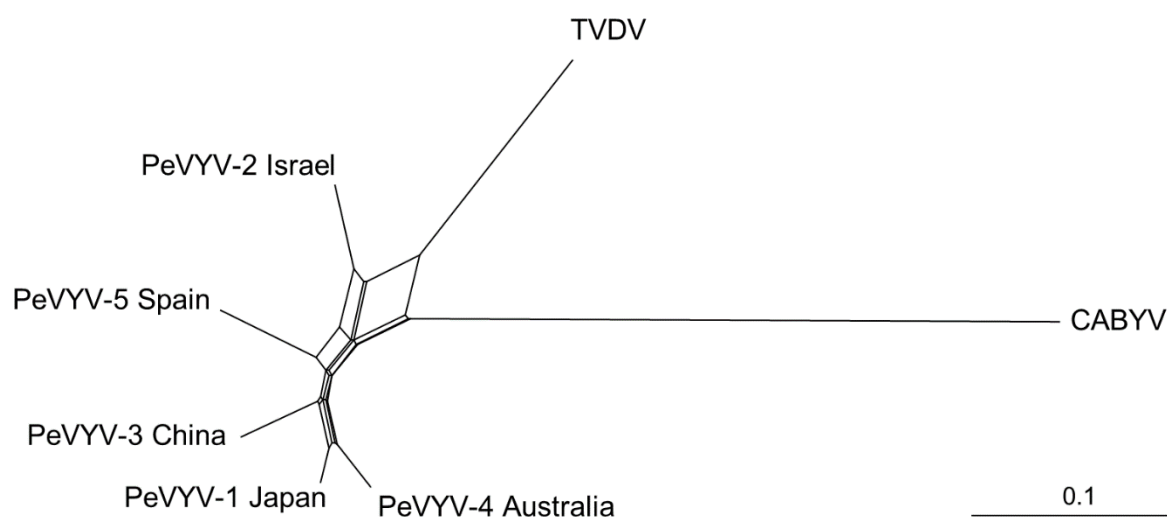


Figure 1.5. Phylogenetic network (neighbour-net method) of the complete genome sequences of the five pepper vein yellows poleroviruses (PeVYV-1 to PeVYV-5), tomato vein distorting virus (TVDV, EF529624) and cucurbit aphid-borne yellows virus (CABYV, X76931) generated using the SplitsTree4 program. Networked relationships among viruses with a box-like structure instead of a bifurcating tree are indicative of recombination. The bar below the network indicates 0.1 nucleotide substitutions per site. See Table 1.2 for names and GenBank accession numbers of the pepper poleroviruses.

The putative major parental virus of the pepper poleroviruses, TVDV, was originally described in China and isolates of CABYV, putative minor parent, are also widely distributed in Asia. This suggests that polerovirus species infecting pepper could have an Asian origin, which is in agreement with the first detection of symptomatic plants in Japan (Yonaha *et al.*, 1995). A recombinant origin for the PYLCV isolate from Israel involving TVDV and CABYV has been suggested previously (Dombrovsky, *et al.*, 2013).

1.3.3 The yellowing disease of pepper emerging worldwide is caused by a complex of different poleroviruses

The current species demarcation criteria for members of the genus *Polerovirus* include

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Table 1.2. Proposed nomenclature for the poleroviruses infecting pepper

Species	Virus name	Exemplar isolate	GenBank accession number	Virus abbreviation	Reference
<i>Pepper vein yellows virus 1</i>	pepper vein yellows virus 1	Japan-Okinawa-2009	AB594828	PeVYV-1-[JP-OkI-09]	Murakami <i>et al.</i> , 2011
<i>Pepper vein yellows virus 2</i>	pepper vein yellows virus 2 (pepper leaf curl virus)	Israel-2002	HM439608	PeVYV-2-[IL-02]	Dombrovsky, <i>et al.</i> , 2013
<i>Pepper vein yellows virus 3</i>	pepper vein yellows virus 3	China-Hunan-2013	KP326573	PeVYV-3-[CN-HN-13]	Liu <i>et al.</i> , 2016
<i>Pepper vein yellows virus 4</i>	pepper vein yellows virus 4	Australia-12KNX1-2015	KU999109	PeVYV-4-[AU-12KNX1-15]	Maina <i>et al.</i> , 2016
<i>Pepper vein yellows virus 5</i>	pepper vein yellow virus 5	Spain-Almería 2-2013	KY523072	PeVYV-5-[ES-Alm2-13]	This work

differences in amino acid sequence identity of any viral gene product higher than 10% (Domier, 2012). The genome of the polerovirus isolate from Spain described here codes for two proteins which are less than 90% identical to equivalent proteins of all previously characterized pepper poleroviruses, P0 (77.5-86.7%) and P5 (74.1-84.1) (Fig 1.3). Thus, according to the above mentioned criterion, the virus isolate characterized in this work belongs to a new species in the genus *Polerovirus*. Also, comparison of the deduced amino acid sequence of all gene products of the five available complete sequences of the poleroviruses causing the yellowing disease in peppers worldwide (PeVYV/PYLCV from Japan, China, Israel, Australia, and Spain) revealed that they belong to five distinct polerovirus species (Fig. 1.3). The sequence of P0 would differentiate the pepper poleroviruses in four species-level units (Australia, Japan, China/Israel and Spain). Additionally, isolates from China and Israel would be separated at the species level according to differences in the sequence of P5.

To date, there is only one recognized species containing pepper-infecting poleroviruses, *Pepper vein yellows virus*, represented by the isolate described in Japan (ICTV, 2015). Given that the symptoms caused by all pepper poleroviruses are similar, if not identical, we propose to name them pepper vein yellows virus 1 to pepper vein yellows virus 5 (PeVYV-1 to PeVYV-5) according to date of description, with PeVYV-5 corresponding to the polerovirus from Spain described in this work (Table 1.2).

Taken together, our results and those published over the last few years (Murakami *et al.*, 2011; Dombrovsky, *et al.*, 2013; Liu *et al.*, 2016; Maina *et al.*, 2016) have shown the emergence of different poleroviruses affecting pepper crops around the world, with a high complexity due to recombination events. The biological and pathological properties of isolates belonging to these species should be investigated in the future. The availability of infectious clones will elucidate whether differences in symptoms, host range or transmission specificity exist between the different species revealed in this work.

CHAPTER 2

TOMATO CHLOROSIS VIRUS INFECTIOUS CLONES: TOWARD INCREASING EFFICIENCY BY INTRODUCING THE HEPATITIS DELTA VIRUS RIBOZYME



2.1. INTRODUCTION

The family *Closteroviridae* includes almost 60 plant virus species grouped in the genera *Ampelovirus*, *Closterovirus*, *Crinivirus*, and *Velarivirus*, plus a number of unassigned species (Fuchs *et al.*, 2020). Closterovirids have several distinguishing characteristics: (i) semi-persistent transmission by insects, (ii) mostly phloem-limited, (iii) elongated particles built of several proteins, (iv) large single-stranded positive-sense RNA (ssRNA) genomes of 15–20 kb, (v) presence of a gene homologous to the HSP70 family of cell chaperones, and (vi) a number of duplicated and diverged genes (Agranovsky, 2016). Some closterovirids cause serious diseases in important crops including citrus, cucurbits, grapevine, sugar beet, and tomato.

Criniviruses have segmented genomes encapsidated in separate long flexuous virions that are restricted to the phloem and companion cells (Kiss *et al.*, 2013). With the sole exception of a tripartite crinivirus, potato yellow vein virus (Livieratos *et al.*, 2004), all members of the genus have a bipartite genome composed of RNA1 and RNA2. Criniviruses are transmitted in nature by whiteflies (Hemiptera: Aleyrodidae) in a semipersistent manner (Tzanetakis *et al.*, 2013).

Tomato chlorosis virus (ToCV) is a crinivirus first detected in Florida, United States, where it has caused a problem in greenhouse-grown tomatoes since 1989, the so-called “yellow leaf disorder” (Wisler *et al.*, 1998b). Symptoms caused by ToCV include interveinal yellow chlorotic areas that initially develop on lower leaves and progress toward the upper leaves of the plant. Bronzing and red patches often occur within the yellow areas, and the leaves become thickened and crispy with the margins slightly curled upward. Fruit ripening is affected, resulting in economic damage. In addition to tomato, ToCV has a wide range of hosts, both natural and experimental, including some important crops such as pepper and potato (Fiallo-Olivé and Navas-Castillo, 2019). ToCV is transmitted in a semipersistent manner by whiteflies of the *Bemisia tabaci* cryptic species complex (at least MEAM1, MED, and NW, formerly biotypes B, Q, and A, respectively), *Trialeurodes vaporariorum*, and *T. abutiloneus* (Wisler *et al.*, 1998a; Navas-Castillo *et al.*, 2000). Since first being detected in Florida, the presence of ToCV has been confirmed in 39 countries or territories of the Americas, Europe, Asia, and Africa (Fiallo-Olivé and Navas-Castillo, 2019; Amer *et al.*, 2020; Kimathi *et al.*, 2020; Raza *et al.*, 2020), constituting a paradigmatic example of an emergent plant pathogen. The ToCV genome is composed of RNA1 (8,593–8,596 nt)-encoding proteins associated with virus replication and RNA2 (8,242–8,247 nt)-encoding proteins associated with encapsidation, cell-

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to-cell movement, and whitefly transmission (Wintermantel *et al.*, 2005; Lozano *et al.*, 2006, 2007; Figure 2.1). Both RNA segments contain gene-encoding proteins with RNA silencing suppression activity, the p22 protein in RNA1 and the major (CP) and minor (CPm) coat proteins in RNA2 (Cañizares *et al.*, 2008).

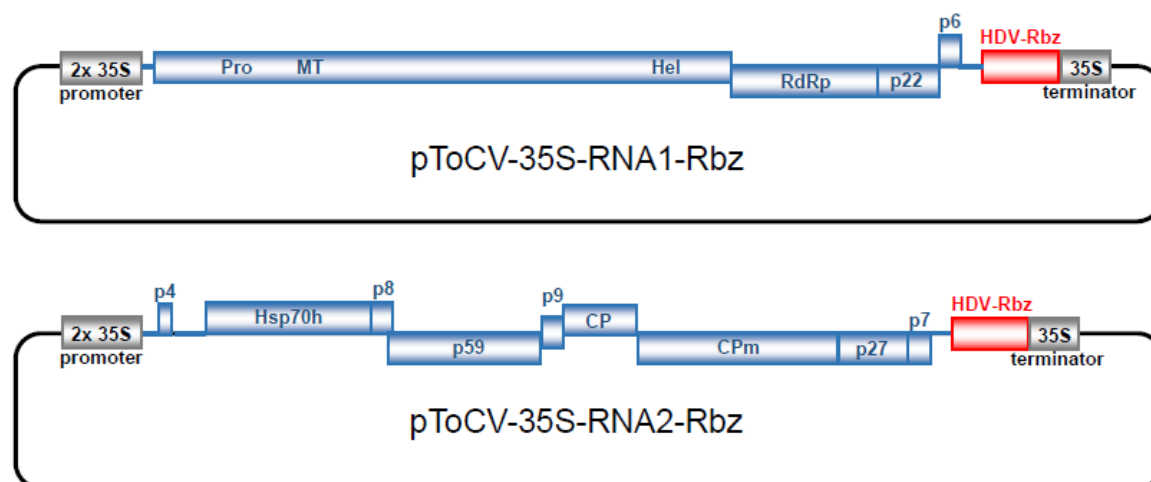


Figure 2.1. Genome organization and construction of infectious full-length cDNA clones of tomato chlorosis virus (Spanish isolate AT80/99-IC; RNA1, pToCV-35S-RNA1-Rbz; RNA2, pToCV-35S-RNA2-Rbz) in the binary vector pJL89 under the control of duplicated 35S cauliflower mosaic virus promoter (2x 35S promoter) and incorporation of hepatitis delta virus ribozyme (HDV-Rbz). Blue boxes represent open reading frames with the putative protein products or protein domains indicated inside or above. PRO, protease; MT, methyltransferase; HEL, helicase; RdRp, RNA-dependent RNA polymerase; Hsp70 h, heat shock protein 70 homolog; CP, coat protein; CPm, minor coat protein.

Infectious plant virus clones are a useful tool for confirming Koch's postulates, performing genetic reverse studies, and selecting plants in breeding programs. Viral DNA (or cDNA for RNA viruses) cloned within bacterial *Escherichia coli* plasmids can be used for *in vitro* transcription of viral RNA for the inoculation of plants (for RNA viruses) or for *in vivo* transcription after transformation into *Agrobacterium tumefaciens* and inoculation of plants by agroinfiltration (for RNA and DNA viruses). *In vivo* transcription from agroinfectious clones is usually under the control of the (duplicated) cauliflower mosaic virus (CaMV) 35S promoter and the *A. tumefaciens* nopaline synthase (NOS) terminator signal.

Currently, agroinfectious clones are available for eight closterovirids, the closteroviruses beet yellows virus (BYV) (Prokhnevsky *et al.*, 2002), citrus tristeza virus (CTV) (Gowda *et al.*, 2005; Ambrós *et al.*, 2011, 2013), and grapevine leafroll-associated virus 2 (GLRaV-2) (Liu *et al.*, 2009; Kurth *et al.*, 2012), the criniviruses lettuce infectious yellows virus (LIYV) (Wang *et*

al., 2009), lettuce chlorosis virus (LCV) (Chen *et al.*, 2012), ToCV (Orílio *et al.*, 2014; Zhao *et al.*, 2016), and cucurbit chlorotic yellows virus (CCYV) (Shi *et al.*, 2016), and the ampelovirus grapevine leafroll-associated virus 3 (GLRaV-3) (Jarugula *et al.*, 2018).

Most of the agroinfectious closterovirid clones are not able to directly infect the natural hosts but require prior infection of *Nicotiana benthamiana* plants or protoplasts as an intermediate host. Then, the infectious (purified) virions can be transferred to the natural hosts to initiate a successful infection by different methods like bark-flap or stem slash inoculation (CTV; Gowda *et al.*, 2005; Ambrós *et al.*, 2011), whitefly transmission by membrane feeding (LIYV; Wang *et al.*, 2009), or grafting (ToCV; Orílio *et al.*, 2014). An exception is the case of an agroinfectious clone of GLRaV-2 that is able to directly infect young micropropagated grapevine plants (Kurth *et al.*, 2012).

In most cases examined, non-viral nucleotides at the 5' extremity of *in vivo* transcripts generated from infectious plant RNA virus clones greatly diminished infectivity (Boyer and Haenni, 1994). Although not as critical as the 5' ends, the presence of non-viral nucleotides at the 3' ends of viral transcripts may also reduce their biological activity. This effect is, in general, dependent on the length of the non-viral extension, with short extensions having little effect on infectivity (Boyer and Haenni, 1994). In an attempt to avoid the negative effects on infectivity caused by extra non-viral nucleotides at the 3' ends of *in vivo* transcripts, the sequences of a number of ribozymes, including hepatitis delta virus (HDV) ribozyme, were positioned behind the viral sequences in the infectious clones to mediate their self-cleavage and produce (quasi) proper viral ends. In some cases where this has been analyzed, a positive influence on infectivity was reported when a ribozyme sequence was introduced in infectious clones. Turpen *et al.* (1993) found a two-fold increase in transfection frequency when a ribozyme based on subterranean clover mottle virus satellite RNA was included in an infectious tobacco mosaic virus clone. Similarly, Scholthof (1999) found the highest levels of infectivity with an infectious tomato bushy stunt virus clone when an HDV ribozyme was used in addition to the NOS terminator signal.

Although highly variable between experiments, typical infection rates of *N. benthamiana* plants with agroinfectious ToCV (Spanish isolate AT80/99-IC) clones rarely exceed 50% and despite our repeated attempts no tomato plants could be agroinfected (Orílio *et al.*, 2014; our unpublished results). In this context, the objectives of this work were (i) to generate infectious

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cDNA clones for ToCV RNA1 and RNA2 containing a ribozyme at the 3' end, (ii) to identify the 3' ends of the virus genomes generated after agroinoculation with clones with or without the ribozyme, (iii) to quantify the effect of the ribozyme on the infection rate of *N. benthamiana* plants, and (iv) to determine whether a possible increase in infectivity due to the presence of the ribozyme would allow direct agroinfection of tomato plants.

2.2. MATERIALS AND METHODS

2.2.1. Virus isolate

Tomato chlorosis virus isolate AT80/99 was obtained from a naturally infected tomato plant collected in Málaga province, southern Spain, in 1999 (Lozano *et al.*, 2006, 2007). Infectious ToCV clones (isolate AT80/99-IC, GenBank acc. nos. KJ740256 and KJ740257) have previously been developed under the control of the SP6 RNA polymerase promoter (pToCV227 [RNA1] and pToCV230 [RNA2]) and the 2x CaMV 35S promoter (pToCV-35S-RNA1 and pToCV-35S-RNA2) (Orílio *et al.*, 2014).

2.2.2. Construction of infectious ToCV clones incorporating a ribozyme sequence

Clones pToCV227 and pToCV230 were used as templates to obtain the full-length sequences of ToCV RNA1 and RNA2 by PCR amplification, respectively. PCR was performed using PfuTurbo DNA polymerase, a mix of cloned Pfu DNA polymerase and ArchaeMaxx polymerase-enhancing factor (Agilent Technologies), and 5' phosphorylated primers MA682/MA683 and MA684/MA685 (Table 2.1) based on AT80/99-IC RNA1 and RNA2 sequences, respectively. PCR products of the expected sizes were purified with a Zymoclean Gel DNA Recovery Kit (Zymo Research) and cloned into SmaI- and StuI-digested pJL89, a binary vector from the pJL series constructed by Lindbo (2007), between the 2x CaMV 35S promoter and HDV ribozyme plus CaMV 35S terminator (Figure 2.1). Ligation products were transformed in *E. coli* strain ElectroMAX Stbl4 by electroporation (25 μ F, 200 Ω , 1,200 V) in a Gene Pulser XCell (Bio-Rad). The presence of the correct inserts in clones selected by restriction analysis was confirmed by sequencing (Macrogen Inc., Seoul, South Korea) using primers based on the 2x CaMV 35S promoter (MA2720) and CaMV 35S terminator (MA2721) (Table 2.1). Clones pToCV-35S-RNA1-Rbz and pToCV-35S-RNA2-Rbz were selected for transformation into *A. tumefaciens* strain GV3101 by electroporation as described above.

Table 2.1. List of primers used in this study.

Primer	Sequence (5'-3')	Position in the ToCV genome ^a	Use
MA682	^P GAAATAGTATTCGTGTGATTACAC	1-24	RNA1 amplification
MA683	^P CGACCTATTTATTTATATACTAGATC	8,569-8,594	RNA1 amplification
MA684	^P GAAATACAAGTCCAGGTGTTTCCTGTG	1-27	RNA2 amplification
MA685	^P GACCTATTTATTTATATACTAAATCTACC	8,244-8,216	RNA2 amplification
MA2720	TAAGGGATGACGCACAATCC	-	RNA1/RNA2 5' end sequencing (CaMV 35S promoter)
MA2721	CCCTTATCTGGGAACACTACTC	-	RNA1/RNA2 3' end sequencing (CaMV 35S terminator)
MA324	GRACDGGDTCDCCRAAYACKTGG	7,120-7,142	RNA1 3' end cloning
MA2813	GGCTTGTCAAGACATTCTTGAGGG	8,012-8,035	RNA1 3' end cloning
MA2798	GGAGTGTTCACAAGATACGGG	7,484-7,505	RNA2 3' end cloning
MA2799	GTTGCTCCATTCTGGGAGTAACAGG	7,976-7,999	RNA2 3' end cloning
MA279	CCGGATCCTCTAGAGCGGCCGCT ₁₇ V	-	RNA1/RNA2 3' end cloning
Oligo(dT)- anchor	GACCACGCGTATCGATGTCGACT ₁₆ V	-	RNA1/RNA2 3' end cloning
Anchor	GACCACGCGTATCGATGTCGAC	-	RNA1/RNA2 3' end cloning

^aNucleotide positions refer to the sequences of ToCV isolate AT80/99-IC (GenBank acc. nos. KJ740256 for RNA1 and KJ740257 for RNA2).

2.2.3. Plant agroinoculation

For agroinoculation experiments, *A. tumefaciens* GV3101 carrying the constructs pToCV-35S-RNA1-Rbz or pToCV-35S-RNA2-Rbz were grown in Luria-Bertani broth containing rifampicin (25 µg/mL), gentamicin (20 µg/mL), tetracycline (2.5 µg/mL), kanamycin (50 µg/mL), 10 mM of 4-morpholineethanesulfonic acid (MES), and 20 mM of acetosyringone with vigorous shaking at 28°C for 48 h. Bacterial cells were harvested by centrifugation and resuspended in induction buffer (10 mM of MgCl₂, 10 mM of MES pH 5.6, 10 mM of MgCl₂, 150 mM of acetosyringone) to a final OD₆₀₀ of 1.0. Both bacterial suspensions were mixed in a 1:1 ratio and then kept at room temperature for 2 h without shaking. In some experiments the OD₆₀₀ = 1.0 cultures were diluted 1/50 and incubated for 2 h prior to inoculation. *A. tumefaciens* cultures carrying the constructs pToCV-35S-RNA1 and pToCV-35SRNA2 (Orilio *et al.*, 2014) or empty vectors (pCambia2300 or pJL89) were used as positive and negative (mock) controls, respectively. *N. benthamiana* plants were agroinfiltrated at the 3–5 leaf stage on the underside of leaves with a 1 mL syringe without a needle. Tomato plants (cv. Moneymaker and cv. Micro-Tom) were inoculated by agroinfiltration at the 2–3 leaf stage as described for *N. benthamiana* or by agroinjection with a 27-gauge needle in the apex of the seedlings. Agroinoculated plants were maintained in a controlled temperature, insect-free growth chamber (25°C day/18°C night, 70% relative humidity, 16 h photoperiod at 250 mmol s⁻¹ m⁻² photosynthetically active radiation).

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2.2.4. Detection of viral RNA in agroinoculated plants

The presence of ToCV RNA in non-inoculated leaves of agroinoculated plants was determined by tissue blot molecular hybridization 30 days post-inoculation (dpi). Freshly cross-sectioned leaf petioles were squash-blotted on positively charged nylon membranes (Roche Diagnostics) and hybridized with a ToCV-specific digoxigenin-labeled negative-sense RNA probe obtained from the coat protein gene (Fortes *et al.*, 2012). The cross-linked RNA was hybridized at 65°C overnight in standard hybridization buffer [5xSSC (750 mM of NaCl, 75 mM of sodium citrate, pH 7.0), 0.1% N-lauryl sarcosine, 0.02% sodium dodecyl sulfate, and 2% blocking reagent (Roche Diagnostics)] with 50% formamide, followed by high stringency washing (65°C in 0.1x SSC, 0.1% sodium dodecyl sulfate) following standard procedures. Chemiluminescent hybridization signals were detected on X-ray film (Mamoray HT, Agfa) after treatment with CDP-Star (Roche Diagnostics).

2.2.5. Cloning and sequencing of the 3' ends of ToCV RNA1 and RNA2 in agroinoculated *N. benthamiana* plants

Total RNA was extracted from infected leaves of six (three agroinoculated with pToCV-35S-RNA1 + pToCV-35S-RNA2 and three with pToCV-35S-RNA1-Rbz + pToCV-35S-RNA2-Rbz) *N. benthamiana* plants 30 dpi using TRIsure Reagent (Bioline) as described by the manufacturer. The sequences of primers used in this study, including those based on the sequence of ToCV isolate AT80/99-IC, are listed in Table 2.1. To generate cDNA of the RNA1 3' end, total RNA was polyadenylated using yeast USB poly (A) polymerase (USB Corporation). Reverse transcription was performed with SuperScript III reverse transcriptase (Invitrogen) and oligo(dT) primer MA297 using the positive-strand RNA as the template. Then, PCR amplification was conducted with Biotaq DNA polymerase (Bioline) using the nested primers MA324/MA279 and MA2813/MA279. cDNA of the RNA2 3' end was obtained using the 5'/3' RACE Kit 2nd Generation (Roche Diagnostics) and oligo(dT)-anchor primer using the negative-strand RNA as the template. The RNA2 cDNA was purified with the High Pure PCR Product Purification Kit (Roche Diagnostics) and the d(A)-tail was added using Recombinant Terminal Transferase. Finally, PCR amplification was conducted with the Expand High Fidelity PCR System (Roche Diagnostics) using the nested primers MA2798/Anchor and MA2799/Anchor. DNA bands with the expected size were extracted from agarose gels, purified using the QIAquick Gel Extraction Kit (QIAGEN), cloned in the pGEM-T Easy System (Promega), and transformed into *E. coli* DH5 α by electroporation (25 μ F, 200

Ω , 1,200 V) with a Gene Pulser XCell electroporation system (Bio-Rad). Three clones were selected for each plant and sequenced at Macrogen Inc. (Seoul, South Korea) using T7 and SP6 promoter primers. DNA sequences were analyzed using the SeqMan software included in the DNASTAR package (Lasergene).

2.3. RESULTS AND DISCUSSION

2.3.1. Construction of ToCV RNA1 and RNA2 cDNA clones incorporating an HDV ribozyme

With the objective of improving the infectivity of available ToCV agroinfectious clones (Orlío *et al.*, 2014), an HDV ribozyme sequence was incorporated at the end of both RNA1 and RNA2 by cloning in the binary vector pJL89 (Lindbo, 2007). This vector is designed to accommodate viral cDNA inserts able to produce *in vivo*-transcribed genome copies without, theoretically, extra non-viral nucleotides, an important point when attempting to obtain highly infectious transcripts (Boyer and Haenni, 1994). pJL89 vector has been successfully used to generate infectious clones of RNA plant viruses including two important pathogens of tomato: tomato marchitez virus (genus *Torradovirus*) (Ferriol *et al.*, 2016) and pepino mosaic virus (genus *Potexvirus*) (Ruiz-Ramón *et al.*, 2019).

In this work, full-length ToCV RNA1 and RNA2 sequences were obtained by PCR amplification using primers corresponding to the exact 5' and 3' ends of the viral sequence using high-fidelity DNA polymerases. To avoid the presence of extra 5' non-viral nucleotides, the duplicated CaMV 35S promoter was positioned immediately upstream of the first nucleotide of the viral sequences, both for RNA1 and RNA2. On the other hand, the HDV ribozyme sequence, followed by the CaMV terminator sequence, was positioned immediately downstream of the viral cDNA with the objective of generating authentic viral 3' ends. In an attempt to avoid the instability and toxicity problems observed with infectious closterovirid cDNA clones (Satyanarayana *et al.*, 2003), ligation products were transformed into *E. coli* strain Stbl4, specifically designed for cloning unstable inserts. Recombinant plasmids pToCV-35SRNA1-Rbz and pToCV-35S-RNA2-Rbz (Figure 2.1) were selected after confirming the size and sequence of the insert ends, and transformed into *A. tumefaciens* to be tested in plants.

2.3.2. Improved infectivity of ToCV agroinfectious clones in *N. benthamiana*

The biological activity of the new ToCV clones containing the HDV ribozyme downstream of the viral genome components (pToCV-35S-RNA1-Rbz C pToCV-35S-RNA2-

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Rbz) was first compared with that of previously available clones lacking the ribozyme sequence (pToCV-35S-RNA1 C pToCV-35S-RNA2) (Orílio *et al.*, 2014), by agroinoculation of *N. benthamiana* plants. For this, systemic infection was assessed by tissue printing hybridization of non-infiltrated apical leaves using a negative sense CP gene probe. Four independent experiments were carried out, each including 6-24 plants per condition. Figure 2.2 shows the results of the molecular hybridization conducted in Experiment 2. In all experiments, the infectivity of the clones carrying the ribozyme, measured as the number of infected plants, was higher than that of the previously available clones. Considerable variability was observed between experiments, with the increase in infectivity ranging from 130 to 300% (Table 2.2). Considering all the experiments together, 82.1% of the plants inoculated with the clones containing the ribozyme became infected, whereas only 40.9% of the plants became infected with the previously available clones, thus resulting in a two-fold increase in infectivity. Similar heterogeneity of results between experiments was reported for infectious clones of the criniviruses LIYV (Wang *et al.*, 2009) and LCV (Chen *et al.*, 2012). A few studies of plant viruses belonging to different families also showed an increase in infectivity, measured as number of local lesions, when a ribozyme was incorporated into infectious clones. Examples include tobacco mosaic virus plus subterranean clover mottle virus satellite RNA ribozyme (Turpen *et al.*, 1993) and tomato bushy stunt virus plus HDV ribozyme (Scholthof, 1999).

2.3.3. ToCV clones containing the HDV ribozyme are still not agroinfectious in tomato

Tomato chlorosis virus clones harboring the HDV ribozyme, which were shown to have increased infectivity in *N. benthamiana* plants, were further tested in tomato plants (Table 2.2). A total of 106 tomato plants were agroinoculated with pToCV-35S-RNA1-Rbz + pToCV-35S-RNA2-Rbz clones in five independent experiments. The experiments also included 70 tomato plants agroinoculated with the clones lacking the ribozyme (ToCV-35S-RNA1 + pToCV-35S-RNA2), previously reported to be agroinfectious in *N. benthamiana* but not in tomato (Orílio *et al.*, 2014). The agroinoculation conditions assayed included leaf agroinfiltration and stem agroinjection, tomato cv. Moneymaker and cv. Micro-Tom, and different *A. tumefaciens* concentrations. None of the tomato plants agroinoculated with either clones became infected with ToCV according to tissue printing hybridization (data not shown).

Similarly to ToCV, most agroinfectious closterovirid clones are not able to directly infect the natural hosts but require prior infection of *N. benthamiana* as an intermediate host.

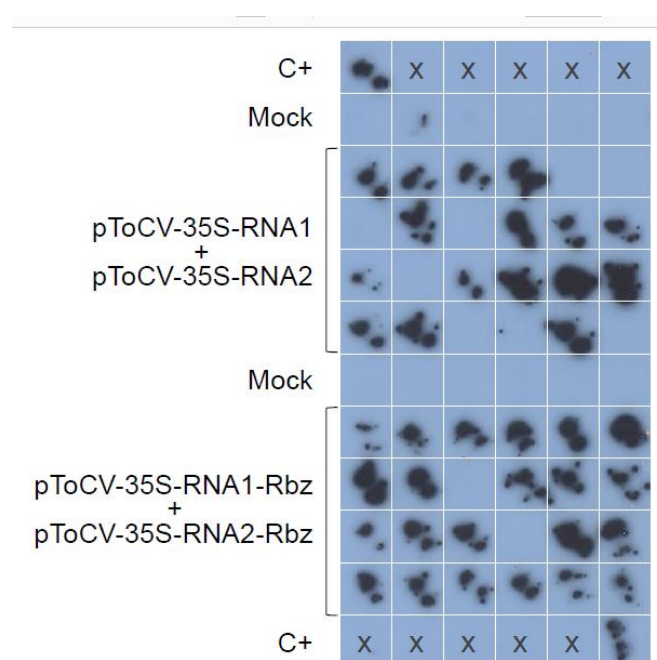


Figure 2.2. Agrobacterium-mediated infection of *Nicotiana benthamiana* plants. *Nicotiana benthamiana* plants were inoculated with *Agrobacterium tumefaciens* GV3101 cultures carrying pToCV-35S-RNA1 + pToCV-35S-RNA2 or pToCV-35S-RNA1-Rbz + pToCV-35S-RNA2-Rbz (Experiment 2 in Table 2.2). A specific digoxigenin-labeled RNA probe was used to analyze the agroinoculated *N. benthamiana* plants by molecular hybridization after tissue printing on nylon membranes. Tissue prints of mock-inoculated plants were included as negative controls. C+, ToCV-infected *N. benthamiana* plant used as positive control; x, no sample.

Subsequently, the infectious virions can be transferred to the natural hosts to initiate an infection by bark-flap or stem slash inoculation (CTV; Gowda *et al.*, 2005; Ambrós *et al.*, 2011), whitefly transmission (LIYV; Wang *et al.*, 2009), or grafting (ToCV; Orílio *et al.*, 2014). It is worth mentioning that the cDNA clones generated for a Chinese ToCV isolate are also agroinfectious in *N. benthamiana* (Zhao *et al.*, 2016) but not in tomato (Tao Zhou, personal communication).

Wang *et al.* (2009a) suggested a number of factors that could contribute to the inability of agroinfectious viral clones to accomplish systemic infection in the natural host plants, including transcript splicing, thus reducing the functional RNA transcripts reaching the cytoplasm, the difference in virulence conferred by different *A. tumefaciens* strains, the plant defense responses caused by the combination of specific *A. tumefaciens* strains and viruses, the inaccessibility of phloem cells, or a strong silencing response at the very early stages of infection. Another possible factor is the difficulty coinfecting a single cell with several genome segments in the case of multipartite viruses such as criniviruses.

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Table 2.2 Agroinoculation experiments carried out with infectious tomato chlorosis virus RNA1 and RNA2 clones (isolate AT80/99-IC) with or without the HDV ribozyme (Rbz).

Host	Experiment	No. infected plants/No. inoculated plants (% infected plants)			Notes
		pToCV-35S-RNA1 +	pToCV-35S- RNA1-Rbz +	Mock (empty vectors)	
		pToCV-35S-RNA2	pToCV-35S- RNA2-Rbz		
<i>N. benthamiana</i>	1	5/12 (41.66)	23/24 (95.83)	0/12 (0)	
	2	16/24 (66.66)	22/24 (91.66)	0/12 (0)	
	3	5/24 (20.83)	15/24 (62.50)	0/12 (0)	
	4	1/6 (16.66)	4/6 (66.66)	0/6 (0)	
Tomato	1	0/12 (0)	0/24 (0)	0/12 (0)	
	2	0/12 (0)	0/24 (0)	0/12 (0)	
	3	0/6 (0)	0/18 (0)	0/6 (0)	
	4	0/10 (0)	0/10 (0)	0/6 (0)	AI ^a
		0/10 (0)	0/10 (0)	0/6 (0)	AI, MT ^b
	5	0/10 (0)	0/10 (0)	0/6 (0)	AI, 1:50 ^c
		0/10 (0)	0/10 (0)	0/6 (0)	AI, MT, 1:50

Systemic infection was determined on agroinoculated and mock-inoculated *Nicotiana benthamiana* and tomato plants by tissue printing hybridization using a negative sense CP gene probe 30 days post-inoculation. Agroinfiltration, tomato cv. Moneymaker and *A. tumefaciens* OD = 1 were used unless otherwise specified.

^aAI, agroinjection; ^bMT, cv. Micro-Tom; ^c1:50, *A. tumefaciens* OD = 1 diluted 1:50.

2.3.4. Determination of 3' end viral progeny sequences

In addition to analyzing the effect of positioning the HDV ribozyme behind the viral sequences in the infectious ToCV RNA1 and RNA2 clones on infectivity, which theoretically would be caused by the elimination of extra non-viral nucleotides at the 3' ends of *in vivo* transcripts, the actual effect of the ribozyme on the viral progeny sequences was assessed. For this, the 3' end viral progeny sequences present in the agroinoculated *N. benthamiana* plants were determined by cloning and sequencing at 30 dpi as a proxy for RNA sequences generated from the cDNA clones after HDV ribozyme self-cleavage. Three plants were analyzed per condition (pToCV-35S-RNA1 + pToCV-35S-RNA1 or pToCV-35S-RNA1-Rbz + pToCV-35S-RNA2-Rbz) and six clones (three for RNA1 and three for RNA2) were sequenced per plant (Figure 2.3). Variability was observed in the 3' ends, mainly for RNA1, even among clones derived from the same plant. When compared with the sequence of the infectious clone (isolate AT80/99-IC), the very end of the RNA2 progeny showed 0–1 nucleotide differences regardless

of whether clones with or without ribozyme were used. In contrast, differences were observed for RNA1 between the viral progeny present in plants agroinoculated with the clones with (0–3 nucleotide changes) or without (1–7 nucleotide changes) the ribozyme. Interestingly, most of the changes observed in the RNA2 progeny of the clones with the ribozyme resulted in sequences present in other ToCV isolates (Table 2.3), which could be considered as alternative proper viral ends. In summary, the presence of the HDV ribozyme produced RNA1 3' ends more similar to those present in the clones used for agroinoculation or in other naturally occurring ToCV isolates. Sequence heterogeneity in the 3' end of sequences of closterovirid isolates is not surprising, as this has been shown in CTV isolates, for example (López *et al.*, 1998; Chen *et al.*, 2018).

		RNA1 (5'–3')	RNA2 (5'–3')
AT80/99-IC		TATATAAATAAATAGGTCG	TATATAAATAAATAGGTC-
pToCV-35S	plant 1	TATATAAATAAA C -----	TATATAAATAAATAGGTC C
		TATATAAATAAATAGGT--	TATATAAATAAATAGGTC C
		TATATAAATAAATAG C ---	TATATAAATAAATAGGTC C
	plant 2	TATATAAATAAATAGG C ---	TATATAAATAAATAGGTC-
		TATATAAATAAA CAGG C ---	TATATAAATAAATAGGTC C
		TATATAAATAAATAGGT T -	TATATAAATAAATAGGTC C
	plant 3	TATATAAATAAATAGGTC C -	TATATAAATAAATAGGTC-
		TATATAAATAAAT TC -----	TATATAAATAAATAGGTC-
		TATATAAATAAATAGG GC -	TATATAAATAAATAGGTC-
pToCV-35S-Rbz	plant 4	TATATAAATAAATAGGTCG	TATATAAATAAATAGGTC-
		TATATAAATAAATAGG C ---	TATATAAATAAATAGGTC-
		C ATATAAATAAATAGGTC C -	TATATAAATAAATAGGTC C
	plant 5	TATATAAATAAATAGGT T -	TATATAAATAAATAGGT T
		TATATAAATAAATAGGTC C -	TATATAAATAAATAGGT T
		TATATAAATAAATAGGT T -	TATATAAATAAATAGGT T
	plant 6	TATATAAATAAATAGGT T	TATATAAATAAATAGGTC C
		TATATAAATAAATAGGT T -	TATATAAATAAATAGGTC C
		TATATAAATAAATAGGT T	TATATAAATAAATAGGTC C

Figure 2.3. Sequencing of tomato chlorosis virus RNA1 and RNA2 3' ends cloned from systemically infected *Nicotiana benthamiana* plants at 30 dpi. Plants were agroinoculated with pToCV-35S-RNA1 + pToCV-35S-RNA1 (pToCV-35S) or pToCV-35S-RNA1-Rbz + pToCV-35S-RNA2-Rbz (pToCV-35S-Rbz). Three plants were analyzed per condition and six clones (three for RNA1 and three for RNA2) were sequenced per plant. Nucleotide changes (deletions, insertions, or substitutions) with respect to the sequence of the infectious clones (isolate AT80/99-IC) are highlighted in bold and red. Of these, those nucleotide changes resulting in sequences present in other ToCV isolates (Table 2.3) are outlined in red.

Chapter 2

ToCV infectious clones: increasing efficiency by introducing the HDV ribozyme

Table 2.3. Tomato chlorosis virus isolates available in GenBank with 3' end nucleotide sequences identical to some of the variants detected in the viral progeny of agroinoculated *Nicotiana benthamiana* plants (Figure 2.3).

Isolate	RNA1		RNA2		Reference
	Acc. no.	Sequence (5'-3')	Acc. no.	Sequence (5'-3')	
AT80/99-IC	KJ740256	TAAATAAATAGGTCG	KJ740257	TAAATAAATAGGTC-	Orlío <i>et al.</i> , 2014
ToC-Br2	JQ952600	TAAATAAATAGGC-	-	-	Albuquerque <i>et al.</i> , 2013
2.5	KJ200304	TAAATAAATAGGT	-	-	Unpublished
MM8	KJ200306	TAAATAAATAGGTC	-	-	Unpublished
Pl-1-2	KJ200308	TAAATAAATAGGTC	-	-	Unpublished
XS	-	-	KY618797	TAAATAAATAGGTCC	Kang <i>et al.</i> , 2018
FERA_160205	KY810786	TAAATAAATAGGTC	-	-	Pecman <i>et al.</i> , 2017

Nucleotide changes present in the mentioned variants with respect to the sequences of the infectious clones (isolate AT80/99-IC, included for comparison) are highlighted in bold and underlined.

CHAPTER 3

CONSTRUCTION OF A SET OF TOMATO CHLOROSIS VIRUS CP_m DELETION MUTANTS TO IDENTIFY WHITEFLY TRANSMISSION DETERMINANTS



3.1 INTRODUCTION

Tomato chlorosis virus (ToCV), belonging to the genus *Crinivirus* in the family *Closteroviridae*, is an emerging plant virus that causes a yellowing disease in tomatoes and other economically important crops. Since its first detection in Florida in 1997 (Wisler *et al.*, 1998b), ToCV has been reported in over 40 countries and territories, infecting plants from 31 botanical families (reviewed by Fiallo-Olivé and Navas-Castillo, 2023). Besides tomatoes, ToCV infects significant crops such as peppers, potatoes, and eggplants, causing severe diseases worldwide. Symptoms of ToCV infection in tomatoes include interveinal chlorosis, starting from older leaves and progressing towards the upper parts of the plant. In advanced stages, interveinal yellow areas may develop reddish-brown necrotic flecks, while lower leaves exhibit rolling, thickening, and brittleness, eventually becoming crispy to the touch. The observed reduction in plant vigor and marketable fruit yield can be attributed to the loss of photosynthetic area.

Criniviruses are semi-persistently transmitted by adult whiteflies. Most criniviruses are transmitted by whiteflies belonging to the *Bemisia tabaci* complex, commonly known as the tobacco/sweet potato/cotton whitefly, or by *Trialeurodes vaporariorum*, also known as the greenhouse whitefly. However, two exceptions to this transmission pattern are observed: sweet potato chlorotic stunt virus, transmitted by *B. tabaci* Middle East-Asia Minor 1 (MEAM1) and *T. abutilonea*, the bandedwinged whitefly, and ToCV, which is transmitted by *B. tabaci* MEAM1, New World 1 (NW1), and Mediterranean (MED), *T. abutilonea*, and *T. vaporariorum* (Navas-Castillo *et al.*, 2000; Sim *et al.*, 2000; Wisler *et al.*, 1998b). The transmission efficiencies follow the order MED > MEAM1 = *T. abutilonea* > NW1 > *T. vaporariorum*.

ToCV possesses a bipartite single-stranded RNA (ssRNA) genome with positive polarity, exhibiting the typical organization of members of the genus *Crinivirus* (Lozano *et al.*, 2006, 2007; Wintermantel *et al.*, 2005). RNA1 consists of four open reading frames (ORFs) flanked by two untranslated regions (UTRs) located at the 5' and 3' ends of the molecule. ORF1a encodes a polyprotein containing conserved protease, methyltransferase, and helicase domains found in all closterovirids (Karasev, 2000). ORF1b encodes the RNA-dependent RNA polymerase, which is translated through a ribosomal +1 frameshift. ORF2 encodes a 22 kDa protein described as a suppressor of RNA silencing (Cañizares *et al.*, 2008). ORF3 encodes a

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putative 6 kDa protein with a predicted transmembrane domain, similar to other proteins located at the 3' end of criniviruses, whose function remains unknown. ToCV RNA2 contains nine ORFs, also flanked by UTRs at both ends. RNA2 includes a cluster of five genes characteristic of the family *Closteroviridae* involved in genome encapsidation, viral movement in plants, and interaction with the insect vector: ORF4, ORF5, ORF7, ORF9, and ORF10, encoding p4, a heat shock protein 70 homologue (Hsp70h), p59, the major capsid protein (CP), and the minor coat protein (CPm), respectively. Both CP and CPm have been identified as suppressors of gene silencing (Cañizares *et al.*, 2008). ORF6 encodes a putative p8 protein with a size, genomic position, and sequence similar to proteins of other criniviruses (Livieratos *et al.*, 2004). ORF8 encodes a putative p9 protein, found exclusively in members of the genus *Crinivirus*, with an unknown function. ORF11 encodes the p27 protein, which appears to be unique to criniviruses (Dolja *et al.*, 2006). Finally, ORF12 encodes a putative p7 with a transmembrane domain that does not exhibit significant similarity to any protein encoded by the genomes of other criniviruses.

Both RNA molecules of ToCV are separately encapsidated in flexuous particles measuring 800-850 nm in length (Liu *et al.*, 2000). These particles are believed to possess a characteristic structure with a long "body" and short "tail" (rattlesnake), similar to that described for the crinivirus lettuce infectious yellows virus (LIYV) (Tian *et al.*, 1999) and other closterovirids (Agranovsky *et al.*, 1995). The virion body consists of the CP, while the tail contains the CPm and units of other viral proteins, including the Hsp70h and p59 proteins. The tail likely encapsidates the 5'-terminal regions of RNA1 and RNA2, which contain a packaging signal recognized by the CPm (Peremyslov *et al.*, 2004a; Satyanarayana *et al.*, 2004). In contrast to other filamentous plant viruses, closterovirids encode two proteins with CP-related domains, CPm and the ~60 kDa protein (Boyko *et al.*, 1992; Napuli *et al.*, 2003). The genes encoding both proteins originated from duplication and divergence of the CP gene, and phylogenetic analyses have suggested that CPms of closterovirids evolved independently in each genus (Dolja *et al.*, 2006).

The CPm protein of criniviruses, aside from forming the virion tail, is believed to be crucial for transmission by whitefly vectors. Several observations support this hypothesis: i) CPm antiserum effectively neutralized LIYV transmission by *B. tabaci* NW1 (Chen *et al.*, 2011; Tian *et al.*, 1999), ii) LIYV virions are retained in the anterior foregut or cibarium of whitefly

vectors through an unknown CPm-mediated mechanism (Chen *et al.*, 2011), and iii) LIYV virions with truncated CPms lacking the C-terminal region, which accumulate in *Nicotiana benthamiana* protoplasts or even systemically spread in *N. benthamiana* plants, were defective in transmission by *B. tabaci* (Chen *et al.*, 2011; Ng and Falk, 2006b; Ng and Zhou, 2015; Stewart *et al.*, 2010).

Based on the available information, we hypothesize that the determinant of dual transmission of criniviruses by members of two whitefly genera is located within the CPm protein. Therefore, in this study, we performed an *in silico* analysis comparing the CPm proteins of all characterized criniviruses to date, aiming to gain insight into the putative CPm regions responsible for ToCV transmission by members of both the genera *Bemisia* and *Trialeurodes*. This analysis identified a central region of the CPm exclusive to criniviruses transmitted by *T. vaporariorum*. Subsequently, using a system of ToCV agroinfectious clones, we generated a set of deletion mutants targeting the CPm central region and its sub-regions. The RNA2 infectious clones derived from these mutants, along with the preliminary results reported in this study, lay the groundwork for future investigations that will elucidate the role of specific regions of the CPm in transmission by different whitefly species.

3.2 MATERIALS AND METHODS

3.2.1 Sequence and phylogenetic analysis

Amino acid sequences of CPm available in the NCBI's Entrez protein database (<https://www.ncbi.nlm.nih.gov/protein/>) for members of the genus Crinivirus were aligned using MUSCLE (Edgar, 2004) and edited with GeneDoc (<https://nrbsc.org/downloads/>). For phylogenetic inference, the maximum likelihood method (500 replicates) was employed, and sequence alignments were performed using MUSCLE in MEGA11 (Tamura *et al.*, 2021). The best-fit model of amino acid substitution was determined based on the corrected Akaike information criterion and Bayesian information criterion, as implemented in MEGA11 (Tamura *et al.*, 2021).

3.2.2 Construction of CPm deletion mutants

Constructs containing partial deletions of the ToCV CPm gene were generated by replacing an AarI-NruI fragment (including most of the CPm gene) in the RNA2 infectious clone (pToCV-

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35S-RNA2) (Orilio *et al.*, 2014) with a DNA fragment lacking specific genome regions (A+B+C, A+B, A, B, or C; Fig. 3.1A.B) (see below). These fragments were obtained using a PCR-driven overlap extension technique, including PCR #1 and PCR #2 (Fig. 3.1C) (Heckman and Pease, 2007). PCR #1 generated both moieties of the AarI-NruI fragment with the deletion mutations using flanking and deletion primers (Table 3.1). The overlapping fragments generated in PCR #1 for each deletion mutant were joined by PCR #2 using the flanking primers. For PCR #1, amplifications were carried out with 100 ng of pToCV-35S-RNA2 (Orilio *et al.*, 2014) using the Expand High Fidelity PCR System (Roche Diagnostics) according to the manufacturer's protocol under the following conditions: initial denaturation at 94 °C for 2 min, followed by 35 cycles of 94 °C for 15 s, 55.3 °C for 15 s, and 72 °C for 2 min, and a final extension at 72 °C for 7 min. For PCR #2, overlapping amplification for each deletion mutant was performed with 1 ng of each gel-purified fragment obtained in PCR #1 following the conditions described above. Fragments obtained in PCR #2 were gel-purified, cloned into the pGEM-T Easy Vector (Promega), and transformed by electroporation (Gene Pulser XCell, Bio-Rad) into *Escherichia coli* DH5 α . The AarI-NruI fragments were excised from the obtained clones, gel-purified, and ligated with T4 DNA ligase (Roche Diagnostics) into pToCV-35S-RNA2 digested with AarI and NruI, dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics), and gel-purified. The recombinant plasmids were transformed into *E. coli* Stbl4 (Invitrogen) by electroporation using a Gene Pulser XCell (Bio-Rad). Putative correct clones, based on restriction analysis and the size of the amplification product obtained by PCR using primers MA2417 and MA2418 (Table 3.1), were sequenced at Macrogen Inc. (Seoul, South Korea) using flanking primers MA2134 and MA2137 (Table 3.1). Two clones per deletion mutant were selected for transformation into *Agrobacterium tumefaciens* GV3101 by electroporation (Gene Pulser Xcell, Bio-Rad).

3.2.3 Plant agroinoculation

For agroinoculation assays, *A. tumefaciens* GV3101 carrying the wild-type ToCV RNA1 (pToCV-35S-RNA1, Orilio *et al.*, 2014) or any of the ToCV RNA2 deletion mutants for the CPm gene (Table 3.2) were added at a 1:1000 dilution to yeast extract peptone liquid medium supplemented with rifampicin (25 μ g/ml), gentamicin (20 μ g/ml), kanamycin (50 μ g/ml), morpholineethanesulfonic acid (MES) (10 mM, pH 5.8), and acetosyringone (20 μ M) with shaking (200 rpm) at 28 °C for 48 h. Bacterial cells were harvested by centrifugation,

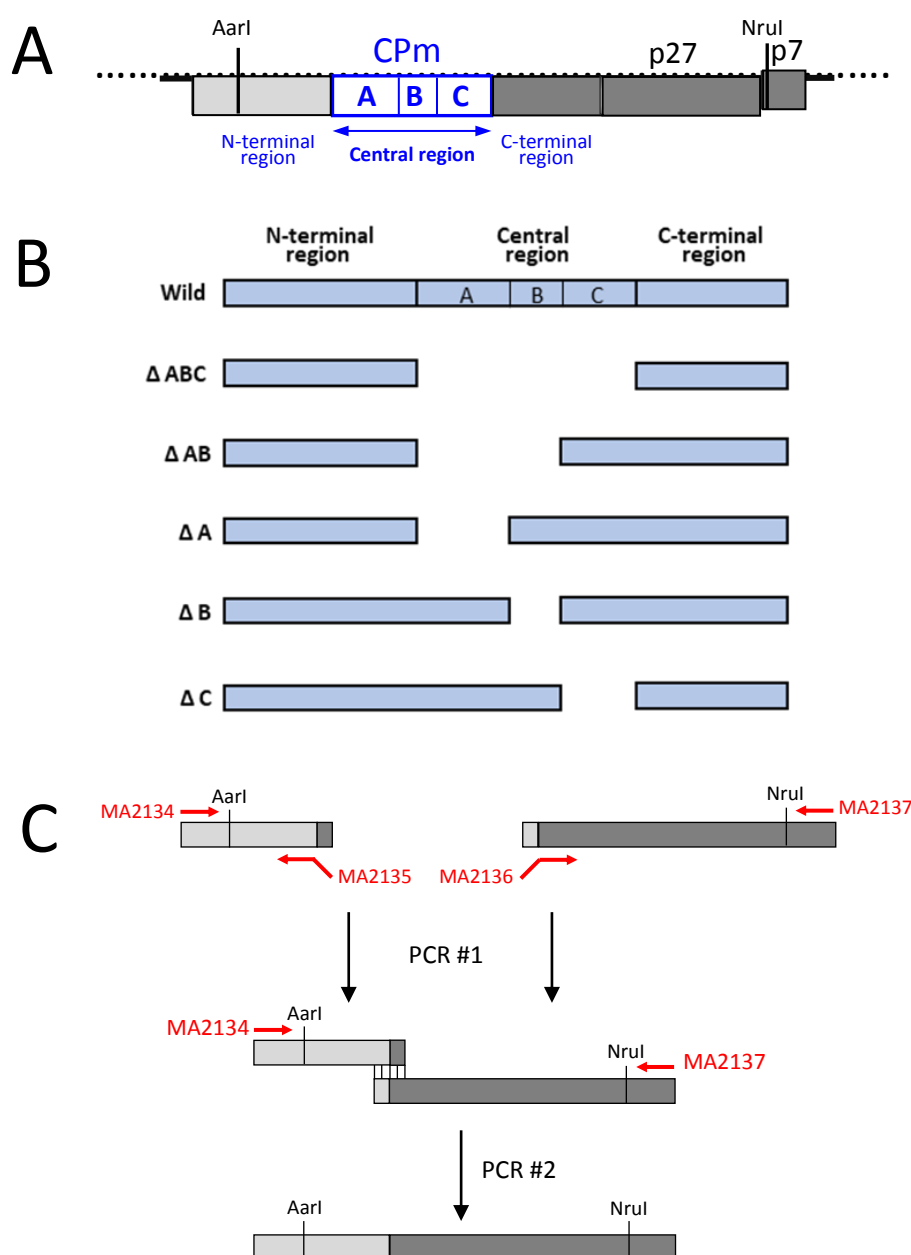


Figure 3.1. Strategy for cloning the tomato chlorosis CPm deletion mutants. Scheme of the RNA2 genomic region containing the CPm gene highlighting the central region and sub-regions A, B, and C (A) and the resulting deletion mutants A+B+C, A+B, A, B, and C (B). C. Schematic representation of the PCR-driven overlap extension technique to generate the deletion mutants, including PCR#1 and PCR#2, exemplified for mutant $\Delta A+B+C$. Both moieties of the resulting mutant are labeled with different shades of gray. Flanking and deletion primers are highlighted in red. Restriction sites AarI and NruI used for replacing the mutant fragment in the RNA2 infectious clone are indicated.

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resuspended in induction medium (10 mM MES pH 5.8, 10 mM MgCl₂, acetosyringone 150 μ M) to a final OD₆₀₀ of 1.0. Both RNA1 and RNA2 bacterial suspensions were mixed in a 1:1 ratio and kept at room temperature for 2 h. *A. tumefaciens* cultures carrying the constructs pToCV-35S-RNA1 and pToCV-35S-RNA2 (Orflio *et al.*, 2014) or empty pCAMBIA2300 vector were used as positive and negative controls (mock), respectively. *N. benthamiana* plants at the 3-5 leaf stage were agroinfiltrated on the underside of leaves using a 1 mL syringe without a needle. Agroinoculated plants were maintained in an insect-proof growth chamber with 16 hours of light (250 μ mol s⁻¹ m⁻² photosynthetically active radiation) at 25 °C, 8 hours of darkness at 18 °C, and 70% relative humidity.

3.2.4 Detection of deletion mutant viral RNA in agroinoculated plants

The presence of viral RNA in non-inoculated leaves of agroinoculated plants was assayed by tissue blot molecular hybridization four weeks post-inoculation. Cross-sectioned leaf petioles were squash-blotted onto positively charged nylon membranes (Roche Diagnostics) and fixed with ultraviolet light (Crosslinker RPN 2500, Amersham Life Science). Membranes were treated with standard hybridization buffer [5xSSC (750 mM NaCl, 75 mM sodium citrate, pH 7.0), 0.2% N-lauryl sarcosine, 0.02% sodium dodecyl sulfate (SDS), 2% blocking agent (Roche Diagnostics), and 50% formamide] at 65 °C for 2 h, hybridized with a digoxigenin-labeled negative sense RNA probe specific to the coat protein gene as described previously (Fortes *et al.*, 2012), diluted in standard hybridization buffer at 65 °C overnight, and washed under high stringency conditions (0.1x SSC, 0.1% SDS at 65 °C). Further hybridization steps and chemiluminescent detection followed standard procedures recommended by the manufacturer (DIG Application Manual for Filter Hybridization, Roche Diagnostics). Membranes were exposed to X-ray film (Mamoray, AGFA) and developed following a conventional photographic process.

The presence of the CPm deletion mutations in the viral progeny generated in ToCV-infected plants after agroinoculation was confirmed by RT-PCR amplification. For that, primers MA2417 and MA2418 (Table 3.1) were designed to amplify a DNA fragment including the deleted regions, thus producing products of different sizes for the different CPm deletions (wild CPm, 1143 bp; A-CPm Δ , 825 bp; B-CPm Δ 939 bp; C-CPm Δ , 885 bp; AB-CPm Δ , 621 bp; and

Table 3.1. Primer used in this work.

Primer	Description	Sequence (5' - 3')	Position in the ToCV genome (nt)
MA2134	Left flanking primer	TTGGGTTCCGGTGATGTGTTTC	5193-5214
MA2135	Rev ABC-PCR CPmΔ (232-491)	CAAAATTGTGACT TCAGCGAATCATTAGGAACATCAGG	5781-5804 6585-6597
MA2136	For ABC-PCR CPmΔ (232-491)	GATTCGCTGA AGTCACAAATTTGTTGCAGCTAATCATTA	5796-5804 6585-6613
MA2137	Right flanking primer	ACGAACGATTGGAGGTTGTGAG	8005-8026
MA2766	Rev A-PCR CPmΔ (232-337)	CAATTTTATGGTC CAGCGAATCATTAGGAACATCAGGC	5780-5804 6123-6135
MA2767	For A-PCR CPmΔ (232-337)	GATTCGCTG GACCATAAAATTGAAAAAGACTCTAAAGGAC	5796-5804 6123-6153
MA2768	Rev B-PCR CPmΔ (338-405)	CTTCAGAACTATC CACGAAACCTGCCTGGAATTG	6103-6122 6327-6339
MA2797	For B-PCR CPmΔ (338-405)	CAGGTTCTGCTGG ATAGTTCTGAAGTTCCTTTGGTAC	6113-6122 6327-6351
MA2770	Rev C-PCR CPmΔ (406-491)	CAAAATTGTGACT TAAACTTGATGTAATTGGGTTTGATGATGTC	6297-6326 6585-6597
MA2771	For C-PCR CPmΔ (406-491)	CATCAAGTTTA AGTCACAAATTTGTTGCAGCTAATC	6317-6326 6585-6609
MA2795	Rev AB-PCR CPmΔ (232-405)	CTTCAGAACTATC CAGCGAATCATTAGGAACATCAGG	5781-5804 6327-6339
MA2796	For AB-PCR CPmΔ (232-405)	GATTCGCTGG ATAGTTCTGAAGTTCCTTTGGTAC	5796-5804 6327-6351
MA2417	For deletion size confirmation	CCCGAGTCTGATATTCAACG	5654-5673
MA2418	Rev deletion size confirmation	GTTCCGGTATCAGTCTTCCAG	6776-6796

Vertical bars (|) in the deletion primers indicate the boundaries of the regions to be deleted. For, forward; Rev, reverse.

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Table 3.2. Agroinoculation experiments carried out with tomato chlorosis virus RNA2 deletions mutants for the CPm gene in *Nicotiana benthamiana* plants.

Construct name	Deleted region (CPm aa positions)	Replicate	No. infected plants/No. inoculated plants (% infected plants)		
			RNA1 + RNA2 CPmΔ	RNA1 + RNA2 wild type	Mock
pToCV-35S-RNA2-CPmΔ-ABC-1	232-491	1	6/20 (30%)	5/20 (25%)	0/10 (0%)
		2	3/20 (15%)	11/20 (55%)	0/10 (0%)
		3	8/20 (40%)	7/20 (35%)	0/10 (0%)
		4	2/20 (10%)	9/20 (45%)	0/10 (0%)
pToCV-35S-RNA2-CPmΔ-ABC-3	232-491	1	4/20 (20%)	5/20 (25%)	0/10 (0%)
pToCV-35S-RNA2-CPmΔ-AB-2	232-405	1	9/20 (45%)	11/20 (55%)	0/10 (0%)
pToCV-35S-RNA2-CPmΔ-AB-80	229-230 / 232-405	1	5/20 (25%)	6/20 (30%)	0/10 (0%)
pToCV-35S-RNA2-CPmΔ-A-8	232-337	1	9/20 (45%)	14/20 (70%)	0/10 (0%)
pToCV-35S-RNA2-CPmΔ-A-10	232-337	1	4/20 (20%)	6/20 (30%)	0/10 (0%)
pToCV-35S-RNA2-CPmΔ-B-17	338-405	1	3/20 (15%)	6/20 (30%)	0/10 (0%)
pToCV-35S-RNA2-CPmΔ-B-38	338-405	1	6/20 (30%)	14/20 (70%)	0/10 (0%)
pToCV-35S-RNA2-CPmΔ-C-4	406-491	1	0/20 (0%)	13/20 (65%)	0/10 (0%)
		2	0/19 (0%)	9/20 (45%)	0/8 (0%)
pToCV-35S-RNA2-CPmΔ-C-5	406-491	1	0/20 (0%)	13/20 (65%)	0/10 (0%)
		2	0/19 (0%)	9/20 (45%)	0/8 (0%)
		3	0/20 (0%)	8/20 (40%)	0/10 (0%)

ABC-CPmΔ, 363 bp). Total RNA was extracted from systemically infected leaves using TRIsure reagent (Bioline) following the manufacturer's instructions. Three microliters of the total RNA were converted to cDNA with primer MA2418 using AMV-RT (Promega) in the presence of dithiothreitol and RNase OUT (Invitrogen) at 42 °C for 50 min. PCR was carried out with 3μL of cDNA, primers MA2417 and MA2418, and BioTaq DNA polymerase (Bioline) with the following conditions: initial denaturation at 94 °C for 3 min, followed by 25 cycles of 94 °C for 15 s, 67 °C for 1 min, and 72 °C for 1 min, and a final extension at 72 °C for 5 min.

The statistical analyses and graphing to compare the percentage of infection of the wild-type and deletion mutant CPms were performed using SigmaPlot 14.5 software (Systat Software Inc.).

3.3 RESULTS AND DISCUSSION

3.3.1 Correlation between crinivirus CPm size and specific whitefly vectors

To investigate the determinants of dual transmission of criniviruses by the whitefly genera *Bemisia* and *Trialeurodes*, we focused on the CPm protein. A comparative analysis of CPm sizes encoded by characterized crinivirus genomes revealed two distinct groups. One group ranged from 452 aa to 474 aa [i.e. bean yellow disorder virus, cucurbit yellow stunting disorder virus (CYSDV), lettuce chlorosis virus, and LIYV], while the other group ranged from 657 aa to 689 aa [i.e. beet pseudoyellows virus, blackberry yellow vein-associated virus, potato yellow vein virus, strawberry pallidosis-associated virus, sweet potato chlorotic stunt virus (SPCSV), ToCV, and tomato infectious chlorosis virus] (Fig. 3.2). Criniviruses with shorter CPm are transmitted by whiteflies of the *B. tabaci* complex, except for a reported but unconfirmed transmission of SPCSV by *B. afer*. Conversely, criniviruses with larger CPm are transmitted by whiteflies of the genus *Trialeurodes*, including *T. vaporariorum* and/or *T. abutilonea*. Notably, two criniviruses transmitted by *Trialeurodes* spp., SPCSV and ToCV, were also transmitted by *B. tabaci*. This suggests that larger CPms are associated with *Trialeurodes* spp. transmission, regardless of *B. tabaci* transmission. Consequently, it can be speculated that certain molecular determinants of ToCV transmission by *Trialeurodes* spp. may be located within a region unique to larger CPms. To identify the genome region(s) responsible for the distribution of CPm sizes, we aligned available crinivirus CPm amino acid sequences (Fig. 3.3). This analysis revealed a central part of the CPm (highlighted by red rectangles in Fig. 3.3) absent in shorter proteins, accounting for the size difference. The alignment excluded the CPm of tetterwort vein chlorosis virus (TwVCV) due to the lack of reported natural vectors. The central region spanned amino acids 291 to 548, flanked by N-terminal (aa 1-290) and C-terminal regions (aa 549-725). The association between this central region and transmission by *Trialeurodes* spp. has not been previously suggested, and no experimental studies have investigated the cause-effect relationship between these factors.

ToCV, being transmitted by both *B. tabaci* and *T. vaporariorum*, served as an excellent candidate for biological experiments aimed at assigning specific transmission features to particular CPm regions. We constructed a series of ToCV CPm mutants by deleting nucleotide sequences encoding the central part of the protein or its sub-regions. Sub-regions A, B, and C

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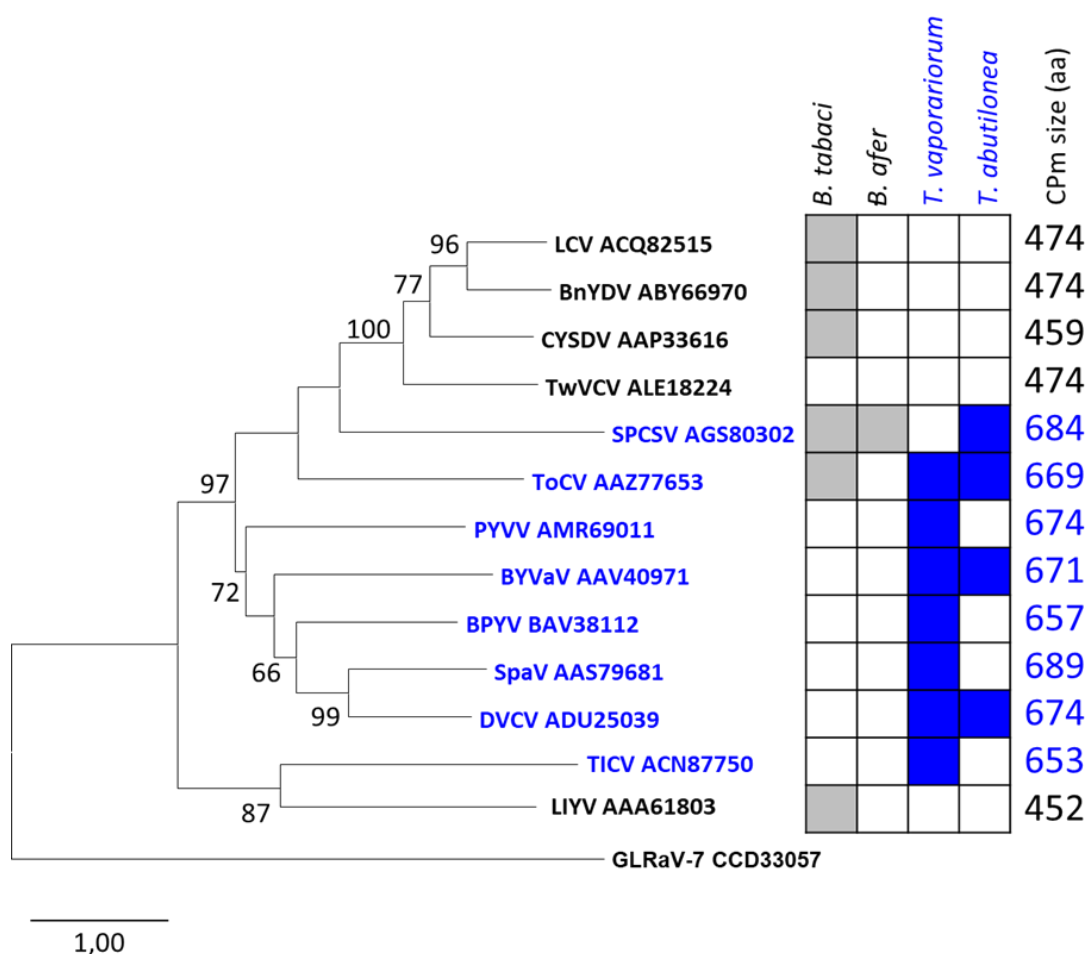


Figure 3.2. Phylogenetic tree illustrating the relationships of the minor capsid protein of members of the genus Crinivirus. The tree was built from an alignment with MUSCLE using the maximum likelihood method (500 replicates) with the MEGA11 program (Tamura et al., 2021) using the best fit model, LG + G + I + F. BnYDV, bean yellow disorder virus; BPYV, beet pseudoyellows virus; BYVaV, blackberry yellow vein-associated virus; CYSDV, cucurbit yellow stunting disorder virus; DVCV, Diodia vein chlorosis virus; LCV, lettuce chlorosis virus; LIYV, lettuce infectious yellows virus; PYVV, potato yellow vein virus; SPaV, strawberry pallidosis-associated virus; SPCSV, sweet potato chlorotic stunt virus; TICV, tomato infectious chlorosis virus; ToCV, tomato chlorosis virus; TwVCV, tetterwort vein chlorosis virus. The bar below the tree indicates the number of amino acid substitutions per site. Bootstrap values higher than 50% are indicated. The table to the right of the tree shows the known whitefly vectors (*Bemisia tabaci*, *Bemisia afer*, *Trialeurodes vaporariorum*, *Trialeurodes abutilonea*) for each crinivirus. Additionally, the CPm size (aa) is indicated for each crinivirus. Data on criniviruses transmitted by *Trialeurodes* spp. are highlighted in blue. References reporting the whitefly vectors for each crinivirus: Berdiales *et al.*, 1999; Celix *et al.*, 1996; Duffus *et al.*, 1965, 1996a,b; Gamarra *et al.*, 2010; Larsen *et al.*, 1991; Liu *et al.*, 1997; Martín *et al.*, 2011; Navas-Castillo *et al.*, 2000; Poudel *et al.*, 2013; Salazar *et al.*, 2000; Segundo *et al.*, 2004; Sim *et al.*, 2000; Tzanetakis *et al.*, 2006, 2011; Wisler *et al.*, 1998a,b.

were defined (Fig. 3.4), and RNA2 infectious clones lacking sub-regions A+B+C, A+B, A, B, or C were generated (Fig. 3.1). These mutant clones, in combination with the wild-type ToCV RNA1 infectious clone, were agroinoculated into *N. benthamiana* plants for further analysis.

During the *in silico* analysis of crinivirus CPms, two predictions emerged. TwVVCV, with a short CPm of 474 aa (Zhao *et al.*, 2015), was predicted to be transmitted by *B. tabaci*. On the other hand, SPCSV, with a large CPm of 684 aa and transmitted by *B. tabaci*, *B. afer*, and *T. abutilonea* (Gamarra *et al.*, 2010; Sim *et al.*, 2000), could also be transmitted by *T. vaporariorum*, considering the transmission pattern of other criniviruses with large CPm. However, both predictions await experimental confirmation.

It is worth noting that the phylogenetic analysis based on complete CPm amino acid sequences did not accurately reflect the separation of the two groups of criniviruses characterized by their large and short CPm sequences. Criniviruses with larger sequences, transmitted by *Trialeurodes* spp., were positioned in distinct branches of the phylogenetic tree. This observation raises questions about the evolutionary history of the CPm gene within the genus, similar to the situation in the family *Closteroviridae* as a whole. Several scenarios have been proposed to explain the appearance and evolution of the CPm gene in different genera, involving gene duplication and adaptation to the insect vector. The occurrence of larger CPm versions multiple times during the evolutionary history of the genus cannot be ruled out.

The availability of additional genomes in the future, facilitated by viral metagenomics technologies (e.g. Liu *et al.*, 2021; Xiao *et al.*, 2019), will contribute to a better prediction of phylogenetic relationships between criniviruses and confirm the association between CPm size and transmission by different whitefly species.

3.3.2 Infectivity of ToCV CPm deletion mutants in *N. benthamiana* plants

The designed CPm deletion mutants were successfully incorporated into the RNA2 infectious clone of ToCV isolate AT80/99-IC (pToCV-35S-RNA2) (Orilio *et al.*, 2014), as confirmed by sequencing the regions flanking the deleted fragments in two clones for each construct (A+B+C, A+B, A, B, and C). In the A+B mutant, one clone (#80) exhibited a 6-nt deletion in the sequence corresponding to one of the deletion primers (Fig. 3.5), resulting in amino acid changes (N228T, D229del, S230del). All A+B+C, A+B, A, and B CPm deletion

```

          *          360          *          380          *          400          *          420
BPYV : CDPIR358NKEIQM360K361Y362RKN363NSTYL--TSYIQKAIPVQ364EKGNLKL365FG366ENYSED367V368FELITAG369TSAT370EKLKIPV371PK372GV373YEFQ : 381
PYVY : MT-GSNKELT374FQ375Y376MKD377RG378RYI--DDFQKSIPI379SV380DKEGYA381EV382IG383ILTN384GK385SE386HVVND387LKIG388PS389FNLRGGN390SF391SEWL : 384
SPCSV : YIHT392PAHRL393FF394HR395GD396SE397YY398K399IEASV400NKVLN401SLP---TE402DF403VT404IR405SG406Q407K408V409SV410NEIV411FLT412IV413LPK414VGS415K416Q417I418WG419IK : 392
BYVaV : RDLNSADNRV420-R421HY422NKH423KGAYL--ED424SKY425Y426DE427SG428PGGNE429NSI430FG431SLKEN432V433ID434IR435NNR436IF437SR438WLKIST439FO440SF441IELQ : 387
ToCV : IGK442N443R444K445FN446V447GV448AW449KDN450SG451FG452H453FK454IG455PTLAKISTIPG---RF456V457HK458IEK459D460SK461GH462LI463VS464ID465NT466VL467VR468ANK469LIV470K471PS472Q473I474W475EFH : 377
SpaV : NNLD476HGRSD477FF478RY479Y480KK481G482RG483KYR--DDLAK484T485MEN486VS487RG488G489FK490Y491FG492SKLMD493NI494HE495K496FN497K498TL499YS500V501KK502HK503IPS504RE505EV506FE507FQ : 403
DVCV : NSEK508N509KSND510V511FR512Y513TKD514KG515KYQ--SNLDR516VL517TD518VL519PS520DG521Y522K523Y524VE525IF526DF527KNN528V529ID530TA531QR532K533FL534YS535IR536LP537IQ538TK539FC540FE541IQ : 388
TICV : YDES542QRDL543TL544Q545MY546TKN547R548WRMF--EEK549V550LT551DE552Q553L554N555K556DD557GS558IF559GL--RK560TK561TV562VV563ANG564K565TL566YS567SD568HAIR569NN570SK571Y572FE573Q : 376
LCV : -----PEPEKQKPDVP----- : 256
CYSDV : -----DEKSK----- : 244
BnYDV : -----PSPQ245PQ246K247PVP----- : 252
LIYV : -----DKG248K249Q250PTDTVP----- : 243

```

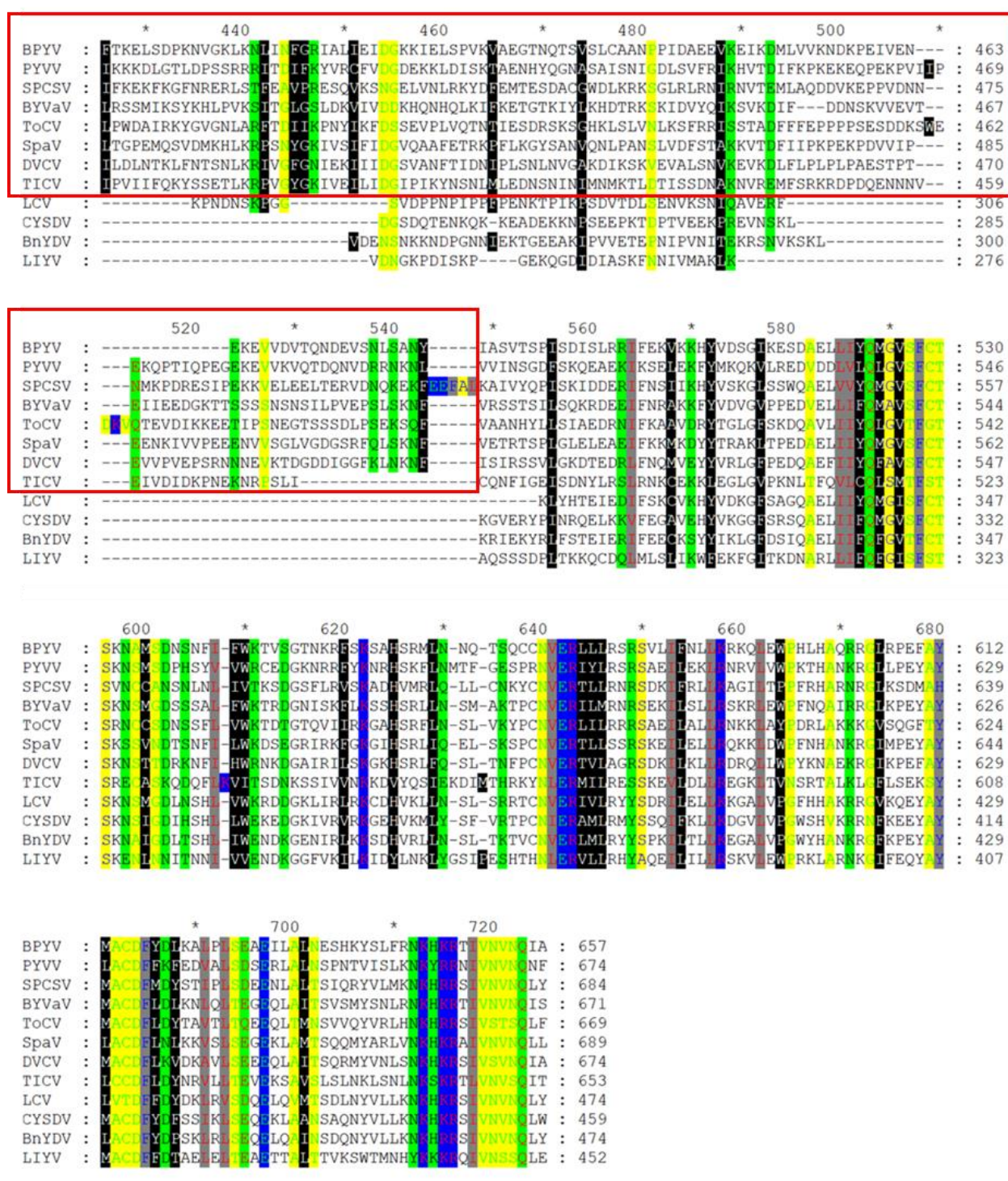


Figure 3.3. Alignment of the Cpm amino acid sequences for criniviruses with known whitefly vector(s) performed with MUSCLE (Edgar, 2004) and edited with GeneDoc (<https://nrbsc.org/downloads>). Residues are colored according to their physicochemical properties. The central part of the Cpm, only present in larger Cpm proteins, is highlighted by red rectangles.



clones (one to four replicates for both clones of each construct) were infectious when agroinoculated into *N. benthamiana* plants, including clone #80 (Table 3.2, Fig. 3.6A). Viral progeny presence was confirmed by tissue blot molecular hybridization, and the size of the CPm deletion was validated by RT-PCR. Although the infection percentages of all three mutants were lower than that of the wild-type CPm-containing construct, the differences were not statistically significant (Fig. 3.6B). However, both C CPm deletion clones (a total of five replicates) were non-infectious when agroinoculated into *N. benthamiana* plants.

```

>CPm-wild    ...AAGCCTGATGTTCTTAATGATTTCGCTGAGTGAAGTCGAATATCATAATCACTCTGATGTTTCC...
              K P D V P N D S L S E V E Y H N H S D V S
>CPmΔ-AB-2   ...AAGCCTGATGTTCTTAATGATTTCGCTGATAGTTCTGAAGTTCCTTTGGTACAACTAATACT...
              K P D V P N D S L D S S E V P L V Q T N T
>CpmΔ-AB-80  ...AAGCCTGATGTTCTTA-----CGCTGATAGTTCTGAAGTTCCTTTGGTACAACTAATACT...
              K P D V P T - - - L D S S E V P L V Q T N T

```

Figure 3.5. Nucleotide and amino acid sequences flanking the deleted A+B region in clone #80 manually aligned with the corresponding sequences of clone #2 and wild-type CPm. Sequences downstream of the deleted A+B region are highlighted in blue, and those mutated in clone #80 are in red.

These results indicate that the central region of the CPm is not essential for ToCV accumulation and systemic spread in *N. benthamiana* plants. Notably, previous studies involving deletion mutants affecting the LIYV CPm demonstrated the loss of transmissibility by *B. tabaci* NW, but did not negatively impact systemic movement in *N. benthamiana* plants (Stewart *et al.*, 2010). Future. Further investigations testing the transmissibility of mutants generated in this study by *B. tabaci* and/or *T. vaporariorum* could provide insights into a similar scenario where virion tail encapsidation, movement within the plant, and dual whitefly transmission functions are located in different regions of the CPm

The observation that CPm deletion mutants affecting sub-regions A+B+C, A+B, A, and B, but not C, can still accumulate and spread systemically in *N. benthamiana* plants strongly suggests that differences in primary structure alone are insufficient to explain these results. Therefore, revealing the three-dimensional structure of wild-type and mutant ToCV CPms would provide further insights. Techniques such as X-ray crystallography could aid in understanding the mechanism of dual transmission of ToCV by its whitefly vectors, *B. tabaci* and *T. vaporariorum*.

Chapter 3

A set of ToCV CPm deletion mutants to identify whitefly transmission determinants

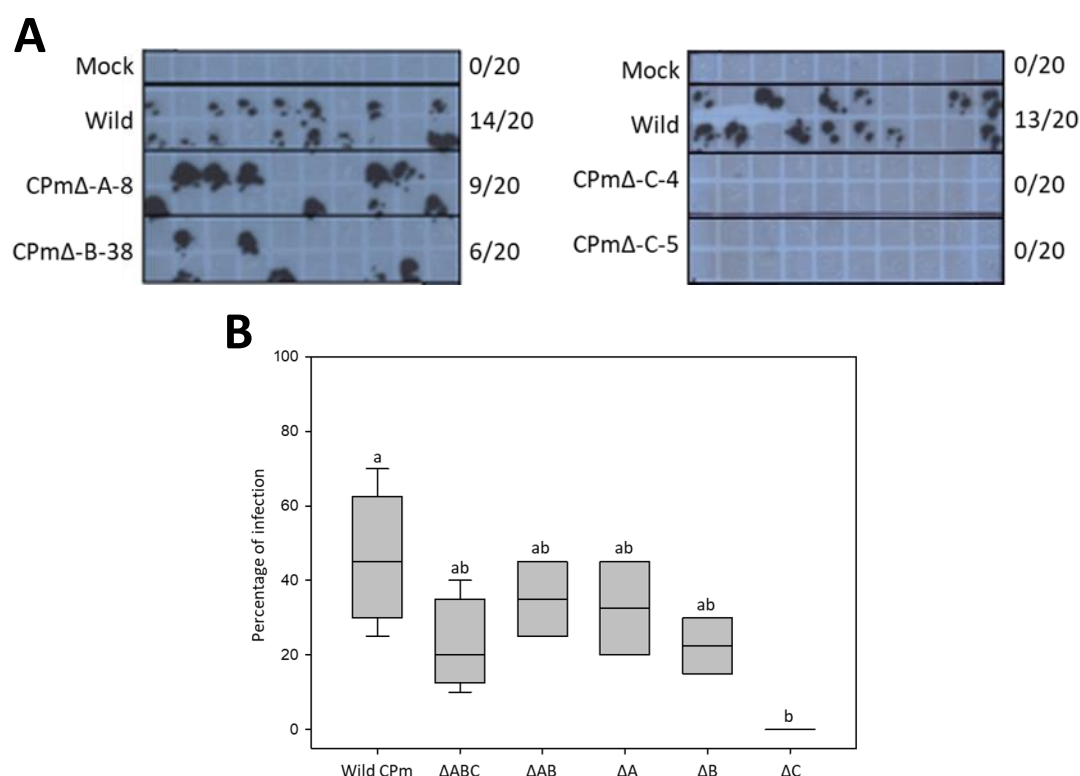


Figure 3.6. Agroinoculation experiments with tomato chlorosis virus (ToCV) CPm deletion mutants in *Nicotiana benthamiana* plants. **A.** Plants agroinoculated with mutants ΔA , ΔB , or ΔC , or with wild-type infectious clones were analyzed for the presence of ToCV by hybridization of cross-sectioned leaf petioles squash-blotted on nylon membranes using a digoxigenin-labeled RNA probe specific to the coat protein gene. Prints of mock-inoculated plants were included as negative controls. The number of infected plants per the number of inoculated plants is indicated on the right of each panel. **B.** SigmaPlot (v 14.5) box plots were used to compare the percentage of infection in *N. benthamiana* plants. The same letters on the boxes indicate no significant differences in the percentage of infection between agroinoculation conditions (wild-type or CPm deletion mutants). Raw data of agroinoculation experiments are in Table 3.2.

The ToCV CPm serves as a paradigmatic example of a multifunctional viral protein. In addition to its putative roles in encapsidation, cell-to-cell movement, and vector transmission suggested by results obtained for other closterovirids (e.g. Alzhanova *et al.*, 2000, 2001; Chen *et al.*, 2011; Satyanarayana *et al.*, 2000; Stewart *et al.*, 2010), it has been shown to function as a gene silencing suppressor (Cañizares *et al.*, 2008). Therefore, it would be interesting to

determine if the constructed mutants are still capable of suppressing gene silencing in *N. benthamiana* plants.

The interaction between CPm and secondary 5'-UTR structures has been identified as a key factor for virion assembly initiation in some closteroviruses (Peremyslov *et al.*, 2004a; Satyanarayana *et al.*, 2004). Additionally, an analog domain within the first 71 N-terminal amino acids of LIYV CPm (Ng and Zhou, 2015) has been proposed. If this holds true for ToCV, it could explain the availability of the constructed deletion mutants for virion tail encapsidation and suggest that they could be transmitted by at least *B. tabaci*.

This research will aid in understanding the detailed interactions between criniviruses and whiteflies that explain the dual transmission of ToCV and CYSDV by members of the genera *Bemisia* and *Trialeurodes*. Furthermore, the results obtained in this study will assist in designing new CPm mutants for fine mapping of putative domains involved in whitefly transmission. Ultimately, this could pave the way for investigations aimed at controlling the serious diseases caused by ToCV and other criniviruses in important crops by hindering the transmission process.

CONCLUSIONS

1. A Spanish isolate of a polerovirus infecting pepper has been shown to be transmitted by the aphid *Aphis gossypii*.
2. Complete genome sequencing of this isolate, Spain-Almería 2-2013, confirmed that this virus is related to pepper vein yellows viruses in the genus *Polerovirus*, with the closest relationships being with the polerovirus described in Israel and named pepper yellow leaf curl virus.
3. Comparison of the sequence from Spain with the complete sequences available for other poleroviruses infecting pepper supports the existence of a complex of polerovirus species.
4. The polerovirus from Spain was proposed to constitute one of these species, which has been officially named *Pepper vein yellows virus 5*.
5. Our results, along with those published over the last few years, have revealed have shown that the emerging poleroviruses threatening pepper crops worldwide are highly complex due to recombination events.
6. The agroinfectious clones generated for the crinivirus tomato chlorosis virus (ToCV) RNA1 and RNA2, using the Spanish isolate AT80/99 and incorporating the hepatitis delta virus ribozyme fused to the 3' ends, showed a two-fold increase in infectivity on *Nicotiana benthamiana* plants compared to previously available clones.
7. This improvement is associated with the generation of a viral progeny with RNA1 3' ends that are more similar to that present in the clone used for agroinoculation.
8. Sequence comparisons revealed a putative role of the central region of the minor coat protein (CPm) in the transmission of criniviruses by the whitefly *Trialeurodes vaporariorum*.
9. The success achieved in the agroinoculation experiments with a number of ToCV RNA2 deletion mutants for this central region and its sub-regions revealed that this region is not necessary for viral accumulation and systemic spread in *Nicotiana benthamiana* plants.
10. The availability of these CPm deletion mutants will pave the way for future investigations aiming to elucidate the role of specific regions of this protein in the transmission of ToCV by different whitefly species.

CONCLUSIONES

1. Se ha demostrado que un aislado español de un polerovirus que infecta pimiento es transmitido por el pulgón *Aphis gossypii*.
2. La secuenciación completa del genoma de este aislado, Spain-Almería 2-2013, confirmó que este virus está relacionado con los virus del amarilleo de las venas del pimiento pertenecientes al género *Polerovirus*, siendo las relaciones más cercanas con el polerovirus descrito en Israel y llamado pepper yellow leaf curl virus.
3. La comparación de la secuencia de España con otras secuencias completas disponibles de polerovirus que infectan al pimiento respalda la existencia de un complejo de especies de polerovirus.
4. Se propuso que el polerovirus de España constituya una de estas especies, la cual ha sido oficialmente nombrada *Pepper vein yellows virus* 5.
5. Nuestros resultados, junto con los publicados en los últimos años, han revelado que los polerovirus emergentes que amenazan los cultivos de pimiento en todo el mundo son altamente complejos debido a eventos de recombinación.
6. Los clones agroinfectivos generados para el RNA1 y RNA2 de tomato chlorosis virus (ToCV), utilizando el aislado español AT80/99 e incorporando el ribozima de hepatitis delta virus fusionado en los extremos 3', mostraron el doble de infectividad en plantas de *Nicotiana benthamiana* en comparación con los clones previamente disponibles.
7. Esta mejora está asociada con la generación de una progenie viral con extremos 3' del RNA1 más similares a los presentes en el clon utilizado para la agroinoculación.
8. Las comparaciones de secuencias revelaron un posible papel de la región central de la proteína menor de la cápsida (CPm) en la transmisión de crinivirus por la mosca blanca *Trialeurodes vaporariorum*.
9. El éxito logrado en los experimentos de agroinoculación con varios mutantes de delección del RNA2 de ToCV para esta región central y sus subregiones reveló que dicha región no es necesaria para la acumulación viral y la propagación sistémica en plantas de *Nicotiana benthamiana*.
10. La disponibilidad de estos mutantes de delección de CPm abrirá el camino para futuras investigaciones con el objetivo de dilucidar el papel de regiones específicas de esta proteína en la transmisión de ToCV por diferentes especies de moscas blancas.

REFERENCES

REFERENCES

Abad P, Jorda C (2000) Characterization of potato Y potyvirus isolates from tomato crops in Islas Canarias (Spain) OEPP/EPPO Bulletin 30:281-287.

Abdel-Salam MMA, Rezk AA, Dawoud RA (2019) Biochemical, serological, molecular and natural host studies on tomato chlorosis virus in Egypt. Pak. J. Biol. Sci. 22:83–94.

Abou-Jawdah Y, El Mohtar C, Atamian H, Sobh H (2006) First report of *Tomato chlorosis virus* in Lebanon. Plant Dis. 90:378.

Accotto GP, Vaira AM, Vecchiati M, Finetti-Sialer MM, Gallitelle D, Davino M (2001) First report of *Tomato chlorosis virus* in Italy. Plant Dis. 85:1208.

Agranovsky AA (2016) Closteroviruses: molecular biology, evolution and interactions with cells. In: Gaur RK, Petrov NM, Patil B, Stoyanova MI, eds. *Plant Viruses: Evolution and Management*. Springer, Singapore, pp. 231-252.

Agranovsky AA, Lesemann DE, Maiss E, Hull R, Atabekov JG (1995) “Rattlesnake” structure of a filamentous plant RNA virus built of two capsid proteins. Proc. Natl. Acad. Sci. USA 92:2470-2473.

Ahmad A, Ashfaq M, Riaz T, Ahsan M, Hyder , Manglli A, Tomassoli LL (2018). First report of pepper vein yellows virus infecting hot pepper in Pakistan. Plant Dis. 102:258-258.

Alabi OJ, Al Rwahnih M, Jifon JL, Gregg L, Crosby KM, Mirkov TE (2015) First report of *Pepper vein yellows virus* infecting pepper (*Capsicum* spp.) in the United States. Plant Dis. 99:1656.

Albuquerque LC, Villanueva F, Resende RO, Navas-Castillo J, Barbosa JC, Inoue-Nagata AK (2013) Molecular characterization reveals Brazilian *Tomato chlorosis virus* to be closely related to a Greek isolate. Trop. Plant Pathol.38:332-336.

Alfaro-Fernández A, Castillo P, Sanahuja E, Rodríguez-Salido MC, Font MI (2021). First report of tomato brown rugose fruit virus in tomato in Spain. Plant Dis. 105:515.

Alfaro-Fernández A, ElShafie EE, Ali MA, El Bashir OOA, Córdoba-Sellés MC, Font San Ambrosio MI (2014) First report of *Pepper vein yellows virus* infecting hot pepper in Sudan. Plant Dis. 98:1446.

Almazán F, González JM, Péntes Z, Izeta A, Calvo E, Plana-Durán J, Enjuanes L (2000) Engineering the largest RNA virus genome as an infectious bacterial artificial chromosome. Proc. Natl. Acad. Sci. USA 97:5516-5521.

Alonso E, García-Luque I, Avila-Rincón JM, Wicke B, Serra TM, Díaz-Ruiz J (1989) A tobamovirus causing heavy losses in protected pepper crops in Spain. J. Phytopathol. 125:67-76.

- Al-Saleh MA, Al-Shahwan IM, Shakeel MT, Amer MA, Orfanidou CG, Katis NI (2014)** First report of *Tomato chlorosis virus* (ToCV) in tomato crops in Saudi Arabia. *Plant Dis.* 98:1590.
- Alvarez-Ruiz P, Jimenez CG, Leyva-López NE, Méndez-Lozano J (2007)** First report of *Tomato chlorosis virus* infecting tomato crops in Sinaloa, Mexico. *Plant Pathol.* 56:1043.
- Alzhanova DV, Hagiwara Y, Peremyslov VV, Dolja VV (2000)** Genetic analysis of the cell-to-cell movement of beet yellows closterovirus. *Virology* 268:192-200.
- Alzhanova DV, Napuli AJ, Creamer R, Dolja VV (2001)** Cell-to-cell movement and assembly of a plant closterovirus: roles for the capsid proteins and Hsp70 homolog. *EMBO J.* 20:6997-7007.
- Alzhanova DV, Prokhnevsky AI, Peremyslov VV, Dolja VV (2007)** Virion tails of *Beet yellows virus*: coordinated assembly by three structural proteins. *Virology* 359:220-226.
- Amari K, González-Ibeas D, Gómez P, Sempere RN, Sánchez-Pina MA, Aranda MA, Díaz-Pendón JA, Navas-Castillo J, Moriones E, Blanca J, Hernandez-Gallardo MD, Anastasio G (2008)** Tomato torrado virus is transmitted by *Bemisia tabaci* and infects pepper and eggplant in addition to tomato. *Plant Dis.* 92:1139.
- Ambrós S, El-Mohtar C, Ruiz-Ruiz S, Peña L, Guerri J, Dawson WO, Moreno P (2011)** Agroinoculation of *Citrus tristeza virus* causes systemic infection and symptoms in the presumed nonhost *Nicotiana benthamiana*. *Mol. Plant Microbe Interact.* 24:1119-1131.
- Ambrós S, Ruiz-Ruiz S, Peña L, Moreno P (2013)** A genetic system for *Citrus tristeza virus* using the non-natural host *Nicotiana benthamiana*: an update. *Front. Microbiol.* 4:165.
- Amer MA, Ibrahim YE, Kheder AA, Hamed AH, Farrag AA, Al-Saleh MA (2020)** Confirmation incidence of *Tomato chlorosis virus* naturally infecting tomato crop in Egypt. *Int. J. Agric. Biol.* 23:963-969.
- Antignus Y, Lachman O, Pearlsman M, Ucko O (2001)** A new pepper yellowing disease of pepper caused by an unidentified luteovirus. *Phytoparasitica* 29:255.
- Aramburu J (2001)** First report of *Parietaria mottle virus* on tomato in Spain. *Plant Dis.* 85:1210.
- Aramburu J, Galipienso L (2005)** First report in Spain of a variant of *Tomato mosaic virus* (ToMV) overcoming the Tm-22 resistance gene in tomato (*Lycopersicon esculentum*). *New Disease Reports* 10:40.
- Aregbesola OZ, Legg JP, Sifsgaard L, Lund OS, Rapisarda C (2019)** Potential impact of climate change on whiteflies and implications for the spread of vectored viruses. *J. Pest Sci.* 92:381-392.
- Arruabarrena A, Rubio L, González-Arcos M, Maeso D, Fonseca MEN, Boiteux LS (2014)** First report of *Tomato chlorosis virus* infecting tomato crops in Uruguay. *Plant Dis.* 98:1445.

Arruabarrena A, Rubio L, González-Arcos M, Sánchez-Campos S, Fonseca MEN, Boiteux LS (2015) First report of *Solanum sisymbriifolium* and *S. americanum* as natural weed hosts of *Tomato chlorosis virus* (Genus *Crinivirus*) in South America. *Plant Dis.* 99:895.

Arteaga LM (1989) Virosis y micoplasmosis del pimiento cultivado al aire libre en España. Identificación de virus y caracterización de cepas. Doctoral Thesis, ETSIA, Madrid.

Barbosa JC, Teixeira APM, Moreira AG, Camargo LEA, Bergamin Filho A, Kitajima EW, Rezende JAM (2008) First report of *Tomato chlorosis virus* infecting tomato crops in Brazil. *Plant Dis.* 92:1709.

Baulcombe DC (1999) Fast forward genetics based on virus-induced gene silencing. *Curr. Opin. Plant Biol.* 2:109-113.

Berdiales B, Bernal JJ, Sáez E, Woudt B, Beitia F, Rodríguez-Cerezo E (1999) Occurrence of cucurbit yellow stunting disorder virus (CYSDV) and beet pseudo-yellows virus in cucurbit crops in Spain and transmission of CYSDV by two biotypes of *Bemisia tabaci*. *Eur. J. Plant Pathol.* 105:211-215.

Bergua M, Luis-Arteaga M, Escriu F (2010) Variabilidad biológica y estructura genética de poblaciones españolas del virus del mosaico de la alfalfa. In: XV Congreso de la Sociedad Española de Fitopatología. Vitoria, p 125.

Bese G, Bóka K, Krizbai L, Takács A (2011) First report of *Tomato chlorosis virus* in tomato from Hungary. *Plant Dis.* 95:363.

Bloom DE, Black S, Rappuoli R (2017) Emerging infectious diseases: A proactive approach. *Proc. Natl. Acad. Sci. USA* 114: 4055-4059.

Boiteux LS, Fonseca MEN, Reis A, Costa AF, Fontes MG, González-Arcos M (2016) Wild radish (*Raphanus* spp.) and garden rocket (*Eruca sativa*) as new brassicaceae hosts of *Tomato chlorosis virus* in South America. *Plant Dis.* 100:1027.

Boiteux LS, Lima MF, Fonseca MEN, Mendonça JL, Costa AF, Silva-Filho JG, Fontes MG, González-Arcos M (2018) Identification of eight solanum (subgenus *Leptostemonum*) species a novel natural hosts of *Tomato chlorosis virus* in Brazil. *Plant Dis.* 102:1673.

Bolou Bi BA, Moury B, Abo K, Kakou DJr, Girardot G, Kouassi N'dri P, Kouadio, EJN, Kouakou, BSM, Kone D (2015) First report of *Pepper vein yellows virus* in field grown pepper in Ivory Coast. *J. Plant Pathol.* 97:S67-S77.

Borges RCF, Fontes MG, Macedo MA, Lima MF, Boiteux LS, Fonseca MEN (2019) First report of tomato chlorosis virus infecting *Tectona grandis* associated with infestation of *Bemisia tabaci* Mediterranean in central Brazil. *Arch. Virol.* 103:2704.

Bos L (1981) Hundred years of Koch's Postulates and the history of etiology in plant virus research. *Eur. J. Plant Pathol.* 87:91-110.

References

- Boyer JC, Haenni AL (1994)** Infectious transcripts and cDNA clones of RNA viruses. *Virology* 198:415-426.
- Boyko VP, Karasev AV, Agranovsky AA, Koonin EV, Dolja VV (1992)** Coat protein gene duplication in a filamentous RNA virus of plants. *Proc. Natl. Acad. Sci. USA* 89:9156-9160.
- Brault V, Herrbach E, Rodriguez-Medina C (2011)** Luteoviruses. In: eLS. Chichester: John Wiley & Sons, Ltd.
- Bruen TC, Philippe H, Bryant D (2006)** A simple and robust statistical test for detecting the presence of recombination. *Genetics* 172:2665-2681.
- Buzkan N, Arpacı BB, Simon V, Fakhfakh H, Moury B (2013)** High prevalence of poleroviruses in field-grown pepper in Turkey and Tunisia. *Arch. Virol.* 158:881-885.
- Cañizares MC, Navas-Castillo J, Moriones E (2008)** Multiple suppressors of RNA silencing encoded by both genomic RNAs of the *Crinivirus*, *Tomato chlorosis virus*. *Virology* 379:168-174.
- Canto T, Aranda MA, Fereres A (2009)** Climate change effects on physiology and population processes of hosts and vectors that influence the spread of hemipteran-borne plant viruses. *Glob. Change Biol.* 15:1884-1894.
- Castro RM, Hernandez E, Mora F, Ramirez P, Hammond RW (2009)** First report of *Tomato chlorosis virus* in tomato in Costa Rica. *Plant Dis.* 93:970.
- Célix A, Lopez-Sese A, Almarza N, Gomez-Guillamon L, Rodriguez-Cerezo E (1996)** Characterization of cucurbit yellow stunting disorder virus, a *Bemisia tabaci*-transmitted closterovirus. *Phytopathol.* 86:1370-1376.
- Çevik B, Erkiş G (2008)** First report of *Tomato chlorosis virus* in Turkey. *Plant Pathol.* 57:767
- Chaudhary P, Kaur A, Singh B, Kumar S, Hallan V, Nagpal AK (2023)** First report of tomato chlorosis virus (ToCV) and detection of other viruses in field-grown tomatoes in North-Western region of India. *Virus Dis.* 34: 56-75
- Chen AY, Walker GP, Carter D, Ng JC (2011)** A virus capsid component mediates virion retention and transmission by its insect vector. *Proc. Natl. Acad. Sci. USA* 108:16777-16782.
- Chen AYS, Pavitrin A, Ng JCK (2012)** Agroinoculation of the cloned infectious cDNAs of *Lettuce chlorosis virus* results in systemic plant infection and production of whitefly transmissible virions. *Virus Res.* 169:310–315.
- Chen AYS, Watanabe S, Yokomi R, Ng JCK (2018)** Nucleotide heterogeneity at the terminal ends of the genomes of two California *Citrus tristeza virus* strains and their complete genome sequence analysis. *Virol. J.* 15:141.
- Chiba M, Reed JC, Prokhnevsky AI, Chapman AJ, Mawassi M, Koonin EV, Carrington JC, Dolja VV (2006)** Diverse suppressors of RNA silencing enhance agroinfection by a viral

replicon. *Virology* 346:7-14.

Dalmon A, Bouyer S, Cailly M, Girard M, Lecoq H, Desbiez C, Jacquemond M (2005) First report of *Tomato chlorosis virus* and *Tomato infectious chlorosis virus* in tomato crops in France. *Plant Dis.* 89:1243.

Dawson WO, Beck DL, Knorr DA, Grantham GL (1986) cDNA cloning of the complete genome of tobacco mosaic virus and production of infectious transcripts. *Proc. Natl. Acad. Sci. USA* 83:1832-1836.

De Barro PJ (1995) *Bemisia tabaci* biotype B: a review of its biology, distribution and control, 2nd edn, CSIRO Division of Entomology, Technical Paper No. 36. CSIRO, Canberra, Australia.

Delatte H, Naze F, Cottineau JS, Lefeuvre P, Hostachy B, Reynaud B, Lett JM (2006) Occurrence of *Tomato chlorosis virus* on tomato in Reunion Island. *Plant Pathol.* 55:289.

Depicker A, Stachel S, Dhaese P, Zambryski P, Goodman HM (1982) Nopaline synthase: transcript mapping and DNA sequence. *J. Mol. Appl. Genet.* 1:561-573.

Díaz-Pendón JA, Cañizares MC, Moriones E, Bejarano ER, Czosnek H, Navas-Castillo J (2010) Tomato yellow leaf curl viruses: ménage à trois between the virus complex, the plant and the whitefly vector. *Mol. Plant Pathol.* 11:441-450.

Dolja VV (2003) *Beet yellows virus*: the importance of being different. *Mol. Plant Pathol.* 4:91-98.

Dolja VV, Kreuze JF, Valkonen JPT (2006) Comparative and functional genomics of closteroviruses. *Virus Res.* 117:38-51.

Dombrovsky A, Glanz E, Lachman O, Sela N, Doron-Faigenboim A, Antignus Y (2013) The complete genomic sequence of *Pepper yellow leaf curl virus* (PYLCV) and its implications for our understanding of evolution dynamics in the genus *Polerovirus*. *PLoS ONE* 8:e70722.

Dombrovsky A, Glanz E, Pearlsman M, Lachman O, Antignus Y (2010) Characterization of *Pepper yellow leaf curl virus*, a tentative new *Polerovirus* species causing a yellowing disease of pepper. *Phytoparasitica* 38:477-486.

Domier LL (2012) Family *Luteoviridae*. In: King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ (eds) *Virus taxonomy*. Ninth report of the International Committee on Taxonomy of Viruses. Elsevier/Academic Press, London, pp 1045-1053.

Domingo E, Perales C (2019) Viral quasispecies. *PLoS Genetics* 15:1-20.

Dovas CI, Katis NI, Avgelis AD (2002) Multiplex detection of criniviruses associated with epidemics of a yellowing disease of tomato in Greece. *Plant Dis.* 86:1345-1349.

Duffus JE (1965) Beet pseudo-yellows virus, transmitted by the greenhouse whitefly,

References

Trialeurodes vaporariorum. Phytopathol. 55:450-453.

Duffus JE, Liu H-L, Wisler GC (1996) Tomato infectious chlorosis virus - a new closterovirus-like virus transmitted by *Trialeurodes vaporariorum*. Eur. J. Plant Pathol. 102:219-226.

Duffus JE, Liu HY, Wisler GC, Li RH (1996b) Lettuce chlorosis virus - a new whitefly-transmitted closterovirus. Eur. J. Plant Pathol. 102:591-596.

Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32:1792-1797.

EPPO (2018a) First report of *Tomato chlorosis virus* in the Netherlands. EPPO Rep. Serv. 2018, 37.

EPPO (2018b) First report of *Tomato chlorosis virus* in the United Kingdom. EPPO Rep. Serv. 2018, 219.

Fariña AE, Rezende JAM, Wintermantel WM (2019) Expanding knowledge of the host range of *Tomato chlorosis virus* and host plant preference of *Bemisia tabaci* MEAM1. Plant Dis. 103:1132-1137.

Fazeli CF, Rezaian MA (2000) Nucleotide sequence and organization of ten open reading frames in the genome of Grapevine leafroll-associated virus 1 and identification of three subgenomic RNAs. J. Gen. Virol. 81:605-615.

Fereres A, Raccach B (2015) Insect transmission of plant viruses. In: eLS. Chichester: John Wiley & Sons, Ltd.

Ferriol I, Turina M, Zamora-Macorra EJ, Falk BW (2016) RNA1-independent replication and GFP expression from *Tomato marchitez virus* isolate M cloned cDNA. Phytopathol. 106:500-509.

Fiallo-Olivé E, Espino AI, Botella-Guillén M, Gómez-González E, Reyes-Carlos JA, Navas-Castillo J (2014) Tobacco: a new natural host of *Tomato chlorosis virus* in Spain. Plant Dis. 98:1162.

Fiallo-Olivé E, Hamed AA, Moriones E, Navas-Castillo J (2011) First report of *Tomato chlorosis virus* infecting tomato in Sudan. Plant Dis. 95:1592.

Fiallo-Olivé E, Navas-Castillo J (2019) Tomato chlorosis virus, an emergent plant virus still expanding its geographical and host ranges. Mol. Plant Pathol. 20:1307-1320.

Fiallo-Olivé E, Navas-Castillo J (2023) Tomato chlorosis virus, a promiscuous virus with multiple host plants and whitefly vectors. Ann. Appl. Biol. 182:29-36.

Fonseca MEN, Boiteux LS, Abreu H, Nogueira I, Pereira-Carvalho RC (2013) *Physalis angulata* : a new natural host of *Tomato chlorosis virus* in Brazil. Plant Dis. 97:692-692.

Fonseca MEN, Boiteux LS, Lima MF, Mendonça JL, Costa AF, Fontes MG, Costa H, González-Arcos M (2016) First report of *Tomato chlorosis virus* infecting eggplant and scarlet eggplant in Brazil. *Plant Dis.* 100:867-868.

Font MI, Juárez M, Martínez O, Jordá C (2004) Current status and newly discovered Natural hosts of *Tomato infectious chlorosis virus* and *Tomato chlorosis virus* in Spain. *Plant Dis.* 88:82.

Font MI, Martínez-Culebras P, Jorda MC, Louro D, Vaira AM, Accott GP (2002) First report of *Tomato infectious chlorosis virus* on tomato in Spain. *Plant Dis.* 86:696.

Font MI, Vaira AM, Accotto GP, Lacasa A, Serra J, Gomila J, Juárez M, Espino AI, Jordá MC (2003) Amarillos en los cultivos de tomate asociados a *Tomato chlorosis virus* (ToCV) y *Tomato infectious chlorosis virus* (TICV) en España. *Bol. San. Veg. Plagas* 29:109-21.

Fortes IM, Moriones E, Navas-Castillo J (2012) Tomato chlorosis virus in pepper: prevalence in commercial crops in south-eastern Spain and symptomatology under experimental conditions. *Plant Pathol.* 6:9941001.

Fortes IM, Navas-Castillo J (2008) Potato (*Solanum tuberosum*) is an experimental and natural host of Tomato chlorosis virus. In: 3rd Conference of the International Working Group on Legume and Vegetable Viruses, p. 56.

Fourment M, Gibbs AJ, Gibbs MJ (2008) SWeBLAST: a Sliding Window Web-based BLAST tool for recombinant analysis. *J. Virol. Methods* 152:98-101.

Fraile A, Alonso-Prados JL, Aranda MA, Bernal JJ, Malpica JM, García-Arenal F (1997) Genetic exchange by recombination or reassortment is infrequent in natural populations of a tripartite RNA plant virus. *J. Virol.* 71:934-40.

Fuchs M, Bar-Joseph M, Candresse T, Maree HJ, Martelli GP, Melzer MJ, Menzel W, Minafra A, Sabanadzovic S, ICTV Report Consortium (2020) ICTV virus taxonomy profile: *Closteroviridae*. *J. Gen. Virol.* 101:364-365.

Galipienso L, Elvira-González L, Velasco L, Herrera-Vásquez JA, Rubio L (2021) Detection of persistent viruses by high-throughput sequencing in tomato and pepper from Panama: Phylogenetic and evolutionary studies. *Plants* 10:2295.

Gal-On A, Meiri E, Huet H, Hua WJ, Raccach B, Gaba V (1995) Particle bombardment drastically increases the infectivity of cloned DNA of zucchini yellow mosaic potyvirus. *J. Gen. Virol.* 76:3223-3227.

Gamarra HA, Fuentes S, Morales FJ, Glover R, Malumphy C, Barker I (2010) *Bemisia afer* sensu lato, a vector of *Sweet potato chlorotic stunt virus*. *Plant Dis.* 94:510-514.

García-Arenal F, Fraile A, Malpica JM (2001) Variability and genetic structure of plant virus populations. *Annu. Rev. Phytopathol.* 39:157-186.

García-Arenal F, McDonald BA (2003) An analysis of the durability of resistance to plant viruses. *Phytopathol.* 93:941-952.

García-Cano E, Navas-Castillo J, Moriones E, Fernández-Muñoz R (2010) Resistance to *Tomato chlorosis virus* in wild tomato species that impair virus accumulation and disease symptom expression. *Phytopathol.* 100:582-592.

García-Luque I, Diaz-Ruiz JR, Rubio-Huertos M, Kaper JM (1983) Cucumovirus survey in Spanish economically important crops. *Phytopath. Mediterr.* 22:127-132.

Gharsallah C, Halima AB, Fakhfakh H, Gorsane F (2015) Insights into the genetic diversity and the phylogenetic analysis of Tunisian isolates of *Tomato chlorosis virus*. *Phytoparasitica* 43:87-96.

Gibbs MJ, Armstrong JS, Gibbs AJ (2001) Recombination in the hemagglutinin gene of the 1918 "Spanish flu". *Science* 293:1842-1845.

Gniffke PA, Shieh SC, Lin SW, Sheu ZM, Chen JR, Ho FI, Tsai WS, Chou YY, Wang JF, Cho MC, Schafleitner R, Kenyon L, Ebert AW, Srinivasan R, Kumar S (2013) Pepper research and breeding at AVRDC-The World Vegetable Center. In: XV EUCARPIA Meeting on Genetics and Breeding of Capsicum and Eggplant; Sep 2–4; Turin, Italy. p.305-311.

Gorbalenya AE, Krupovic M, Mushegian A, Kropinski AM, Siddell SG, Varsani A, Adams MJ, Davison AJ, Dutilh BE, Harrach B, Harrison RL, Junglen S, King AMQ, Knowles NJ, Lefkowitz EJ, Nibert ML, Rubino L, Sabanadzovic S, Sanfaçon H, Simmonds P, Walker PJ, Zerbini FM, Kuhn JH (2020) The new scope of virus taxonomy: partitioning the virosphere into 15 hierarchical ranks. *Nat. Microbiol.* 5:668-674.

Gowda S, Satyanarayana T, Ayllón MA, Moreno P, Flores R, Dawson WO (2003) The conserved structures of the 5' nontranslated region of *Citrus tristeza virus* are involved in replication and virion assembly. *Virology* 317:50-64.

Gowda S, Satyanarayana T, Robertson CJ, Garnsey SM, Dawson WO (2005) "Infection of citrus plants with virions generated in *Nicotiana benthamiana* plants agroinfiltrated with a binary vector based *Citrus tristeza virus*," in *Proceedings of the 16th Conference of the International Organization of Citrus Virologists*, eds ME Hilf, N Duran-Vila, and MA Rocha-Peña (Riverside, CA: IOCV), 23-33.

Grimsley N, Hohn B, Hohn T, Walden (1986) Agroinfection, an alternative route for viral infection of plants by using the Ti plasmid. *Proc. Natl. Acad. Sci. USA* 83:3282-3286.

Hanafi A (2002) Invasive species. A real challenge to IPM in the Mediterranean region? *EWSN Newsletter* 13:4.

Hanssen IM, Lapidot M (2012) Major tomato viruses in the Mediterranean basin. In Gad L, Hervé L (Eds.) *Advances in Virus Research*. Academic Press. Piscataway, NJ, USA, pp.31-66.

Hanssen IM, Lapidot M, Thomma BPHJ (2010) Emerging viral diseases of tomato crops.

Mol. Plant Microbe In. 23:539-548.

Harun Kimathi R, Wilisiani F, Mashiko T, Neriya Y, Elijah Miinda A, Nishigawa H, Natsuaki T (2020). First report of *Tomato chlorosis virus* infecting tomato in Kenya. Scientific African e00286.

Heckman KL, Pease LR (2007) Gene splicing and mutagenesis by PCR-driven overlap extension. Nat. Protoc. 2:924-932.

Hirota T, Natsuaki T, Murai T, Nishigawa H, Niibori K, Goto K, Hartono S, Suastika G, Okuda S (2010) yellowing disease of tomato caused by *Tomato chlorosis virus* newly recognized in Japan. J. Gen. Plant Pathol. 76:168-171.

Huson DH, Bryant D (2006) Application of phylogenetic networks in evolutionary studies. Mol. Biol. Evol. 23:254-267.

Ibiza VP, Blanca J, Cañizares J, Nuez F (2012) Taxonomy and genetic diversity of domesticated *Capsicum* species in the Andean region. Genet. Resour. Crop. Ev. 59:1077-1088.

International Committee on Taxonomy of Viruses (ICTV). Virus Taxonomy: (2016) Release. <https://talk.ictvonline.org/> Accessed 13 Jul 2022.

Janssen D, Saez E, Segundo E, Gil GMF, Cuadrado IM (2005) *Capsicum Annuum* - a new host of *Parietaria mottle virus* in Spain. Plant Pathol. 54:567.

Jarugula S, Gowda S, Dawson WO, Naidu RA (2018) Development of infectious cDNA clones of *Grapevine leafroll-associated virus 3* and analyses of the 5' non-translated region for replication and virion formation. Virology 523:89-99.

Jones DR (2001) Pest risk analysis of *Tomato chlorosis virus*. York: CSL.

Jones RA, Naidu RA (2019) Global dimensions of plant virus diseases: Current status and future perspectives. Annu. Rev. Virol. 6:387-409.

Jordá C, Lázaro Pérez A, Martínez Culebras PV, Lacasa A (2001) First report of *Pepino mosaic virus* on natural hosts. Plant Dis. 85:1292-1292.

Kamran A, Lotos L, Amer MA, Al-Saleh MA, Alshahwan IM, Shakeel MT, Ahmad MH, Umar M, Katis NI (2018) Characterization of pepper leafroll chlorosis virus, a new polerovirus causing yellowing disease of bell pepper in Saudi Arabia. Plant Dis. 102:318-326.

Kang YC, Wang YC, Hsia CM, Tsai WS, Huang LH, Yeh SD, Chen TC, (2018) Molecular characterization and detection of a genetically distinct tomato chlorosis virus strain in Taiwan. Plant Dis. 102:600-607.

Karasev AV (2000) Genetic diversity and evolution of closteroviruses. Annu. Rev. Phytopathol. 38:293-324.

Kataya ARA, Stavridou A, Farhan K, Livieratos IC (2008) Nucleotide sequence analysis and detection of a Greek isolate of *Tomato chlorosis virus*. *Plant Pathol.* 57:819-824.

Kenyon L, Kumar S, Tsai WS, Hughes JDA (2014) Virus diseases of peppers (*Capsicum* spp.) and their control. *Adv. Virus. Res.* 90:297-354.

Kil EJ, Kim S, Lee YJ, Kang EH, Lee M, Cho SH, Kim MK, Lee KY, Heo NY, Choi HS, Kwon ST, Lee (2015a) Advanced loop-mediated isothermal amplification method for sensitive and specific detection of *Tomato chlorosis virus* using a uracil DNA glycosylase to control carry-over contamination. *J. Virol. Methods* 213:68-74.

Kil EJ, Lee JJ, Cho S, Auh CK, KimD, Lee KY, Kim MK, Choi HS, Kim CS, Lee S (2015b) Identification of natural weed hosts of *Tomato chlorosis virus* in Korea by RT-PCR with root tissues. *Eur. J. Plant Pathol.* 142:419-426

Kimathi RH, Wilisiani F, Mashiko T, Neriya Y, Miinda AE, Nishigawa H, Natsuaki T (2020) First report of *Tomato chlorosis virus* infecting tomato in Kenya. *Sci. Afr.* 7:e00286.

Kiss ZA, Medina V, Falk B (2013) *Crinivirus* replication and host interactions. *Front. Microbiol.* 4:99.

Klaassen VA, Mayhew D, Fisher D, Falk BW (1996) *In vitro* transcripts from cloned cDNAs of the lettuce infectious yellows closterovirus bipartite genomic RNAs are competent for replication in *Nicotiana benthamiana* protoplasts. *Virology* 222:169-175.

Knierim D, Tsai WS, Kenyon L (2013) Analysis of sequence from field samples reveals the presence of the recently described pepper vein yellows virus (genus *Polerovirus*) in six additional countries. *Arch. Virol.* 158:1337-1341.

Koonin EV, Dolja VV, Krupovic M (2015) Origins and evolution of viruses of eukaryotes: The ultimate modularity. *Virology* 479:2-25.

Kreuze JF, Savenkov EI, Valkonen JPT (2002) Complete genome sequence and analyses of the subgenomic RNAs of *Sweet potato chlorotic stunt virus* reveal several new features for the genus *Crinivirus*. *J. Virol.* 76:9260-9270.

Kurth EG, Peremyslov VV, Prokhnevsky AI, Kasschau KD, Miller M, Carrington JC, Dolja VV (2012) Virus-derived gene expression and RNA interference vector for grapevine. *J. Virol.* 86:6002-6009.

Lai MMC (2000) The making of infectious viral RNA: No size limit in sight. *Proc. Natl. Acad. Sci. USA* 97:5025-5027.

Larsen RC, Kim KS, Scott HA (1991) Properties and cytopathology of diodia vein chlorosis virus- A new whitefly-transmitted virus. *Phytopathol.* 81:277-232.

Laviña A, Aramburu J, Moriones E (1996) Occurrence of tomato spotted wild and cucumber mosaic viruses in field-grown tomato crops and associated weeds in northeastern Spain. *Plant Pathol.* 45:837-842.

- Lee YJ, Kil EJ, Kwak HR, Kim M, Seo JK, Lee S, Choi HS (2018)** Phylogenetic characterization of *Tomato chlorosis virus* population in Korea: evidence of assortment between isolates from different origins. *Plant Pathol. J.* 34:199-207.
- Lett JM, Hoareau M, Reynaud B, Saison A, Hostachy B, Lobin K, Benimadhu SP (2009)** First report of *Tomato chlorosis virus* in tomato on Mauritius Island. *Plant Dis.* 93:111.
- Lindbo JA (2007)** TRBO: a high-efficiency tobacco mosaic virus RNA-based overexpression vector. *Plant Physiol.* 145:1232-1240.
- Liu HY, Li RH, Wisler GC, Duffus JE (1997)** Characterization of *Abutilon yellows virus* - A new clostero-like virus transmitted by banded-wing whitefly (*Trialeurodes abutilonea*). *Phytopathol.* 87:S58-S59.
- Liu HY, Wisler GC, Duffus JE (2000)** Particle length of whitefly-transmitted criniviruses. *Plant Dis.* 84:803-805.
- Liu M, Liu X, Li X, Zhang D, Dai L, Tang Q (2016)** Complete genome sequence of a Chinese isolate of pepper vein yellows virus and evolutionary analysis based on the CP, MP and RdRp coding regions. *Arch. Virol.* 161:677-683.
- Liu Q, Zhang S, Mei S, Zhou Y, Wang J, Han GZ, Chen L, Zhou C, Cao M (2021)** Viromics unveils extraordinary genetic diversity of the family *Closteroviridae* in wild citrus. *PLoS Pathog.* 17:e1009751.
- Liu YP, Peremyslov VV, Medina V, Dolja VV (2009)** Tandem leader proteases of *Grapevine leafroll-associated virus 2*: host-specific functions in the infection cycle. *Virology* 383:291-299.
- Livieratos IC, Eliasco E, Müller G, Olsthoorn RCL, Salazar LF, Pleij CWA, Coutts RHA (2004)** Analysis of the RNA of *Potato yellow vein virus*: evidence for a tripartite genome and conserved 30-terminal structures among members of the genus *Crinivirus*. *J. Gen. Virol.* 85:2065-2075.
- Lole KS, Bollinger RC, Paranjape RS, Gadkari D, Kulkarni SS, Novak NG, Ingersoll R, Sheppard HW, Ray SC (1999)** Full-length Human immunodeficiency virus type 1 genomes from subtype C-infected seroconverters in India with evidence of intersubtype recombination. *J. Virol.* 73:152-160.
- López C, Ayllón MA, Navas-Castillo J, Guerri J, Moreno P, Flores R (1998)** Molecular variability of the 5'- and 3'-terminal regions of citrus tristeza virus RNA. *Phytopathol.* 88:685-691.
- López-Moya JJ, García JA (2000)** Construction of a stable and highly infectious intron-containing cDNA clone of plum pox potyvirus and its use to infect plants by particle bombardment. *Virus Res.* 68:99-107.
- Lotos L, Olmos A, Orfanidou C, Efthimiou K, Avgelis A, Katis NI, Maliogka VL (2017)** Insights into the etiology of polerovirus-induced pepper yellows disease.

References

Phytopathol.107:1567-1576.

Louro D, Accotto GP, Vaira AM (2000) Occurrence and diagnosis of *Tomato chlorosis virus* in Portugal. Eur. J. Plant Pathol. 106:589-592.

Lozano G, Moriones E, Navas-Castillo J (2004) First report of sweet pepper (*Capsicum annuum*) as a natural host plant for *Tomato chlorosis virus*. Plant Dis. 88:224.

Lozano G, Moriones E, Navas-Castillo J (2006) Complete nucleotide sequence of the RNA2 of the crinivirus tomato chlorosis virus. Arch. Virol. 151:581-587.

Lozano G, Moriones E, Navas-Castillo J (2007) Complete nucleotide sequence of the RNA1 of a European isolate of tomato chlorosis virus. Arch. Virol. 152:839-841.

Maina S, Edwards OR, Jones RAC (2016) First complete genome sequence of *Pepper vein yellows virus* from Australia. Genome Announc. 4:e00450-16.

Martin DP, Murrell B, Golden M, Khoosal A, Muhire B (2015) RDP4: Detection and analysis of recombination patterns in virus genomes. Virus Evol. 1:1-5.

Martín G, Cuadrado IM, Janssen D (2011) *Bean yellow disorder virus*: Parameters of transmission by *Bemisia tabaci* and host plant range. Insect Sci. 18:50-56.

Martínez-Zubiaur Y, Fiallo-Olivé E, Carrillo-Tripp J, Rivera-Bustamante R (2008) First report of *Tomato chlorosis virus* infecting tomato single and mixed infections with *Tomato yellow leaf curl virus* in Cuba. Plant Dis. 92:836.

Massé D, Lefeuvre P, Delatte H, Abdoul Karime AL, Hostachy B, Teynaud B, Lett JM (2008) *Tomato chlorosis virus*: first report in Mayotte Island. Plant Pathol. 57:388.

Mohammed IU, Yakub AM, Yusuf I, Muhammad A, Navas- Castillo J, Fiallo-Olivé E (2018) First report of *Tomato chlorosis virus* infecting tomato in Nigeria. Plant Dis. 102:257.

Monci F, García-Andrés S, Maldonado JA, Moriones E (2005) Resistance to monopartite begomoviruses associated with the bean leaf crumple disease in *Phaseolus vulgaris* controlled by a single dominant gene. Phytopathol. 95:819-826.

Mongkolsiriwattana C, Chen AYS, Ng JCK (2011) Replication of *Lettuce chlorosis virus* (LCV), a crinivirus in the family *Closteroviridae*, is accompanied by the production of LCV RNA 1-derived novel RNAs. Virology 420:89-97.

Mongkolsiriwattana C, Zhou JS, Ng JCK (2016) A 3'-end structure in RNA2 of a crinivirus is essential for viral RNA synthesis and contributes to replication-associated translation activity. Sci. Rep.6:1-13.

Moodley V, Gubba A, Mafongoya PL (2019a) A survey of whitefly-transmitted viruses on tomato crops in South Africa. Crop Prot. 123:21-29.

- Moodley V, Gubba A, Mafongoya PL (2019b).** Prevalence, epidemiology and molecular studies of *Tomato chlorosis virus* (ToCV) in South Africa. PLoS ONE 14, e0220298.
- Morilla G, Janssen D, García-Andrés S, Moriones E, Cuadrado IM, Bejarano ER (2005)** Pepper (*Capsicum annuum*) is a dead-end host for *Tomato yellow leaf curl virus*. Phytopathol. 95:1089-1097.
- Moriones E, Navas-Castillo J (2000)** Tomato yellow leaf curl virus, an emerging virus complex causing epidemics worldwide. Virus Res. 71:123-134.
- Morris J, Steel E, Smith P, Boonham N, Spence N, Barker I (2006)** Host range studies for *Tomato chlorosis virus*, and *Cucumber vein yellowing virus* transmitted by *Bemisia tabaci* (Gennadius). Eur. J. Plant Pathol. 114:265-273.
- Moury B, Verdin E (2012)** Viruses of pepper crops in the Mediterranean basin: a remarkable stasis. Adv. Virus. Res. 84:127-162.
- Muhire BM, Varsani A, Martin DP (2014)** SDT: A virus classification tool based on pairwise sequence alignment and identity calculation. PLoS One 9:e108277.
- Murakami R, Nakashima N, Hinomoto N, Kawano S, Toyosato T (2011)** The genome sequence of pepper vein yellows virus (family *Luteoviridae*, genus *Polerovirus*). Arch. Virol. 156:921-923.
- Nagy PD, Simon AE (1997)** New insights into the mechanisms of RNA recombination. Virology 235:1-9.
- Napuli AJ, Alzhanova DV, Doneanu CE, Barofsky DF, Koonin EV, Dolja VV (2003)** The 64-kilodalton capsid protein homolog of *Beet yellows virus* is required for assembly of virion tails. J. Virol. 77:2377-2384.
- Nault LR (1997)** Arthropod transmission of plant viruses: A new synthesis. Ann. Entomol. Soc. Am. 90:521-541.
- Navas-Castillo J, Camero R, Bueno M, Moriones E (2000)** Severe yellowing outbreaks in tomato in Spain associated with infections of *Tomato chlorosis virus*. Plant Dis. 84:835-837.
- Navas-Castillo J, Fiallo-Olivé E, Sánchez-Campos S (2011)** Emerging virus diseases transmitted by whiteflies. Annu. Rev. Phytopathol. 49, 219-248.
- Ng JC, Falk BW (2006a)** *Bemisia Tabaci* transmission of specific *Lettuce infectious yellows virus* genotypes derived from in vitro synthesized transcript-inoculated protoplasts. Virology 352:209-215.
- Ng JC, Zhou JS (2015)** Insect vector-plant virus interactions associated with non-circulative, semi-persistent transmission: current perspectives and future challenges. Curr. Opin. Virol. 15:48-55.

References

Ng JCK, Falk BW (2006b) Virus-vector interactions mediating non-persistent and semipersistent transmission of plant viruses. *Annu. Rev. Phytopathol.* 44:183-212.

Oerke EC, Dehne HW (2004) Safeguarding production-losses in major crops and the role of crop protection. *Crop Prot.* 23:275-285.

Orfanidou CG, Cara M, Merkuri J, Katis NI, Maliogka VL (2022) First report of tomato chlorosis virus in tomato in Albania. *J. Plant. Pathol.* doi: 10.1007/s42161-022-01148-5.

Orfanidou CG, Dimitriou C, Papayiannis LC, Maliogka VI, Katis NI (2014) Epidemiology and genetic diversity of criniviruses associated with tomato yellows disease in Greece. *Virus Res.* 186:120-129.

Orílio AF, Fortes IM, Navas-Castillo J (2014) Infectious cDNA clones of the crinivirus *Tomato chlorosis virus* are competent for systemic plant infection and whitefly-transmission. *Virology* 46:365-374.

Orílio AF, Navas-Castillo J (2009) The complete nucleotide sequence of the RNA2 of the crinivirus tomato infectious chlorosis virus: isolates from North America and Europe are essentially identical. *Arch. Virol.* 154:683-687.

Owen CA, Moukarzel R, Huang X, Kassem MA, Eliasco E, Aranda MA, Coutts RHA, Livieratos IC (2016) *In vitro* synthesized RNA generated from cDNA clones of both genomic components of *Cucurbit yellow stunting disorder virus* replicates in cucumber protoplasts. *Viruses* 8:170.

Pantoja KFC, De Marchi BR, Krause-Sakate R, Mituti T, Rezende JAM, Ghanim M, Ghosh S, Boari AJ (2019) First report of a putative new pepper vein yellows virus species associated with a vein yellows disease of bonnet pepper plants in Brazil. *Plant Dis.* 103:2972.

Papayiannis LC, Ioannou N, Dovas CI, Maliogka VI, Katis NI (2006) First report of *Tomato chlorosis virus* on tomato crops in Cyprus. *Plant Pathol.* 55:567.

Parrella G, Gognalons P, Gebre-Selassie K, Marchouz G, Vovlas C (2003) An update of the host range of *Tomato spotted wilt virus*. *J. Plant Pathol.* 85:227-264.

Pecman A, Kutnjak D, Gutiérrez-Aguirre I, Adams I, Fox A, Boonham N, Ravnika M (2017) Next generation sequencing for detection and discovery of plant viruses and viroids: comparison of two approaches. *Front. Microbiol.* 8:1998.

Peralta IE, Knapp S, Spooner DM (2005) New species of wild tomatoes (*Solanum* Section *Lycopersicon*: Solanaceae) from Northern Peru. *Syst. Bot.* 30:424-434.

Peremyslov VV, Andreev IA, Prokhnevsky AI, Duncan GH, Taliansky ME, Dolja VV (2004a) Complex molecular architecture of beet yellows virus particles. *Proc. Natl. Acad. Sci. USA* 101:5030-5035.

Peremyslov VV, Hagiwara Y, Dolja VV (1998) Genes required for replication of the 15.5-kilobase RNA genome of a plant closterovirus. *J. Virol.* 72:5870-5876.

-
- Peremyslov VV, Hagiwara Y, Dolja VV (1999)** HSP70 homolog functions in cell-to-cell movement of a plant virus. *Proc. Natl. Acad. Sci. USA* 96:14771-14776.
- Peremyslov VV, Pan YW, Dolja VV (2004b)** Movement protein of a closterovirus is a type III integral transmembrane protein localized to the endoplasmic reticulum. *J. Virol.* 78:3704-3709.
- Poudel B, Wintermantel WM, Cortez AA, Ho T, Khadgi A, Tzanetakis IE (2013)** Epidemiology of *Blackberry yellow vein associated virus*. *Plant Dis.* 97:1352-1357.
- Prokhnevsky AI, Peremyslov VV, Dolja VV (2005)** Actin cytoskeleton is involved in targeting of a viral Hsp70 homolog to the cell periphery. *J. Virol.* 79:14421-14428.
- Prokhnevsky AI, Peremyslov VV, Napuli AJ, Dolja VV (2002)** Interaction between long-distance transport factor and Hsp70-related movement protein of Beet yellows virus. *J. Virol.* 76:11003-11011.
- Pruss GJ, Nester EW, Vance V (2008)** Infiltration with *Agrobacterium tumefaciens* induces host defense and development-dependent responses in the infiltrated zone. *Mol. Plant Microbe Interact.* 21:1528-1538.
- Raza A, Shakeel MT, Umar UUD, Tahir MN, Hassan AA, Katis NI, Wang X (2020)** First report of tomato chlorosis virus infecting tomato in Pakistan. *Plant Dis.* 104:2036.
- Reina J, Morilla G, Bejarano E, Rodríguez M, Janssen D (1999)** First report of *Capsicum annuum* plants infected by *Tomato yellow leaf curl virus*. *Plant Dis.* 83:1176.
- Rohwer F (2003)** Global Phage Diversity. *Cell* 113:141.
- Roossinck MJ (1997)** Mechanisms of plant virus evolution. *Annu. Rev. Phytopathol.* 35:191-209.
- Rubio L, Luis-Arteaga M, Cambra M, Serra J, Moreno P, Guerri J (2002)** First report of *Broad bean wilt virus 1* in Spain. *Plant Dis.* 86:698
- Ruiz ML, Simón A, Velasco L, García MC, Janssen D (2015)** First report of *Tomato leaf curl New Delhi virus* infecting tomato in Spain. *Plant Dis.* 99:894-894
- Ruiz-Ramón F, Sempere RN, Méndez-López E, Sánchez-Pina MA, Aranda MA (2019)** Second generation of pepino mosaic virus vectors: improved stability in tomato and a wide range of reporter genes. *Plant Methods* 15:58.
- Salazar LF, Müller G, Querci M, Zapata JL, Owens RA (2000)** Potato yellow vein virus : its host range, distribution in South America and identification as a crinivirus transmitted by *Trialeurodes vaporariorum*. *Ann. Appl. Biol.* 137:7-19.
- Salem NM, Mansour AN, Abdeen AO, Araj S, Khurfan I (2015)** First report of *Tomato chlorosis virus* infecting tomato crops in Jordan. *Plant Dis.* 9:1286.
- Satyanarayana T, Bar-Joseph M, Mawassi M, Albiach-Martí MR, Ayllón MA, Gowda S,**

References

Hilf ME, Moreno P, Garnsey SM, Dawson WO (2001) Amplification of *Citrus tristeza virus* from a cDNA clone and infection of *Citrus* trees. *Virology* 280:87-96.

Satyanarayana T, Gowda S, Ayllón MA, Albiach-Martí MR, Dawson WO (2002) Mutational analysis of the replication signals in the 3'-nontranslated region of *Citrus tristeza virus*. *Virology* 300:140-152.

Satyanarayana T, Gowda S, Ayllón MA, Dawson WO (2003) Frameshift mutations in infectious cDNA clones of *Citrus tristeza virus*: a strategy to minimize the toxicity of viral sequences to *Escherichia coli*. *Virology* 313:481-491.

Satyanarayana T, Gowda S, Ayllón MA, Dawson WO (2004) Closterovirus bipolar virion: Evidence for initiation of assembly by minor coat protein and its restriction to the genomic RNA 5' region. *Proc. Natl. Acad. Sci. USA* 101:799-804.

Satyanarayana T, Gowda S, Boyko VP, Albiach-Martí MR, Mawassi M, Navas-Castillo J, Karasev AV, Dolja VV, Hilf ME, Lewandowski DJ, Moreno P, Bar-Joseph M, Garnsey SM, Dawson WO (1999) An engineered closterovirus RNA replicon and analysis of heterologous terminal sequences for replication. *Proc. Natl. Acad. Sci. USA* 96:7433-7438.

Satyanarayana T, Gowda S, Mawassi M, Albiach-Martí MR, Ayllón MA, Robertson C, Garnsey SM, Dawson WO (2000) Closterovirus encoded HSP70 homolog and p61 in addition to both coat proteins function in efficient virion assembly. *Virology* 278:253-265.

Sau AR, Nazmie NMF, Yusop MSM, Akbar MA, Saad MFM, Baharum SN, Talip N, Goh HH, Kassim H, Bunawan H (2020) First report of pepper vein yellows virus and pepper yellow leaf curl virus infecting chilli pepper (*Capsicum annuum*) in Malaysia. *Plant Dis.* 104:9-10.

Scheets K, Miller WA, Sömera, M (2020) Abolish the family *Luteoviridae* (*Tolivirales*) and move its genera to the families *Tombusviridae* (*Tolivirales*) and *Solemoviridae* (*Sobelivirales*). https://ictv.global/ictv/proposals/2020.026P.R.Abolish_Luteoviridae.zip.

Scholthof HB (1999) Rapid delivery of foreign genes into plants by direct rub-inoculation with intact plasmid DNA of a tomato bushy stunt virus gene vector. *J. Virol.* 73:7823-7829.

Scholthof HB (1999) Rapid delivery of foreign genes into plants by direct rub-inoculation with intact plasmid DNA of a tomato bushy stunt virus gene vector. *J. Virol.* 73:7823-7829.

Segev L, Wintermantel WM, Polston JE, Lapidot M (2004) First report of *Tomato chlorosis virus* in Israel. *Plant Dis.* 88:1160.

Segundo E, Lesemann DE, Martín G, Carmona MP, Ruiz L, Cuadrado IM, Velasco L, Janssen D (2007) *Amaranthus leaf mottle virus*: 3'-end RNA sequence proves classification as distinct virus and reveals affinities within the genus *Potyvirus*. *Eur. J. Plant Pathol.* 117:81-87.

Segundo E, Martín G, Cuadrado IM, Janssen D (2004) A new yellowing disease in *Phaseolus vulgaris* associated with a whitefly-transmitted virus. *Plant Pathol.* 53:517.

Shakeel MT, Al-Saleh MA, Amer MA, Al-Shahwan IM, Umar M, Dimou N, Orfanidou

-
- CG, Zakri AM, Katis NI (2017)** Molecular characterization and natural host range of *Tomato chlorosis virus* in Saudi Arabia. *J. Plant Pathol.* 99:415-421.
- Shi Y, Shi Y, Gu Q, Yan F, Sun X, Li H, Chen L, Sun B, Wang Z (2016)** Infectious clones of the crinivirus cucurbit chlorotic yellows virus are competent for plant systemic infection and vector transmission. *J. Gen. Virol.* 97:1458-1461.
- Sim J, Valverde RA, Clark CA (2000)** Whitefly transmission of *Sweetpotato chlorotic stunt virus*. *Plant Dis.* 84:1250.
- Simón A, García C, Pascual F, Ruiz L, Janssen D (2016)** The influence of crop habitat and control strategies on pepper viruses in Andalusia (Spain). *Horticulturae* 2:1-9.
- Smirnova E, Firth AE, Miller WA, Scheidecker D, Brault V, Reinbold C, Rakotondrafara AM, Chung BYW, Ziegler-Graff V (2015)** Discovery of a small non-AUG-initiated ORF in poleroviruses and luteoviruses that is required for long-distance movement. *PLoS Pathog.* 11:e1004868.
- Smith KM, Machalaba CC, Seifman R, Feferholtz Y, Karesh WB (2019)** Infectious disease and economics: The case for considering multi-sectoral impacts. *One Health* 7:100080.
- Solórzano-Morales A, Barboza N, Hernández E, Mora-Umaña F, Ramirez P, Hammond RW (2011)** Newly discovered natural hosts of *Tomato chlorosis virus* in Costa Rica. *Plant Dis.* 95:497.
- Sõmera M, Fargette D, Hébrard E, Sarmiento C, ICTV Report Consortium (2021).** ICTV Virus Taxonomy Profile: *Solemoviridae* 2021. *J. Gen. Virol.* 102: 001707.
- Souza TA, Macedo MA, Inoue-Nagata A (2019)** Natural infection of apple-of-Peru (*Nicandra physaloides*) with *Tomato chlorosis virus* in Brazil. *Plant Dis.* 103:593
- Stewart LR, Medina V, Tian T, Turina M, Falk BW, Ng JC (2010)** A mutation in the *Lettuce infectious yellows virus* minor coat protein disrupts whitefly transmission but not *in planta* systemic movement. *J. Virol.* 84:12165-12173.
- Suastika G, Hartono S, Nishigawa H, Natsuaki T (2011)** Yellowing disease outbreaks in tomato in Indonesia associated with infection of *Tomato chlorosis virus* and *Tomato infectious chlorosis virus*. *J. ISSAAS.* 17:233.
- Sun Q-Y, Ding L-W, He L-L, Sun Y-B, Shao J-L, Luo M, Xu Z-F (2009)** Culture of *Escherichia coli* in SOC medium improves the cloning efficiency of toxic protein genes. *Anal. Biochem.* 394:144-46.
- Tamura K, Stecher G, Kumar S (2021)** MEGA11: molecular evolutionary genetics analysis version 11. *Mol. Biol. Evol.* 38:3022-3027.
- Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013)** MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* 30:2725-2729.

- Tan WP, Dong YZ, Sun XH, Liang YC, Liu HX, Zhu XP (2015)** The first identification of *Pepper vein yellows virus* in Shandong Province, China. *Plant Dis.* 99:1288.
- Tang X, Shi X, Zhang D, Li F, Yan F, Zhang Y, Liu Y, Zhou X (2017)** Detection and epidemic dynamic of ToCV and CCYV with *Bemisia tabaci* and weed in Hainan of China. *Virology J.* 14:169.
- Tian T, Rubio L, Yeh HH, Crawford B, Falk BW (1999)** Lettuce infectious yellows virus: *in vitro* acquisition analysis using partially purified virions and the whitefly *Bemisia tabaci*. *J. Gen. Virol.* 80:1111-1117.
- Tomassoli L, Manglli A, Ahmad A, Tiberini A, Barba M (2016)** First report of *Pepper vein yellows virus* infecting chilli pepper (*Capsicum* spp.) in Italy. *New Dis. Rep.* 33:22.
- Trenado HP, Fortes IM, Louro D, Navas-Castillo J (2007)** *Physalis ixocarpa* and *P. peruviana*, new natural hosts of *Tomato chlorosis virus*. *Eur. J. Plant Pathol.* 118:193-96.
- Tsai WS, Shih SL, Green SK, Hanson P, Liu HY (2004)** First report of the occurrence of *Tomato chlorosis virus* and *Tomato infectious chlorosis virus* in Taiwan. *Plant Dis.* 88:311.
- Turpen TH, Turpen AM, Weinzettl N, Kumagai MH, Dawson WO (1993)** Transfection of whole plants from wounds inoculated with *Agrobacterium tumefaciens* containing cDNA of tobacco mosaic virus. *J. Virol. Methods* 42:227-240.
- Tzanetakis IE, Postman JD, Martin RR (200)** Identification, detection and transmission of a new vitivirus from *Mentha*. *Arch Virol.* 152:2027-2033.
- Tzanetakis IE, Wintermantel WM, Cortez AA, Barnes JE, Barrett SM, Bolda MP, Martin RR (2006)** Epidemiology of strawberry pallidosis-associated virus and occurrence of pallidosis disease in North America. *Plant Dis.* 90:1343-1346.
- Tzanetakis IE, Wintermantel WM, Poudel B, Zhou J (2011)** Diodia vein chlorosis virus is a group-1 crinivirus. *Arch. Virol.* 156:2033-2037.
- Tzanetakis, IE, Martin RR, Wintermantel W (2013).** Epidemiology of criniviruses: an emerging problem in world agriculture. *Front.Microbiol.* 4:119.
- Verbeek M, Dulleman AM, Van Den Heuvel JFJM, Maris PC, Van Der Vlugt RAA (2007)** Identification and characterisation of tomato torrado virus, a new plant picorna-like virus from tomato. *Arch. Virol.* 152:881-890.
- Villanueva F, Castillo P, Font MI, Alfaro-Fernández A, Moriones E, Navas-Castillo J (2013)** First report of *Pepper vein yellows virus* infecting sweet pepper in Spain. *Plant Dis.* 97:1261.
- Wang J, Turina M, Stewart LR, Lindbo JA, Falk BW (2009)** Agroinoculation of the *Crinivirus*. Lettuce infectious yellows virus, for systemic plant infection. *Virology* 392:131-136.

Wang XY, Feng J, Zang LY, Yan YL, Yang YY, Zhu XP (2018) Natural occurrence of *Tomato chlorosis virus* in cowpea (*Vigna unguiculata*) in China. *Plant Dis.* 102:254.

Wintermantel WM, Polston JE, Escudero J, Paoli ER (2001) First report of *Tomato chlorosis virus* in Puerto Rico. *Plant Dis.* 85:228.

Wintermantel WM, Wisler GC (2006) Vector specificity, host range, and genetic diversity of *Tomato chlorosis virus*. *Plant Dis.* 90:814-819.

Wintermantel WM, Wisler GC, Anchieta AG, Liu HY, Karasev AV, Tzanetakis IE (2005) The complete nucleotide sequence and genome organization of tomato chlorosis virus. *Arch. Virol.* 150:2287-2298.

Wisler GC, Duffus JE, Liu H, Li RH (1998a) Ecology and epidemiology of whitefly-transmitted closteroviruses. *Plant Dis.* 82:270-280.

Wisler GC, Li RH, Liu HY, Lowry DS, Duffus JE (1998b) Tomato chlorosis virus: a new whitefly-transmitted, phloem limited, bipartite closterovirus of tomato. *Phytopathol.* 88:402-409.

Wu F, Zhao S, Yu B, Chen YM, Wang W, Gang Song ZG, Hu Y, Tao ZW, Tian JH, Pei YY, Yuan ML, Zhang YL, Dai FH, Liu Y, Wang QM, Zheng JJ, Xu L, Holmes EC, Zhang YZ (2020) A new coronavirus associated with human respiratory disease in China. *Nature* 579:265-269.

Xiao H, Li C, Al Rwahnih M, Dolja V, Meng B (2019) Metagenomic analysis of Riesling grapevine reveals a complex virome including two new and divergent variants of grapevine leafroll-associated virus 3. *Plant Dis.* 103:1275-1285.

Xu S, Jiang L, Qiao G, Chen J (2020) The bacterial flora associated with the polyphagous aphid *Aphis gossypii* glover (Hemiptera: Aphididae) is strongly affected by host plants. *Microb. Ecol.* 79:971-984.

Yonaha T, Toyosato T, Kawano S, Osaki T (1995) Pepper vein yellows virus, a novel luteovirus from bell pepper plants in Japan. *Ann. Phytopathol. Soc. Jpn.* 61:178-184.

Zhang H, Zhao X, Cao X, Khan LU, Zhao R, Wang H, Huang X (2022) Transmission of areca palm velarivirus 1 by mealybugs causes yellow leaf disease in betel palm (*Areca catechu*). *Phytopathol.* 112:700-707.

Zhang SB, Zhao ZB, Zhang DY, Liu Y, Zhang SB, Zhang DY, Liu Y, Luo XW, Liu J, Wu LF, Peng J (2015) First report of *Pepper vein yellows virus* infecting red pepper in mainland China. *Plant Dis.* 99:1190.

Zhao F, Yoo RH, Lim S, Igori D, Lee SH, Moon JS (2015) Nucleotide sequence and genome organization of a new proposed crinivirus, tetterwort vein chlorosis virus. *Arch. Virol.* 160:2899-2902.



References

Zhao R, Wang N, Liu S, Ling KS, Fan Z, Zhou T (2016) P22 of tomato chlorosis virus, an RNA silencing suppressor, is naturally expressed in the infected plant. *Acta Virol.* 60:423-425.

Zhao RN, Wang R, Wang N, Fan ZF, Zhou T, Shi YC, Chai M (2013) First report of *Tomato chlorosis virus* in China. *Plant dis.* 8:1123.

Zhao X, Zhu M, Wu Q, Zhang J, Xu Y, Tao X (2018) Complete genome sequence of a lettuce chlorosis virus isolate from China and genome recombination/rearrangement analysis. *Arch. Virol.* 163:751-754.

Zhou Y, Yan JY, Qiao GH, Liu M, Zhang W, Li XH (2015) First report of *Tomato chlorosis virus* infecting eggplant (*Solanum melongena*) in China. *Plant Dis.* 99:1657.