Characterization and epidemiology of members of the genus *Torradovirus*

Martin Verbeek

Thesis committee

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Prof. dr. J.M. Vlak
Personal chair at the Laboratory of Virology

Co-promotors
Dr. ir. R.A.A. van der Vlugt
Senior researcher, Plant Research International

Dr. ir. R.J.M. Kormelink
Assistant professor, Laboratory of Virology

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Dr. J.W. Roenhorst, National Plant Protection Organization, Wageningen

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Martin Verbeek

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M. Verbeek
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Voor mijn vader † en moeder,

voor Rian,

Toni, Jesse en Daan

Chapter 1

General introduction

The discovery of a so-far unknown virus is a rare and exciting event, at least to a virologist. The work described in this thesis began with such a discovery: the causal agent of the 'torrado' disease in tomato appeared to be a plant virus that was not described before. When other 'new', but related, viruses were found, the International Committee on Taxonomy of Viruses (ICTV) decided to create a separate genus to harbour these viruses (King et al., 2012; Sanfaçon et al., 2009). Torradoviruses, as these members of the genus *Torradovirus* are called, are able to cause serious economic losses in important crops, such as in tomato. These viruses possess some unique characteristics and are therefore interesting subjects to study. However, the study presented in this thesis can only be the beginning of the exploration of this new group of plant viruses.

Tomato and emerging diseases

Tomato (*Solanum lycopersicum* L.) is the second economically most important vegetable crop (after potato) and is grown worldwide in the open field or in screen- / greenhouses. The tomato originates from the New World (South America) and was brought to Europe in the 1600s, where it was first treated as an ornamental plant (Hanssen and Lapidot, 2012). Later, the people in Europe accepted to eat the fruits as a vegetable. From Europe tomato was distributed to many countries, and even back to the American continent. Tomato plants can be cultivated in various climate zones, ranging from tropical to moderate (Hanssen and Lapidot, 2012). With the distribution of tomato to various geographical locations, the crop was exposed to new pathogens. Moreover, diseases could spread easily through the dissemination of seeds, transplants and insect vectors. When circumstances were in favour of the pathogen, e.g. a suitable host, a good environment and the presence of a vector, an economically important disease could occur.

Emerging diseases are new or previously known diseases that have become more of a problem, often due to a change in some aspect of the pathogen, pathogen vector, and host and/or environment (Gilbertson and Batuman, 2013). New emerging diseases, which were found in tomato crops during the last 10-20 years, comprise diseases caused by fungi (e.g. *Oidium neolycopersici*), viroids (e.g. *Tomato apical stunt viroid*), and plant viruses. Examples of the emerging tomato-infecting plant viruses are *Pepino mosaic*

virus (PepMV), new species of the genus *Tospovirus*, begomoviruses, Tomato necrotic spot virus (a new ilarvirus) and torradoviruses (Gilbertson and Batuman, 2013; Hanssen and Lapidot, 2012).

Figure 1.1: Marchitez, an emerging disease in tomato crops in Mexico (courtesy of J.F.J.M. van den Heuvel)

Plant viruses

The current virus taxonomy (2012) lists 7 Orders, 96 Families, 22 Subfamilies, 420 Genera and 2618 Species as recognised taxa (http://www.ictvonline.org/). Viruses are obligate parasites, which can be found in all living organisms. They only consist of nucleic acid (RNA or DNA), which is surrounded by a protein coat, and in some cases an additional envelope. Viruses have to make use of the 'machinery' of their host (cell) to replicate themselves and turn hosts and vectors to their account to facilitate the spread of the virus. Infection with a virus often results in a disease of the host, but the virus can also exist in a slumbering or latent stage, causing the host no or little trouble.

Viruses belonging to approximately 90 genera have plants as their hosts. Most of them only infect plants, but some are also able to replicate in their insect vector. As plants are quite immobile and plant viruses are not capable to enter the plant cell actively, plant viruses use a range of typical strategies to infect and spread. Infection can occur when plant cells are damaged upon contact by humans or animals, but viruses can also be actively delivered to the plant cells by vector organisms, such as insects, nematodes and fungi (see Bos (1983) and Gergerich and Dolja (2006) for further reading).

The taxonomy of plant viruses

In the early days of plant virology, plant viruses were described and named mainly on the basis of their symptomatology in host- and indicator plants. The virus name was chosen in such a way that the host in which the virus was found and the symptoms it induces in this host were presented (e.g. *Tobacco mosaic virus*). Viruses were grouped into genera mainly based on their morphology, which could be investigated by the means of an electron microscope. Members of the same genus share the same morphology (the appearance of virus particles), which can be rod-shaped, filamentous, spherical, bullet-shaped, enveloped, etc. Later, when more advanced techniques became available for virologists, viruses were grouped also according to other intrinsic properties (e.g. number of coat proteins, number of genome segments, type of nucleic acid, etc.). Nowadays, the nucleotide sequence and the organization of the viral genome has become of utmost importance in the taxonomy of viruses. Virus taxonomy is changing

continuously due to new data and new insights. A nice example of such a change in taxonomy can be found in the group of plant-infecting picorna-like viruses (Sanfaçon et al., 2012).

The family Secoviridae

Until 2009, the plant-infecting picorna-like viruses in the order *Picornavirales* were classified into the families *Comoviridae*, (containing the genera *Comovirus*, *Fabavirus* and *Nepovirus*) and *Sequiviridae* (containing the genera *Sequivirus* and *Waikavirus*), and into the unassigned genera *Cheravirus* and *Sadwavirus* (Fauquet et al., 2005). In 2009, the ICTV working group on plant *Picornavirales* proposed a new taxonomy on the plant-infecting picornaviruses. They proposed to place them all in the newly created family *Secoviridae* and to place the genera *Comovirus*, *Fabavirus* and *Nepovirus* into the new subfamily *Comovirinae*. They also proposed to create a new genus within the family *Secoviridae* to accommodate the recently identified *Tomato torrado virus* (ToTV) and *Tomato marchitez virus* (ToMarV). The new genus was named *Torradovirus*, after the first virus discovered, and *Tomato torrado virus* was chosen to be the type species of this genus (Sanfaçon et al., 2009).

Demarcation criteria

To be able to distinguish the different genera within the family *Secoviridae*, so-called genus demarcation criteria were set. These demarcation criteria have been determined for each family separately, and therefore may differ according to the family. For the family *Secoviridae* the genus demarcation criteria are: (1) the number of genomic RNAs; (2) the number of protein domains and/or processing sites within the polyprotein(s); (3) the number of coat proteins; (4) the presence of additional Open Reading Frames (ORFs) and/or subgenomic RNAs (Sanfaçon et al., 2012).

The ICTV study group determined also demarcation criteria for the species level. A virus represents a new species when the following criteria are met: (1) Coat protein (CP) amino acid sequence with less than 75% identity (for viruses with two or three CPs, combined CP sequences are considered); (2) Conserved Protase-Polymerase region, amino acid sequence with less than 80% identity; (3) Differences in antigenic determinants; (4) Distinct host range; (5) Distinct vector specificity; (6) Absence of cross-protection; (7) For

viruses with a bipartite genome, absence of reassortment between RNA1 and RNA2 (Sanfaçon et al., 2012).

When analysing the four tomato-infecting torradoviruses which were characterized until now, and applying the above demarcation criteria, it became clear that these criteria did not fit exactly with the situation within this genus. Looking at all available full genome sequences, it became clear that four tomato-infecting species should be proposed. However, according to strict application of the demarcation criteria, which were set for other members of the family *Secoviridae* when no information was available on the torradoviruses yet, only two species could be defined. From the work described in this thesis some additional demarcation criteria were developed allowing the recognition of all four tomato-infecting torradovirus species. These new demarcation criteria were proposed to the ICTV working group (Verbeek et al., 2010a) and are currently under evaluation.

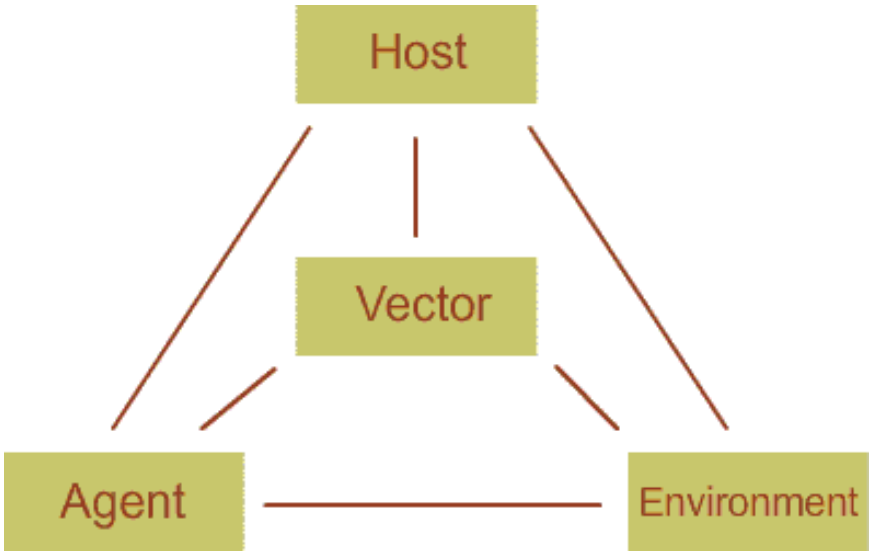

Figure 1.2: The epidemiological triangle

Epidemiology of torradoviruses

Besides the characterization and description of the torradoviruses that were isolated from tomato crops, and recently from lettuce, also the epidemiology will receive a prominent place in this thesis. For understanding the epidemiology of a virus disease, the interactions between de causal agent (in this case the virus), the host, and the environment have to be investigated. These three interactions are depicted in the epidemiological triangle (McNew, 1960). However, there is another important factor: the vector, which is placed centrally with interactions to all three vertices of this triangle (Fig. 1.2). The vector is essential for the spread of plant viruses, as plant hosts do not generally move except with the aid of other agents (e.g. man, animals, wind, water). Understanding the way of transmission (seed-transmitted, pollen-transmitted, vector-borne, mechanical transmission) is essential to understand the epidemiology of a disease. When a disease is vector-borne, the knowledge on the mode of vector transmission is equally important, as this will give insight into the persistence of the virus in the vector and the possibility of spread over large distances. So, first of all, to understand the mechanism with which torradoviruses are transmitted was a significant goal in this work. Observations of large numbers of whiteflies in greenhouses with ToTV-infected tomato crops, already led to the assumption that ToTV was spread by whiteflies. Research on ToTV in Poland and Spain revealed indeed that ToTV was vectored by two species of whiteflies: the greenhouse whitefly *Trialeurodes vaporariorum* and the tobacco or silverleaf whitefly *Bemisia tabaci* (Amari et al., 2008; Pospieszny, 2005; Pospieszny et al., 2010). However, that work did not lead to an indication of the mode of transmission by the whitefly vectors, in other words whether ToTV was transmitted in a non-persistent, semi-persistent or persistent manner.

The emergence of ToTV in Australia, a country where only the import of seeds is allowed due to phytosanitary regulations, led to the suspicion of torradoviruses to be seed-transmitted. Evidence for seed transmission is still lacking in literature, however preliminary results indicating possible seed-transmission were presented at a congress in 2013 (Pospieszny et al., 2013). To fully understand the epidemiology of torradoviruses it is essential to know in what manner the viruses are transmitted in order to predict efficiency and distance of spread.

Aim and scope of this thesis

This thesis describes the discovery and characterization of a new group of plant infecting members of the *Picornavirales* superfamily, which have been assigned to the new genus *Torradovirus*. Four new viruses were characterized within this genus (and a fifth by another research group) and specific and generic detection tools were developed. This thesis also focusses on the epidemiology of torradoviruses. In this light, the mode of vector transmission by three species of whiteflies was determined. This work will give insight in the current knowledge and knowledge gaps within the field of torradovirology.

Chapter 2 is the report on the discovery and characterization of the causal agent of the 'Torrado' disease in tomato. In the early 2000s, this disease caused serious economic losses in the Southeast part of Spain, in the area around Murcia. However, at that time it was unknown what agent caused this disease. Samples that were analysed were all also infected with PepMV. Electron microscopic analysis on one of these samples revealed the co-infection of a small spherical virus (Fig. 1.3). Classical virological methods were used to separate this virus from the virus complex with PepMV, to propagate and purify the virus and to extract RNA from the purified particles. The entire, bi-partite, genome was sequenced and analysed. Based on this analysis a new virus was reported with the name *Tomato torrado virus* (ToTV), referring to the Spanish name of the disease symptoms.

Chapter 3 covers the characterization of three new torradoviruses and the expansion of the newly formed genus *Torradovirus*. In **Section 3A**, the causal agent of a torrado-like disease in Mexico was isolated and characterized. In Mexico, this disease was called 'marchitez', meaning 'withered'. This virus is clearly related to ToTV, but the nucleotide sequence and biological properties showed that this virus belonged to another species, which has now been named *Tomato marchitez virus* (ToMarV).

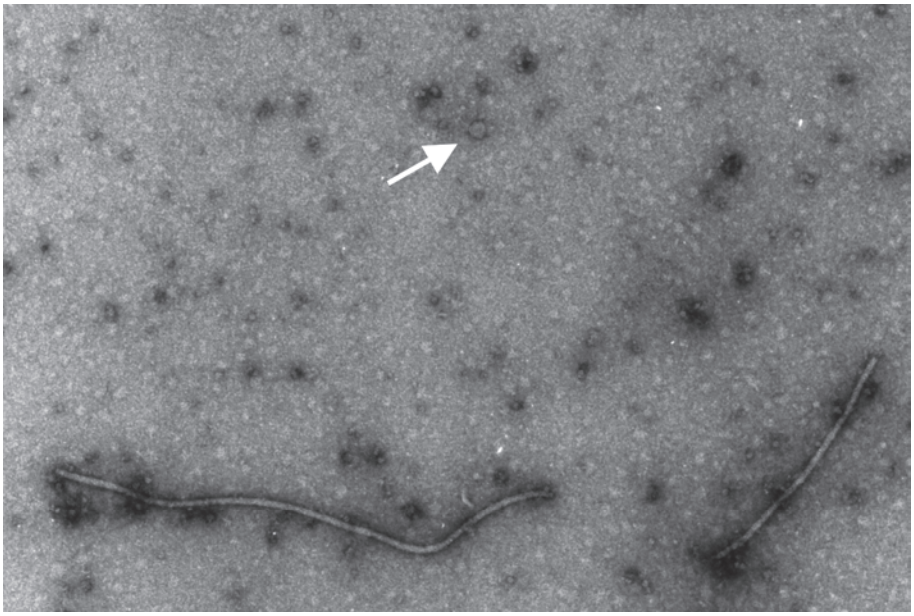

Figure 1.3: Electron micrograph of a leaf-dip preparation of a tomato leaf with torrado disease. Filamentous particles represent *Pepino mosaic virus*, the spherical virus particle (arrow) represents *Tomato torrado virus.*

Section 3B is the report on a third torradovirus isolated from tomatoes grown in Guatemala. A torrado-like disease, called 'chocolàte', was causing serious economic losses. A torradovirus was isolated from tomato samples originating from Guatemala and the complete nucleotide sequence was elucidated. This virus was named Tomato chocolàte virus (ToChV) and we proposed it as a new species within the genus *Torradovirus*. In this chapter comparison was made between ToChV and another torradovirus that had also been isolated from Guatemalan tomatoes showing chocolàte disease. This virus was named Tomato chocolate spot virus and appears to be yet another species than ToTV, ToMarV and ToChV (Batuman et al., 2010).

In **Section 3C** the recent finding of a lettuce-infecting torradovirus is reported. The infected lettuce plant was found in an open field in the Netherlands and it was showing necrosis and leaf curling. Diagnostic tests did not reveal the causal agent of this syndrome. Unfortunately, the original sample was lost, but the virus was maintained in test plants by mechanical inoculation. It was decided to analyse total RNA extracted from test plants by

Next Generation Sequencing. This revealed the presence of a virus with a typical torradovirus genome organization, but with low sequence identities compared to the tomato-infecting torradoviruses. This virus was named Lettuce necrotic leaf curl virus (LNLCV) and will be proposed to the ICTV as a separate species within the genus *Torradovirus*.

Chapter 4 focuses on the generic detection of torradoviruses. Two sets of PCR primers targeting highly conserved regions within the torradovirus genome were designed and tested. The two primer sets, Torrado-1F / Torrado-1R and Torrado-2F / Torrado-2R, were successfully evaluated in RT-PCR using 15 isolates belonging to 4 tomato-infecting torradovirus species.

In **Chapter 5** the focus is on the transmission of torradoviruses by whiteflies as vectors. First of all, in addition to *Trialeurodes vaporariorum* and *Bemisia tabaci*, the banded-winged whitefly (*Trialeurodes abutilonea*) was identified as vector for torradoviruses. This chapter also contains a study to find out which of the three modes of virus transmission operates in these vectors: persistent, semi-persistent and non-persistent. To achieve this the acquisition access period (AAP), the retention period and the inoculation access period (IAP) have been determined for torradoviruses and their vectors. Finally, also the place where virus particles are retained within the vector was localized.

In **Chapter 6** the results described in the experimental chapters are discussed and an outlook is given for future directions in the research on torradoviruses.

Chapter 2

Identification and characterization of tomato torrado virus, a new plant picorna-like virus from tomato

M. Verbeek[1], A. M. Dullemans[1], J. F. J. M. van den Heuvel[2],

P. C. Maris[2], and R. A. A. van der Vlugt[1]

[1]Plant Research International, part of Wageningen UR, Wageningen, The Netherlands

[2]De Ruiter Seeds, currently known as Monsanto Vegetable Seeds Division, Bergschenhoek, The Netherlands

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Summary

A new virus was isolated from tomato plants from the Murcia region in Spain which showed symptoms of 'torrado disease'; very distinct necrotic, almost burn-like symptoms on leaves of infected plants. The virus particles are isometric with a diameter of approximately 28 nm. The viral genome consists of two (+)ssRNA molecules of 7793 (RNA1) and 5389 nts (RNA2). RNA1 contains one open reading frame (ORF) encoding a predicted polyprotein of 241 kDa that shows conserved regions with motifs typical for a protease-cofactor, a helicase, a protease and an RNA-dependent RNA polymerase. RNA2 contains two, partially overlapping ORFs potentially encoding proteins of 20 and 134 kDa. These viral RNAs are encapsidated by three proteins with estimated sizes of 35, 26 and 23 kDa. Direct protein sequencing mapped these coat proteins to ORF2 on RNA2. Phylogenetic analyses of nucleotide and derived amino acid sequences showed that the virus is related to but distinct from viruses belonging to the genera *Sequivirus*, *Sadwavirus* and *Cheravirus*. This new virus, for which the name Tomato torrado virus is proposed, most likely represents a member of a new plant virus genus.

The nucleotide sequences reported in this article are available under GenBank accession numbers DQ388879 (RNA1) and DQ388880 (RNA2).

Introduction

In the past three years, tomato plants with severe necrotic leaf symptoms were observed in the area of Murcia in the South-East of Spain. The symptoms of this new emerging disease consisted initially of necrotic spots, surrounded by a light green or yellow area beginning at the base of leaflets (Fig. 2.1). This syndrome developed into a severe necrosis of leaves and fruits and overall growth reduction of the plant, resulting in serious economic damage. The disease was named 'torrado' by the local farmers, meaning burnt or roasted. Primary diagnostics always revealed the presence of *Pepino mosaic virus* (PepMV, genus Potexvirus) (Van der Vlugt et al., 2002), but the symptoms could not be attributed to this virus alone. In addition to PepMV, a virus with isometric particles was found in symptomatic samples from the area of Murcia. In this paper, we describe the identification and characterization of this new picorna-like plant virus, which we tentatively named tomato torrado virus (ToTV).

Figure 2.1: Typical initial symptoms of tomato torrado virus on tomato leaves: necrotic spots, surrounded by a light green or yellow area, beginning at the base of a leaflet

Material and methods

Virus transmission and propagation

ToTV was isolated from a tomato plant showing typical symptoms of 'torrado' (i.e. severe leaf necrosis) from the Murcia region, Spain. The isolate was designated PRI-ToTV0301. For mechanical transmission of ToTV to alternative hosts or indicator plants, a standard inoculation buffer (e.g. 0.03M sodium/potassium phosphate buffer, pH 7.7) proved suitable. The virus was mechanically inoculated to and maintained in *Nicotiana glutinosa* 'PRI' or *N. benthamiana*.

DAS-ELISA

Double antibody sandwich (DAS)-ELISA was carried out according to standard DAS-ELISA protocols, using antisera to PepMV, tomato aspermy virus (genus *Cucumovirus*), cucumber mosaic virus (genus *Cucumovirus*), Andean potato mottle virus (genus *Comovirus*), Andean potato latent virus (genus *Tymovirus*), carnation etched ring virus (genus *Caulimovirus*), potato black ringspot virus (genus *Nepovirus*), pelargonium flower break virus (genus *Carmovirus*), pelargonium line pattern virus (genus *Carmovirus*), melon necrotic spot virus (genus *Carmovirus*), carnation mottle virus (genus *Carmovirus*), and carnation ringspot virus (genus *Dianthovirus*). All antisera were obtained from Prime Diagnostics, Wageningen, The Netherlands.

Virus purification

All centrifugation steps were performed at 6 ^{o}C. Infected leaves of *N. glutinosa* 'PRI' or *N. benthamiana* were harvested approximately 14 days after inoculation of the virus and homogenized in 5 parts (w/v) 0.1M Tris–HCl, pH 8.0, containing 20mM Na_2SO_3, 10mM Na-DIECA and 5mM Na-EDTA (homogenization buffer). The homogenate was centrifuged for 30min at 49,000 g. The supernatant was placed on a sucrose cushion (20% in homogenization buffer) and centrifuged at 70,000 g for 1.5 h. The pellet was resuspended in 2ml Tris–HCl, pH 8.0, and the suspension was placed onto a sucrose gradient (10–40% in homogenization buffer) and centrifuged at 110,000 g for 2 h. Gradient fractions were tested for the presence of virus by inoculation experiments on *N. hesperis* '67A'. The virus-containing fraction

was pipetted from the gradient, placed onto a 10–40% cesium sulfate gradient (in Tris–HCl, pH 8.0) and centrifuged at 125,000 g for 16 h. The virus bands were collected and dialyzed against 0.1M Tris–HCl, pH 8.0.

Electron microscopy

Virus suspensions were mounted on formvar carbon-coated grids, stained with 2% uranyl acetate and examined in a Philips CM12 electron microscope.

Polyacrylamide gel electrophoresis

Viral proteins were separated by subjecting purified virus particles to 12% denaturing polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and visualized by silver staining.

Nucleic acid isolation and evaluation

Purified virus was concentrated by centrifugation (at 115,000 g for 2 h). Pellets were subjected to RNA extraction using a Qiagen RNeasy kit (Qiagen) according to the manufacturer's instructions. RNA concentration was determined in a Beckmann DU-530 UV-spectrophotometer. Viral RNA integrity and size was checked by running 1 µg of each RNA preparation on a 1% agarose gel using a formaldehyde/formamide/HEPES buffer system. After electrophoresis, the RNA was stained using ortho-toluidine blue.

Protein identification by mass spectrometry

Viral capsid proteins were subjected to mass spectrometry analysis essentially as described by Volpicella et al. (2003). Purified virus particles were separated by SDS-PAGE. After Coomassie Brilliant Blue (CBB R-250, Bio-Rad laboratories) staining, the protein bands of interest were excised from the gel. Prior to in-gel trypsin digestion (Seq. Grade Modified Porcine Trypsin, Promega), the proteins were reduced with dithiothreitol and alkylated with iodoacetamide (Shevchenko et al., 1996). After overnight tryptic digestion, the peptides were extracted with 50% acetonitrile, 0.5% formic acid and concentrated in a vacuum centrifuge. The resuspended peptides were then loaded onto a C18 Atlantis column (15 cm x 75 µm ID, Waters). Peptides were eluted by a gradient from 0.5% formic acid in water to 0.5% formic acid in 50% acetonitrile at a speed of approximately 0.2 µl/min. The C18 column was

connected to the electro-spray of a Q-Tof-2 mass spectrometer (Waters) by a PicoTip (New Objective). The resulting MS/MS spectra contained the sequence information for a single peptide per spectrum. The ProteinLynx GlobalServer package V2.1 (Waters) was used to process MS/MS data, which were automatically selected for blasting against the protein translation of the ORF2 sequence on RNA2.

cDNA synthesis and cloning

cDNAwas synthesized using the SuperScript Choice System for cDNA Synthesis (Invitrogen) according to the manufacturer's instructions. First-strand cDNA was primed using either oligo(dT) or random hexamer primers. After second-strand synthesis, *Eco*RI adapters were ligated to facilitate cloning into pBluescript II IR Predigested Vector (Stratagene). The resulting constructs were transformed to TOP10 competent cells (Invitrogen), and insert lengths of recombinant colonies were determined by colony PCR using both T3 and T7 cloning-vector-specific primers. Clones containing inserts over 1500 nucleotides (nts) were used for further sequence analysis. Additional cDNA fragments were obtained by one-tube RT-PCR (Access RT-PCR system, Promega) with ToTV-specific primers derived from earlier obtained sequence data.

The 5' terminus of the ToTV sequence was determined using the 5' RACE System for Rapid Amplification of cDNA Ends (LifeTechnologies) using dCTP according to the manufacturer's instructions.

Nucleotide sequencing and sequence analysis

Cloned cDNA fragments and PCR products were sequenced directly. Sequence analysis was performed using an Applied Biosystems 3100 Genetic Analyser, with a DYEnamic ET Terminator Cycle Sequencing Kit (Amersham) and the primers that had been used for amplification. For additional PCR fragments, ToTV-specific primers were used for primer-walking sequencing.

Nucleotide and amino acid sequence data were analyzed and assembled using the DNASTAR package (Lasergene). Blast searches were carried out using the NCBI Blast server (www.ncbi.nlm.nih.gov) with all available databases. Sequence comparisons with other viruses were performed with programs from the PHYLIP package. Multiple alignments and phylogenies

were performed with the CLUSTAL X program after bootstrapping in 1000 replicates. Neighborjoining consensus phylogenies were viewed by the NJplot program (Thompson et al., 1997) and printed using TreeView (Page, 1996).

Results and discussion

Virus characterisation

Tomato plants showing very typical burn-like necrotic symptoms ('torrado') on leaves and fruits were found in the Murcia area in Spain. Initially, infected tomato plants were examined by electron microscopy, which revealed the presence of two distinct virus particles: filamentous particles of approximately 550 nm in length and isometric particles of about 28 nm in diameter. ELISA showed the presence of PepMV in infected plants but no reactions were observed with available antisera to carmovirus, caulimovirus, comovirus, cucumovirus, dianthovirus, nepovirus and tymovirus isolates (results not shown).

Mechanical inoculation experiments onto indicator plants showed that *Physalis floridana* and *Nicotiana glutinosa* 'PRI' were hosts for the spherical virus, but not for PepMV. These alternative host plants could be used as filter hosts, and a pure isolate of the unknown virus, ToTV, was obtained. Other test plant reactions to ToTV inoculations are presented in Table 2.1.

To verify that the virus encountered is the causal agent of the 'torrado' disease, purified virions were mechanically inoculated onto tomato plants. Necrosis beginning at the base of a leaflet, which is typical for 'torrado' disease, emerged two weeks after inoculation. The virus could be re-isolated from the inoculated tomato plants, and its identity was verified by RT-PCR with virus-specific primers (nts 4141–4161 and 4705–4724 on RNA2; results not shown).

Development of a purification protocol allowed further characterization of the virus, however yields were low (15–20 µg/100 g leaf material). The final virus purification step employing Cs_2SO_4 buoyant density gradient centrifugation yielded two types of particles, identical in size (28 nm) but different in density. The top band in the gradient (T) always contained only a 5.5-kb RNA (RNA2). RNA isolated from the bottom band (B) was always resolved into two RNA bands on a denaturing agarose gel; a 5.5-kb band and a

band of about 8 kb (RNA1; see Fig. 2.2). SDS-PAGE revealed that both particle types were composed of three coat proteins with estimated sizes of 35, 26 and 23 kDa, respectively named Vp35, Vp26 and Vp23 (Fig. 2.3A).

Table 2.1: Symptoms of Tomato torrado virus on alternative host plants.

Tested alternative host plants for ToTV	Symptoms (local/systemic)
Chenopodium quinoa	- / -
Gomphrena globosa	- / -
Nicotiana benthamiana	- / c, mf
Nicotiana clevelandii	- / c
Nicotiana glutinosa 'PRI'	- / c
Nicotiana hesperis '67A'	nl / c, n, mf
Nicotiana occidentalis 'P1'	nl / c, n, mf
Nicotiana rustica	la
Nicotiana tabacum 'White Burley'	la
Physalis floridana	nl / c, n, mf, do

c: chlorosis; do: die off; la: latent infection; mf: malformation; n: necrosis; nl: necrotic lesions; -: no symptoms

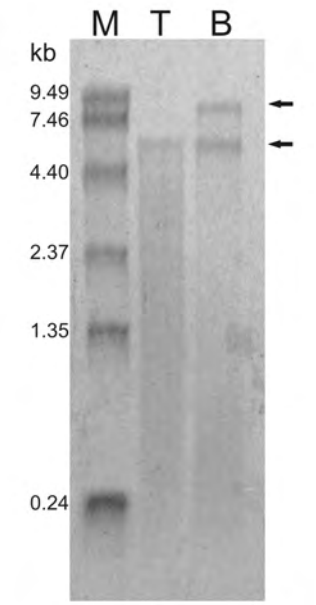


Figure 2.2: Denaturating agarose gel electrophoresis of RNAs extracted from ToTV virions and stained with orthotoluidine blue. 1 (M): molecular size standard (Invitrogen 0.24–9.5-kb RNA Ladder); 2 (T): RNA purified from ToTV top component; 3 (B): RNA purified from ToTV bottom component. Arrows indicate positions of the ~5.5-kb and ~8-kb RNA bands.

Viral RNA analysis

Generally, the band containing the bottom component was more diffuse than the band of the top component. Also, cDNA libraries derived from particles from the bottom fraction of the Cs_2SO_4 gradient always contained sequences typical for RNA1 and RNA2 and, in addition, were contaminated with plant RNA sequences. This is in contrast to cDNA libraries derived from the top component band, which only contained sequences typical for RNA2 and virtually no plant-specific sequences (results not shown). This indicates that the bottom band after Cs_2SO_4 buoyant density gradient centrifugation contained both types of particles, each separately encapsidating an RNA1 or RNA2 and, in addition, a minor contamination with plant components.

RNA isolated from each Cs_2SO_4 gradient component was used to create a cDNA library. cDNA synthesis and cloning of RNA1 from the bottom component and RNA2 from the top component yielded 15 and 14 clones, respectively. Sequence analysis and assembly resulted in two contigs for each of the two RNAs. The missing regions between the contigs were amplified by RT-PCR with ToTV-specific primers derived from the contig sequences already obtained.

The sequence analysis resulted in contigs of ~7.7 kb and ~5.2 kb for RNA1 and RNA2, respectively. Determination of the 5' terminal sequences of the two ToTV RNAs, using a 5' RACE kit, from 10 individual clones indicated that RNA1 consists of 7793 nt and RNA2 of 5389 nt [both excluding the poly(A) tail]. The genome sequence is A/U rich: A(26.9%), U(28.9%), C(20.5%), G(23.7%).

RNA1

RNA1 [7793 nt, without poly(A) tail] contains one open reading frame (RNA1-ORF1) encoding a predicted polyprotein of 2158 amino acids (aa) with a molecular mass of 241 kDa (Fig. 2.4). The first in-frame AUG is at nt positions 107–109. The ORF has an UGA stop codon at nt positions 6581–6583. The polyprotein contains conserved regions with motifs typical for a protease cofactor, helicase, protease and RNA-dependent RNA polymerase (RdRp).

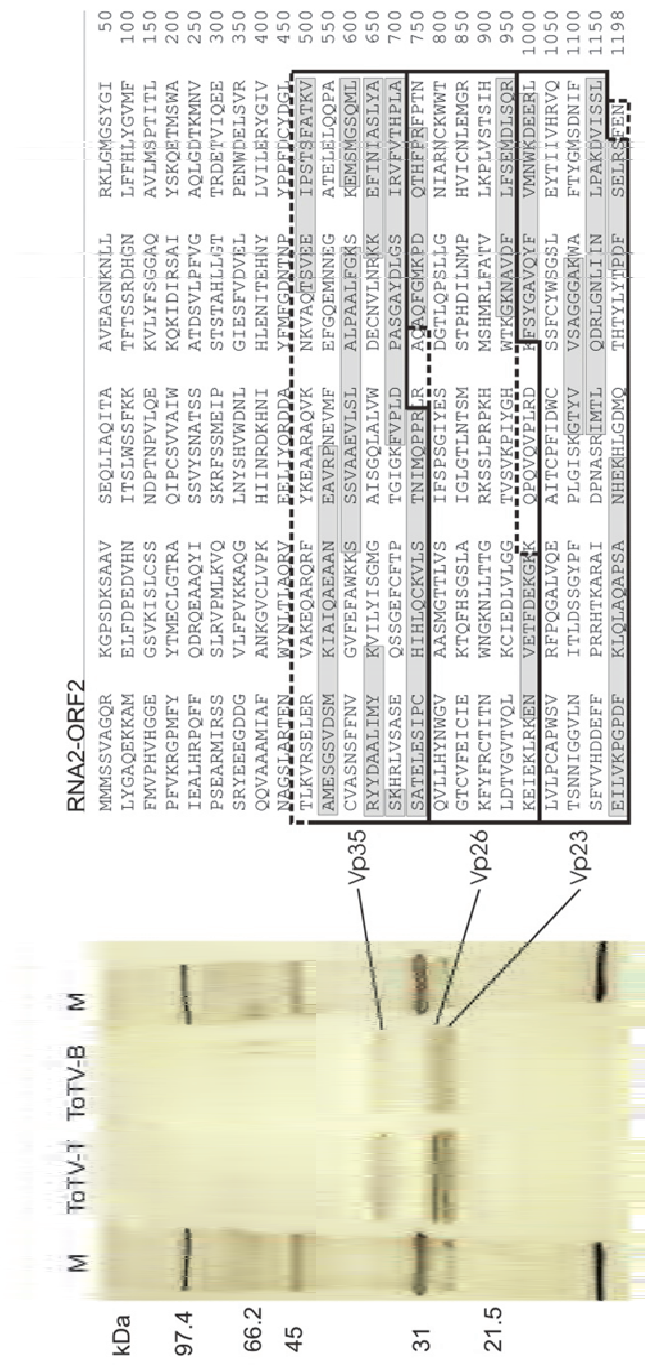
The closest identities of the complete polyprotein sequence were found with members of the family *Sequiviridae* and the floating (unassigned) genera

Cheravirus and *Sadwavirus*: rice tungro spherical virus (RTSV, genus *Waikavirus*; CAA67042), with 29% identity between aa positions 897 and 1647 of the ToTV translated sequence; maize chlorotic dwarf virus (MCDV, genus *Waikavirus*; AAV86083), with 28% identity between aa positions 905 and 1646; parsnip yellow fleck virus (PYFV, genus *Sequivirus*; BAA03151.1), with 33% identity between aa positions 1155 and 1655; apple latent spherical virus (ALSV, genus *Cheravirus*; BAA90870.1), with 32% identity between aa positions 1155 and 1626; strawberry mottle virus (SMoV, genus *Sadwavirus*; NP_599086), with 30% identity between aa positions 1039 and 1718; and cherry rasp leaf virus (CRLV, genus *Cheravirus*; CAF21713.1), with 33% identity between aa positions 1155 and 1619.

In the C-terminal part of the RNA1-ORF1, a low level of aa sequence similarity (22%) was observed with a protease cofactor (PRO-co) of patchouli mild mosaic virus (PatMMV; NP_647592.1, a strain of *Broad bean wilt virus* 2, genus *Fabavirus*), for the aa positions 106–338.

Typical helicase motifs A (GKS), B (D), C (N) were identified at aa positions 398–400, 444 and 495 of the putative polyprotein. The closest identities in the helicase region were found with RTSV (NP_042507.1; 42% identical between aa positions 381 and 520), MCDV (AAB58882.1; 43% identical between aa positions 383 and 519), SMoV (NP_599086.1; 42% identical between aa positions 386 and 520) and PYFV (NP_619734.1; 42% identical between aa positions 383 and 520). The highest similarity in the protease region (PRO) was found for aa 1000–1100, 25% identity is found with potato virus V (PVV, genus *Potyvirus*; NP_659008.1; NIa protease between aa positions 1003 and 1088). The RdRp region could be identified between aa 1303 and 1554 by the presence of the typical motifs I (KDE) to VII (FLSR) (Koonin, 1991).

Figure 2.3: A: Silver-stained capsid proteins after denaturing polyacrylamide gelelectrophoresis (SDS-PAGE). 1 and 4 (M): molecular weight markers (Bio-Rad silver stain markers, low range), 2: ToTV-T (top band of purified virions in Cs_2SO_4 buoyant density gradient centrifugation) and 3: ToTV-B (bottom band of purified virions after Cs_2SO_4 buoyant density gradient centrifugation). B: Amino acid sequence of RNA2-ORF2. Positions of the three coat proteins (Vp35, Vp26 and Vp23) are indicated by boxes. Putative cleavage site areas are indicated by dotted lines. Peptides found in MS/MS analysis are shaded


RNA2-ORF2
Vp35
Vp26
Vp23
B
M
ToTV-T
ToTV-B
M
kDa
97.4
66.2
45
31
21.5
A

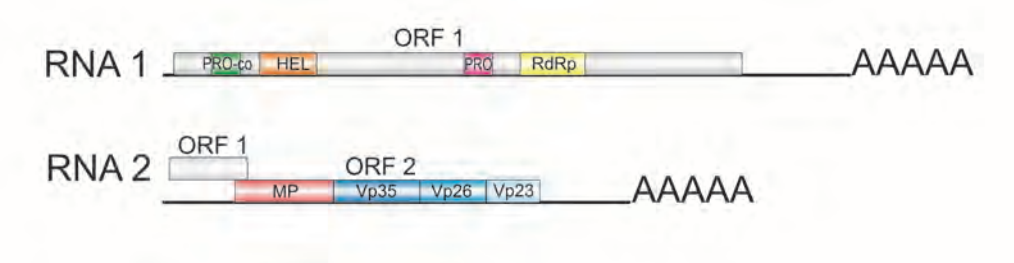


Figure 2.4: Genome organization of the tomato torrado virus isolate PRI-ToTV0301. Relative positions of regions containing motifs of protease-cofactor (PRO-co), helicase (HEL), protease (PRO), and RNA-dependent RNA polymerase (RdRp) on RNA1 and of the three coat proteins (Vp35, Vp26 and Vp23) and putative movement protein (MP) on RNA2 are indicated

RNA2

RNA2 [5389 nt, without poly(A) tail] contains two potential ORFs. The first ORF (RNA2-ORF1) encodes a predicted protein of 187 aa with a molecular mass of 20 kDa (Fig. 2.4). The first in-frame AUG of RNA2-ORF1 is at nt position 182–184. RNA2-ORF1 has a UGA stop codon at positions 743–745 nt. In Blast searches, the RNA2-ORF1 polyprotein shows no apparent homologies with proteins in sequence databases.

The position of ORF1 on RNA2 of ToTV was verified by sequence analysis of three independently obtained RT-PCR fragments generated from total RNA isolated from three individual ToTV infected host plants. Each RT-PCR fragment (nt pos 140–861 on RNA2) was identical in sequence to the sequences obtained initially from five different cDNA clones. This confirmed the presence of both the ORF1 AUG start codon at position 182–184 and the ORF1 UGA stop codon at position 743–745 as well as the three in-frame AUG start codons of ORF2 at positions 702–710 (see below).

The second ORF (RNA2-ORF2) encodes a predicted protein of 1198 aa with a molecular mass of 134 kDa. The first in-frame AUG of RNA2-ORF2 is at nt positions 702–704, and thus it partly overlaps with RNA2-ORF1. The first AUG is immediately followed by two other AUG start codons in frame. An UAA stop codon is found at nt positions 4296–4298. The ORF2 polyprotein contains a movement protein (MP) motif and shows low levels of similarity with viral coat proteins (CPs). The N-terminal region of the RNA2-ORF2 polyprotein most likely codes for the putative MP since a motif LRVPML highly

similar to the proposed movement protein consensus sequence LxxPxL (Mushegian, 1994) was found at aa position 262–267. No other sequence homologies were found in the N-terminus of the RNA2-ORF2.

To verify that the three CPs are encoded by the RNA2-ORF2, the three separated CPs were analysed using a tandem mass spectrometer (MS/MS). This resulted in aa sequences of small peptides, each of which was aligned with the aa sequence deduced from the RNA2 nucleotide sequence. Fragments of the largest CP (Vp35; ~35 kDa) could be aligned with an area in the RNA2-ORF2 between aa 487–729. Fragments of the Vp26 (~26 kDa) could be aligned with an area of RNA2-ORF2 between aa 730 and 983, while fragments of the smallest CP (Vp23; ~23 kDa) could be aligned with the C-terminus of RNA2-ORF2 (aa 983–1195). These results suggested that the coding sequences of the three CPs of ToTV are located in the RNA2-ORF2 and are in the order Vp35, Vp26 and Vp23 (Fig. 2.3). Thus, ORF2 potentially encodes four proteins which are likely to be cleaved from the polyprotein precursor by proteolytic cleavage. Since detailed alignments did not reveal any homologies with known protease cleavage sites, the exact cleavage site between the putative movement protein and Vp35 remains unknown.

On the basis of the protein sequencing results, the cleavage site between Vp35 and Vp26 is likely to be located in the aa region 730–733 (LRAQ) and that between Vp26 and Vp23 in the aa region 971–982 (KQPQVQVPLRDK) (Fig. 2.3b). However, no apparent homologies with known polyprotein cleavage sites were identified. Protease recognition sites in plant picorna-like viruses are known to be very diverse, and the exact positions of these cleavage sites remain to be determined experimentally.

For the aa positions 483–981 (Vp35 and Vp26) of RNA2-ORF2, a 21% identity was found with RTSV (AAB17090.1) between aa positions 614 and 930. The Vp35 aa sequence alone showed a 21% identity between aa positions 602 and 704 with human parechovirus (HPeV, genus *Parechovirus*; BAD05057.1). The closest identities of the Vp26 aa sequence were found with Rhopalosiphum padi virus (genus Cripavirus; NP_046156.1; with 25% identity between aa positions 750 and 917), avian encephalomyelitis virus (genus *Hepatovirus*; NP_653151.1; with 33% between aa positions 760 and 833), black queen cell virus (unassigned; AAF72338.1; with 43% identity between aa positions 786 and 822), and Solenopsis invicta virus (unassigned;

AAU85376.1; with 30% identity between aa positions 772 and 822). For the Vp23 aa sequence, no significant homologies were found.

5'- and 3'-untranslated regions (UTRs)

The 5'-UTR sizes of RNA1 and RNA2 are 106 nt and 181 nt, respectively. There is 31% overall sequence identity in the first 106 nucleotides. For the first 17 nt, both RNAs share a sequence identity of 82%.

The 3'-UTRs of RNA1 and RNA2 are 1210 nt and 1092 nt, respectively, in length and have an overall level of identity of 90.2%. Interestingly, however, the 988 most 3'-terminal nucleotides of both RNAs are almost perfectly conserved (98% identity). To confirm that the 3'-regions of the 3'-UTRs of both RNAs are nearly identical and to exclude cloning artefacts, RT-PCRs were performed on total viral RNA isolated from purified virions using one reverse primer derived from the identical 3'-UTR region (RNA1 at nt 7109–7128 and RNA2 at nt 4705–4724) and two forward primers located upstream of the 3'-UTR region and specific for either RNA1 (nts 6515–6534) or RNA2 (nts 4141–4161). This resulted in PCR fragments of the expected sizes. Sequence analysis of these RT-PCR fragments confirmed the presence of RNA1- or RNA2-specific sequences at their 5'-end, followed by nearly identical regions in the 3'-part of both 3'-UTRs.

Taxonomic position of ToTV

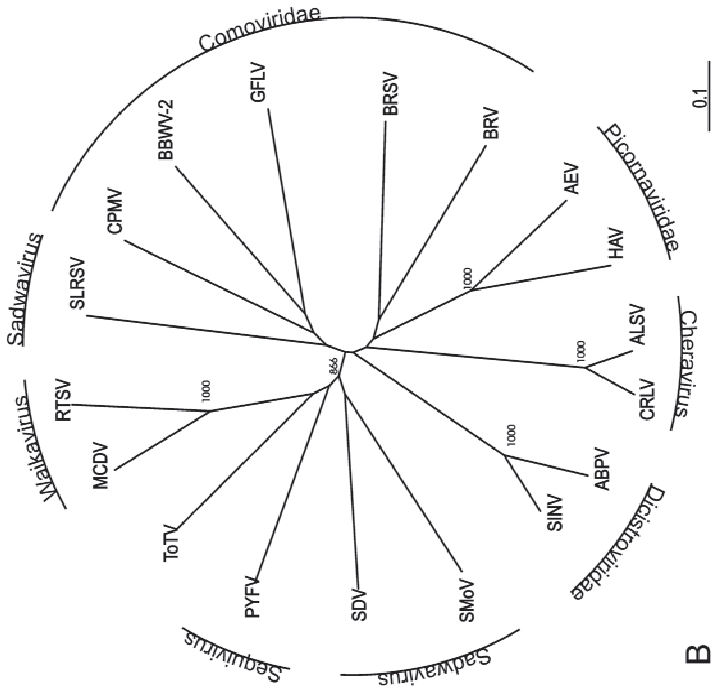
ToTV shares virion characteristics and sequence similarities with viruses of the genera *Sequivirus* and *Waikavirus* (family *Sequiviridae*) and the unassigned genera *Cheravirus* and *Sadwavirus*. Viruses assigned to these genera are distinguished on the basis of the number of genomic RNAs (members of the *Sequiviridae* have a monopartite genome, whereas those of chera- and sadwaviruses are bipartite) and the number of CPs (two in sadwaviruses vs. three CPs in sequi- and cheraviruses). The virus characterized in this study possesses icosahedral virions measuring 28 nm in diameter and containing three CPs, which encapsidate two single-stranded, positive-sense, polyadenylated RNAs. These data suggest that ToTV shares most structural characteristics with viruses of the genus *Cheravirus* (Le Gall et al., 2005).

The latest[1] ICTV report (Fauquet et al., 2005) states: *'Cheraviruses were previously considered as atypical but tentative members of the genus* Nepovirus *(family* Comoviridae*), but were distinguished on the basis of their genomic organization, in particular the number of CP species, as well as sequence homologies and, for some of them, natural transmission by insects'*. Both cheraviruses and sadwaviruses are now considered members of unassigned genera with affinities to members of the family *Sequiviridae*. So far, the genus *Cheravirus* contains two assigned species, CRLV (James and Upton, 2002; James and Upton, 2005; Thompson et al., 2004) and ALSV (Li et al., 2000), while a third virus (stocky prune virus; StPV) has recently been proposed as a possible new member of this genus (Candresse et al., 2006). The aa region between the CG protease motif (Bazan and Fletterick, 1988) and the GDD RdRp active site (Argos, 1988) in the RNA1-ORF1 is proposed to be a good taxonomic predictor for classifying picorna-like viruses (Ikegami et al., 2002). Because ToTV showed similarities to viruses of the picornavirus 'superfamily', this region of ToTV was used in a phylogenetic analysis including comparable regions of viruses from the genera *Sadwavirus*, *Cheravirus* and the families *Sequiviridae*, *Comoviridae*, *Dicistroviridae* and *Picornaviridae*. The resulting dendrogram (Fig. 2.5A) shows that ToTV does not cluster with cheraviruses. A similar phylogenetic analysis on the basis of the helicase region between the motifs A and C (Gorbalenya et al., 1990) (aa 382–495) confirms the separate taxonomic position of ToTV from the cheraviruses (Fig. 2.5B).

Interestingly, the 3'-UTRs of ToTV (1210 and 1092 nt) are much longer than those of the cheraviruses ALSV and CRLV and the proposed cheravirus StPV (246–145 nt). Moreover, the largest capsid protein of ToTV (Vp35) is significantly larger than that of cheraviruses (~25 KDa). StPV and CRLV are likely to be transmitted in a soil-borne fashion, most likely by nematodes (Candresse et al., 2006; Nyland et al., 1969; Stace-Smith and Hansen, 1976). The presence of ToTV in the field suggested an association with an insect vector, and preliminary experiments indicated the involvement of whiteflies in ToTV transmission (results not shown).

[1] As the article was written in 2007, the 'latest ICTV report' refers to the Eighth Report, published in 2005

Figure 2.5: Phylogenetic analysis of ToTV and related viruses based on the alignment of A) the region between the protease CG motif and GDD RdRp motif (aa 1041–1498 of RNA1-ORF1) and B) the helicase region between motifs A and C (aa 382–495 of RNA1-ORF1). Sequences included in the analysis are those of (with virus acronyms, genus and accession numbers in parentheses): acute bee paralysis virus (ABPV; unassigned species in the family *Dicistroviridae*; NP_066241), apple latent spherical virus (ALSV; *Cheravirus* NP_620568), avian encephalomyelitis virus (AEV; *Hepatovirus*; NP_653151), beet ringspot virus (BRSV; *Nepovirus*; NP_620112), blackcurrant reversion virus (BRV; *Nepovirus*; NP_612604), broad bean wilt virus-2 (BBWV-2; *Fabavirus*; AAK27841), cherry rasp leaf virus (CRLV; *Cheravirus*; YP_081444), cowpea mosaic virus (CPMV; *Comovirus*; NP_613283), grapevine fanleaf virus (GFLV; *Nepovirus*; NP_619689), hepatitis A virus (HAV; *Hepatovirus*; NP_041008), maize chlorotic dwarf virus (MCDV; *Waikavirus*; NP_619716), navel orange infectious mottling virus (NIMV; *Sadwavirus*; BAA74537), parsnip yellow fleck virus (PYFV; *Sequivirus*; NP_619734), rice tungro spherical virus (RTSV; *Waikavirus*; NP_042507), satsuma dwarf virus (SDV; *Sadwavirus*; NP_620566), Solenopsis invicta virus (SinV; unassigned species in the family *Dicistroviridae*; YP_164440), strawberry mottle virus (SMoV; *Sadwavirus*; NP_599086). strawberry latent ringspot virus (SLRSV; *Sadwavirus*; NC_006764), stocky prune virus (StPV; *Cheravirus*; DQ143874). Potato virus Y (PVY; *Potyvirus*; ABA28320) was used as an outgroup sequence in the analyses. The numbers at each node are the bootstrap values for 1000 replicates. The scale bar represents the number of residue substitutions per site


Comoviridae
GFLV
BBMV-2
CPMV
SLRSV
Sadwavirus
RTSV
Waikavirus
MCDV
ToTV
PYFV
SDV
SMoV
Sequiviridae
Sadwavirus
SINV
ABPV
Dicistroviridae
ALSV
CRLV
Cheravirus
HAV
AEV
Picornaviridae
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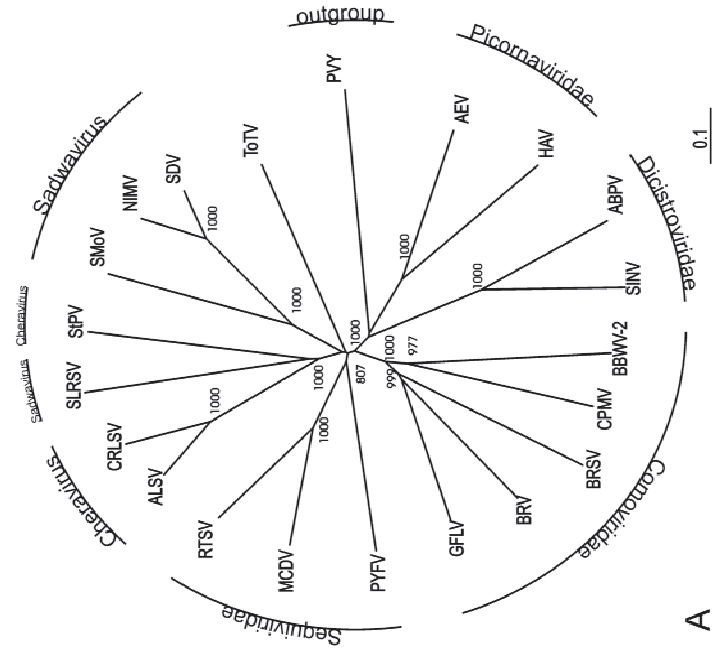
BRSV
0.1
B


outgroup
PVY
Picornaviridae
AEV
HAV
ToTV
SDV
NIMV
Sadwavirus
SMoV
SlPV
Cheravirus
SLRSV
Sadwavirus
CRLSV
ALSV
Cheravirus
RTSV
MCDV
PYFV
Sequiviridae
GFLV
BRV
BRSV
CPMV
BBMV-2
Comoviridae
SINV
ABPV
Dicistroviridae
0.1
A

The data presented in this paper identify ToTV as a new picorna-like virus naturally infecting tomato. Although it shares the same number of genomic RNA molecules (i.e. two) and capsid proteins (i.e. three) with viruses from the genus *Cheravirus*, its sequence characteristics clearly separate it from members of this and other plant virus genera. Therefore, ToTV most likely represents a member of a new plant virus genus.

Acknowledgements

We thank Dr A. H. P. America and Dr J. H. G. Cordewener (Plant Research International,Wageningen, The Netherlands) for the analysis of the coat proteins to determine partial amino acid sequences.

Chapter 3

Characterization of three new torradoviruses and the expansion of the newly formed genus *Torradovirus*

Section A: *Tomato marchitez virus* (ToMarV)

Section B: Tomato chocolàte virus (ToChV)

Section C: Lettuce necrotic leaf curl virus (LNLCV)

Chapter 3, section A

Tomato marchitez virus, a new plant picorna-like virus from tomato related to tomato torrado virus

M. Verbeek[1], A. M. Dullemans[1], J. F. J. M. van den Heuvel[2],

P. C. Maris[2], and R. A. A. van der Vlugt[1]

[1]Plant Research International, part of Wageningen UR, Wageningen, The Netherlands

[2]De Ruiter Seeds, currently known as Monsanto Vegetable Seeds Division, Bergschenhoek, The Netherlands

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Summary

A new virus was isolated from a tomato plant from the state of Sinaloa in Mexico. This plant showed symptoms locally known as 'marchitez disease': severe leaf necrosis, beginning at the base of the leaflets, and necrotic rings on the fruits. A virus was isolated from the infected plant consisting of isometric particles with a diameter of approximately 28 nm. The viral genome consists of two (+)ssRNA molecules of 7221 (RNA1) and 4898 nts (RNA2). The viral capsid contains three coat proteins of 35, 26 and 24 kDa, respectively. The abovementioned characteristics: symptoms, morphology, number and size of coat proteins, and number of RNAs are similar to those of the previously described tomato torrado virus (ToTV). Sequence analysis of the entire viral genome shows that this new virus is related to, but distinct from, ToTV and that these members of two obviously new virus species belong to the recently proposed plant virus genus Torradovirus. For this new virus, the name tomato marchitez virus (ToMarV) is proposed.

Nucleotide sequence data reported are available in the GenBank database under accession numbers EF681764 (RNA1) and EF681765 (RNA2).

Introduction

In 2003 a new disease was found in tomatoes grown in the fields and screenhouses in the state of Sinaloa in Mexico. Typical symptoms observed were leaf necrosis, beginning at the base of the leaflets (Fig. 3.1A), as well as dark necrotic rings on the fruits, already visible on the unripe green fruits (Fig. 3.1B). In severely infected plants, the top of the plant showed necrosis and malformation. Economic losses are substantial due to the inhibited growth of the tomato plant and to the unmarketable fruits. Locally, this disease is known as 'marchitez', meaning wilted or withered.

Because of the resemblance in leaf symptoms, it was initially assumed that Marchitez disease was caused by the recently described tomato torrado virus (ToTV) (Verbeek et al., 2007). However, ToTV could not be detected in tomato plants showing symptoms of Marchitez disease with the aid of ToTV-specific primers in PCR (Verbeek et al., 2007). In infected material, a spherical virus was found that shares several characteristics with ToTV. In this paper, we describe and characterize this new virus, related to but clearly distinct from ToTV, which we tentatively name tomato marchitez virus (ToMarV), a member of the newly proposed genus Torradovirus.

Material and methods

Virus transmission and propagation

ToMarV was isolated in 2005 from a tomato plant from Culiacán, Sinaloa, Mexico, that showed severe necrosis. The isolate was designated PRI-TMarV0601. Using 0.03M sodium/potassium phosphate buffer, pH 7.7, the virus was mechanically inoculated to and maintained in *Nicotiana glutinosa* 'PRI', *Physalis floridana* or *N. benthamiana*.

Virus purification

All centrifugation steps were carried out at 6 ^{o}C. Systemically infected leaves of *N. benthamiana* were harvested approximately 14 days post-inoculation, homogenized in 5 volumes (w/v) extraction buffer (0.1 M Tris–HCl, pH 8, containing 20 mM Na_2SO_3, 10 mM Na-DIECA and 5 mM Na-EDTA) and squeezed through cheesecloth. Three volumes of a 1:1 mixture of chloroform/butanol were added and, after mixing, the suspension was

centrifuged at 16,500 g for 10 min. Triton X-100 was added up to a concentration of 1% to the water phase and the suspension was stirred for 30 min. Then, PEG 6000 and NaCl were added to concentrations of 5% and 2.3%, respectively, and solubilized by stirring for 1 h. The suspension was left to settle for 1 h and centrifuged for 15 min at 21,500 g. The pellets were

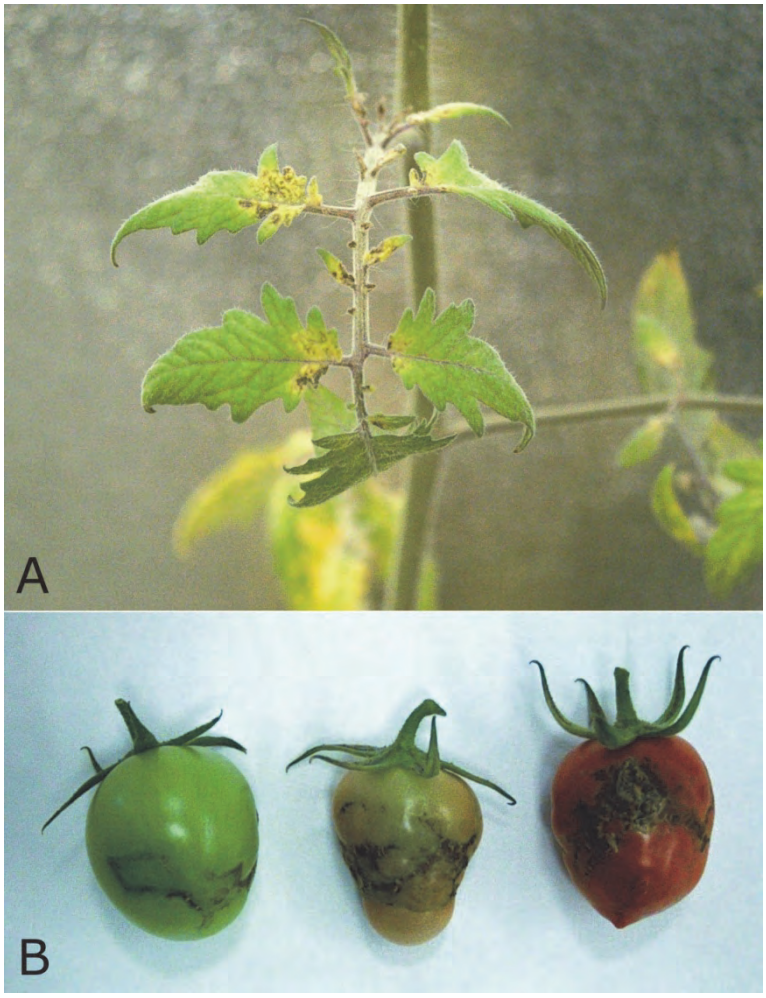


Figure 3.1: Typical symptoms of tomato marchitez virus in (A) tomato leaves: necrosis surrounded by a yellow or bright green area, beginning at the base of the leaflets, and (B) tomato fruits: necrotic rings and patches

resuspended in a total of 80 ml extraction buffer and centrifuged for 10 min at 15,000 g, and the supernatant was placed on a 30% sucrose cushion. After centrifugation for 3 h at 70,500 g, the pellets were resuspended in a total of 2 ml Tris buffer, pH 8.0, and centrifuged for 2 min at 14,000 g. The virus suspension was loaded onto a 10–40% cesium sulfate gradient and centrifuged for 16 h at 126,000 g. Virus bands were collected and dialyzed against 0.1M Tris–HCl, pH 8.0.

Electron microscopy

Virus suspensions were mounted on formvar-carbon coated grids, stained with 2% uranyl acetate and examined using a Philips CM12 electron microscope.

Polyacrylamide gel electrophoresis

Viral proteins were separated by subjecting purified virus particles to 12% denaturing polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970), and visualized by silver staining.

Nucleic acid isolation and evaluation

Purified virus was concentrated by centrifugation (at 115,000 g for 2 h). RNA was extracted from pelleted virus particles using a Qiagen RNeasy kit (Qiagen) according to the manufacturer's instructions. The RNA concentration was determined in a Beckmann DU 530UV-spectrophoto-meter. Viral RNA integrity and size were checked on a 1% agarose gel using a formaldehyde/formamide/HEPES buffer system. After electrophoresis, the RNA was stained using ortho-toluidine blue. Total RNA for RT-PCR was isolated from ToMarV infected *Nicotiana occidentalis* 'P1' plants using an RNeasy plant mini kit (Qiagen).

RT-PCR and 5' RACE

PCR fragments were obtained by one-tube RT-PCR (Access RT-PCR system, Promega). RT-PCRs were initiated using a universal oligo(dT) primer (Van der Vlugt et al., 1999) and various primers derived from the ToTV RNA1 and RNA2 sequences (GenBank accession numbers DQ388879 and DQ388880, respectively). The 5' regions of the ToMarV RNAs were determined by walking

towards the 5' end of the viral genome through repeated use of a 5' RACE kit (Roche) in combination with the Expand high-fidelity PCR system (Roche), essentially as described previously (Ongus et al., 2004; Valles et al., 2004). cDNA primers for the 5'-RACE strategy and primer sets for additional RT-PCR reactions were based on newly obtained ToMarV sequence data.

Nucleotide sequencing and sequence analysis

All PCR products (also those resulting from the 5' RACE) were purified using the QIAquick PCR Purification Kit (Qiagen) and directly sequenced. Sequence analysis was performed with an Applied Biosystems 3100 Genetic Analyser, using a DYEnamic ET Terminator Cycle Sequencing Kit (Amersham) and the primers that were used for amplification. For longer PCR fragments, ToMarV-specific primers were used for primer walking sequencing. Nucleotide and amino acid sequence data were analyzed and assembled using the DNASTAR package (Lasergene). Sequence comparisons with other viruses were performed with programs from the PHYLIP package. Multiple alignments and phylogenies were performed with the CLUSTAL X program after bootstrapping in 1000 replicates. Neighbourjoining consensus phylogenies were viewed using the NJplot program (Thompson et al., 1997) and printed by using TreeView (Page, 1996).

Results and discussion

Virus characterization

ToMarV was easily transmitted to a number of indicator plants by mechanical inoculation. Table 3.1 gives an overview of the experimental hosts used and their reactions to ToMarV and ToTV. Most symptoms of ToMarV and ToTV in indicator plants resemble each other, except for the reactions in two host plants. In *P. floridana*, ToMarV induces a systemic mottle and only occasionally necrosis in the locally infected leaves, whereas ToTV causes severe necrosis and die-off in this plant. In *Chenopodium quinoa*, ToMarV causes necrotic pin point lesions in inoculated leaves which remain symptomless when inoculated with ToTV.

Table 3.1: Symptoms of tomato marchitez virus (ToMarV) and tomato torrado virus (ToTV) on experimental host plants.

Tested experimental host plants	**Symptoms (local/systemic)**	
	ToMarV	**ToTV**
Chenopodium quinoa	nl / noi	- / noi
Gomphrena globosa	n.t.	- / noi
Nicotiana benthamiana	- / c, mf	- / c, mf
Nicotiana clevelandii	- / c	- / c
Nicotiana glutinosa 'PRI'	occasionally cl / c	- / c
Nicotiana hesperis '67A'	nl / c, n, mf	nl / c, n, mf
Nicotiana occidentalis 'P1'	nl / c, n, mf	nl / c, n, mf
Nicotiana rustica	- / la	- / la
Nicotiana tabacum 'White Burley'	occasionally cl / la	- / la
Physalis floridana	nl / mo	nl / c, n, mf, do

c: chlorosis; cl: chlorotic lesions/rings; do: die off; la: latent infection (verified by inoculation on indicator plants); mf: malformation; mo; mottle; n: necrosis; noi: no infection (verified by inoculation on indicator plants); nl: necrotic lesions; n.t.: not tested; -: no symptoms.

Electron microscopic examinations of leaf extracts from tomato showing marchitez symptoms and from systemically infected leaves of *Nicotiana occidentalis* 'P1' revealed the presence of spherical virus particles with a diameter of 28–30 nm. The particles of ToMarV clearly resembled the particles of ToTV in shape and size.

In initial attempts at ToMarV purification, the protocol designed for ToTV was used (Verbeek et al., 2007). This protocol did not lead to visible virus bands in the final Cs_2SO_4 gradient. Electron microscopic analysis of gradient fractions revealed that virus particles were present in the bottom part of the gradient. However, this part of the gradient also contained plant constituents, veiling the virus bands. Therefore, another purification protocol was designed in which the separation of the virus from plant components was aided by the use of a chloroform-butanol mixture. This protocol resulted in one diffuse band in the Cs_2SO_4 gradient. This result is in contrast with that obtained for ToTV purifications, which always yielded two distinct bands following Cs_2SO_4 gradient centrifugation. The ToMarV band was collected from the gradient and

examined by electron microscopy. Virions of the expected size of 28 nm were present in the collected band and were infectious when mechanically inoculated to test plants. Purified virions were also mechanically transmitted to tomato plants, which showed characteristic symptoms of marchitez disease two weeks after inoculation. The presence of ToMarV in these plants was confirmed by electron microscopy and RT-PCR.

Purified virions were subjected to SDS-PAGE, and three viral proteins were detected with estimated sizes of 35, 26 and 24 kDa, named Vp35, Vp26 and Vp24, respectively (Fig. 3.2A). The number and estimated molecular sizes of the viral coat proteins of ToMarV are the same as previously found for ToTV (Verbeek et al., 2007).

When purified virus preparations of ToMarV were analysed on a denaturing RNA gel, two RNA molecules with estimated sizes of 7.5 kb (RNA1) and 4 kb (RNA2) were visualized (Fig. 3.2B). The number of RNAs found is in accordance with ToTV, but their estimated sizes are smaller (ToTV RNA1 [8.5 kb] and RNA2 [5.5 kb]).

Viral RNA analysis

Based on biological and structural data like indicator plant symptoms, particle sizes and morphology, number and sizes of coat proteins, and number of RNAs obtained for ToMarV, a possible relationship with ToTV was suspected. Therefore, different upstream primers were derived from the ToTV RNA1 (DQ388879) and RNA2 (DQ388880) sequences and used in combination with a general oligo(dT) primer for RT-PCR. This resulted in a limited number of PCR fragments, indicating possible differences in RNA sequences between the two viruses. Sequence analyses of these fragments revealed low levels of similarity with the ToTV RNAs 1 and 2. Based on the obtained sequence information, new cDNA primers were generated and used to obtain additional sequence information in a 5'-RACE sequence-walking strategy.

ToMarV-specific primers derived from newly obtained sequences were used for RT-PCR to confirm sequences of both RNAs in two orientations.

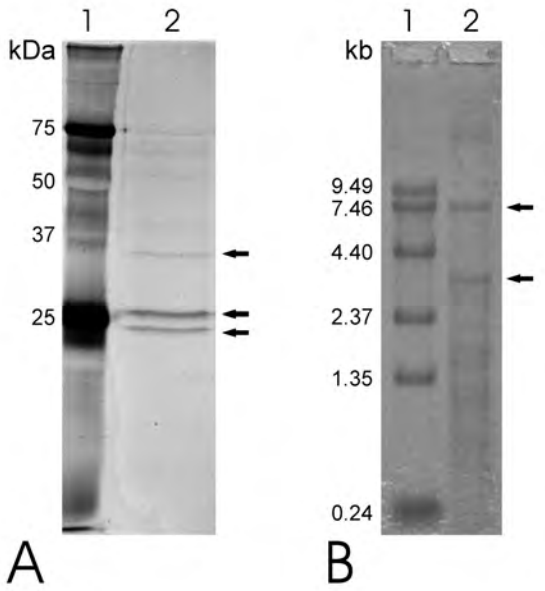


Figure 3.2: (A) Denaturing polyacrylamide gel electrophoresis (SDS-PAGE) of ToMarV capsid proteins. Proteins were visualized by silver staining. 1 Molecular weight markers (Bio-Rad Precision Plus Protein Standards (note: the prestained 37-kDa and 50-kDa standards do not stain in the silver staining method used); 2 ToMarV purified virions after Cs_2SO_4 buoyant density gradient centrifugation. Arrows indicate positions of the coat protein bands. (B) Denaturing agarose gel electrophoresis of RNA extracted from ToMarV virions and stained with ortho-toluidine blue. 1 Molecular size standard (Invitrogen 0.24–9.5-kb RNA Ladder); 2 RNA purified from ToMarV virions. Arrows indicate positions of the RNA bands

RNA1

RNA1 [7221 nucleotides (nts), excluding poly(A) tail] contains one open reading frame (RNA1-ORF1) of 6453 nts encoding a predicted polyprotein of 2151 amino acids (aa) with a molecular mass of 237 kDa (Fig. 3.3). The first in-frame AUG is found at nt positions 141–143. The ORF has an UGA stop codon at positions 6594–6596. The putative polyprotein sequence contains several conserved regions with motifs typical for helicase and RNA-dependent RNA polymerase (RdRp). The ORF1-encoding region shows 65% overall identity with the ToTV-RNA1 (DQ388879) at both the nucleotide level (6474 nts) and the amino acid level (2158 aa).

Typical helicase motifs A (GKS), B (D), C (N) were identified at aa positions 397–399, 443 and 494 of the putative ORF1-encoded protein. The RdRp region could be identified between aa 1305 and 1553 by the presence of the typical motifs I (KDE) to VII (FLSR) (Koonin, 1991).

The helicase region between motifs A and C in the C-terminal part of RNA1-ORF1 showed 95.6% aa identity with the corresponding region of ToTV and significantly lower levels of identity with other viruses ranging from 46.9% for maize chlorotic dwarf virus (MCDV, genus *Waikavirus*, AAV86083) to 25.4% for acute bee paralysis virus (ABPV, unassigned species in the family *Dicistroviridae*, NP_066241).

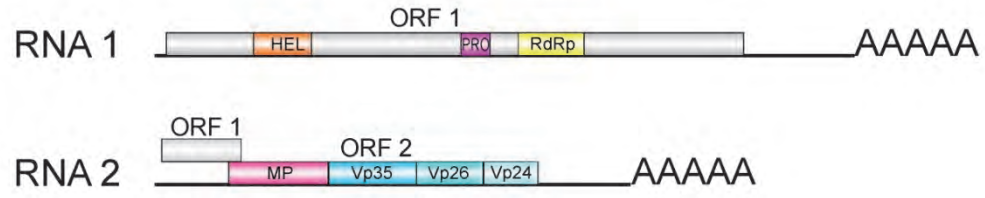


Figure 3.3: Genome organization of tomato marchitez virus. Relative positions of regions containing motifs of helicase (HEL), protease (Pro), and RNA-dependent RNA polymerase (RdRp) on RNA1 and of the putative movement protein (MP) and the three coat proteins (Vp35, Vp26 and Vp24) encoded on RNA2 are indicated

Levels of aa identity for the RdRp region, motifs I to IV in ORF1 of RNA1, were relatively high with ToTV (85%) and significantly lower with other viruses: 40% for cherry rasp leaf virus (CRLV, genus *Cheravirus*, YP_081444) to 38% for strawberry latent ringspot virus (SLRSV, genus *Sadwavirus*, NC_006764). These levels of identity indicate a close level of relationship between ToMarV and ToTV.

A BLAST search with our available data for ToMarV RNA1 revealed a remarkably high level of overall sequence identity (95%) with a partial sequence in the NCBI data base submitted under the name of tomato apex necrosis virus (ToANV, accession number EF0636641), which only became available during the course of our work. In the coding region of RNA1, the overall level of identity between ToMarV and ToANV is 96% at the nt level and 99% at the protein level with levels of aa sequence identity of 100% for both the RdRp and helicase motifs. These percentages suggest a close relationship between ToMarV and the partial sequence deposited as ToANV RNA1.

RNA2

As with ToTV, RNA2 [4898 nts, excluding poly(A) tail] of ToMarV contains two open reading frames (RNA2-ORF1 and RNA2-ORF2) encoding predicted polyproteins of 190 and 1191 amino acids (aa) with molecular masses of 21 and 131 kDa, respectively (Fig. 3.3). The first in-frame AUG is at position 139–141. It is likely that this start codon maps the start of ORF1 given the lack of significant sequence identity of the ToMarV sequence directly upstream of this AUG start codon with the 5'-UTR of ToTV and the significant levels of aa sequence identity with ORF1 of ToTV just downstream from this AUG start. The ORF has an UAA stop codon at positions 709–711, implying a partial overlap with ORF2. The overall level of sequence identity between ORF1 of ToMarV and ToTV is around 63% at both the nt level (570 nts) and the aa level (190 aa). A BLAST search revealed no significant homologies with other viruses either at the nt or the aa level.

For the second ORF (RNA2-ORF2), the first in-frame AUG is at position 709–711. A UAA stop codon is found at nt positions 4244–4246. ORF2 encodes a putative protein of 1191 aa. This ORF shows 66.1% identity with the ToTV RNA2-ORF2 (DQ388880) at the nt level (3573 nts) and 69.9% at the aa level (1191 aa). The only other significant similarity is found with the incomplete sequence of ToANV RNA2 (EF0636642). In comparable regions of ToMarV RNA2-ORF2, the nt and aa sequence identities are 78% and 90%, respectively.

By analogy with ToTV, the RNA2-ORF2-encoded protein is likely to be a polyprotein coding for a putative movement protein (MP) and three structural coat proteins (CPs). Indeed the N-terminal region of the RNA2-ORF2

polyprotein most likely codes for an MP since the motif LRVPTL, which is highly similar to the proposed movement protein consensus sequence LxxPxL (Mushegian, 1994), was found at aa position 263–268. The overall level of identity in the movement protein region (aa 1–470) between ToMarV and ToTV is 62.1%.

The relative order of the three putative coat proteins of ToMarV in the RNA2-ORF2 was derived from direct aa sequence comparisons with the ToTV CPs. The first CP (ToMarV-Vp35; aa 477–718) shows 72% identity with Vp35 of ToTV, the second CP (ToMarV-Vp26; aa 723–959) shows 86% identity with Vp26 of ToTV, and the third CP (ToMarV-Vp24; aa 975–1191) shows 71% identity with Vp23 of ToTV.

From direct aa sequence comparison of the ToMarV CP region with the corresponding region of the ToANV RNA2 it is likely that the relative order of the ToANV CPs is similar to that of ToMarV and ToTV. The ToANV CPs of 38.4, 28 and 23.8 kDa (Turina et al., 2007) show aa sequence identities of 89%, 98% and 94% with ToMarV Vp35, Vp26 and Vp24, respectively. In ToTV we identified putative cleavage regions between the different proteins encoded on ORF2 on the basis of direct protein sequencing of CPs (Verbeek et al., 2007). No homologous regions were apparent in the ToMarV ORF2, and no homologies with known protease cleavage sites were found, leaving the exact polyprotein cleavage sites to be determined.

Since the RNA2 sequence 5' of the three putative CPs of ToANV, including the putative movement protein region and the ORF1 is not available, no direct comparison with this region of ToMarV could be made.

5'- and 3'-untranslated regions (UTRs)

The 5'-UTR sizes of RNA1 and RNA2 are 140 and 138 nts, respectively, and share an overall level of identity of 58%, with the eight 5'-terminal nucleotides of RNA1 and RNA2 being identical.

The 3'-UTRs of RNA1 and RNA2 are 628 and 655 nts in length, respectively, and share an overall level of identity of 91%. The 553 most 3'-terminal nucleotides of both RNAs are almost perfectly conserved (99% identity). The nearly identical regions in the 3'-part of both 3'-UTRs are a characteristic that ToMarV shares with ToTV and the sequence of ToANV. However, direct sequence comparisons between the total 3'-UTRs of RNA1

and RNA2 of ToMarV and ToTV reveals only 49.0% and 48.9% sequence identity, respectively, between the two viruses, with a large difference in length of the 3'-UTRs of ToMarV (628 and 655 nts) and ToTV (1210 and 1092 nts). A comparison with the 3'-UTRs of ToMarV and ToANV RNA1 (630 nts) and RNA2 (650 nts), showed 88.5% and 85.6% sequence identity.

Taxonomic position of ToMarV

Tomato marchitez virus (ToMarV) showed similar host plant symptoms and shared virion characteristics and its genome organization with ToTV, but based on levels of nt and aa sequence identities, the two viruses should be considered related but distinct. Remarkably high levels of identity were observed between both RNAs of ToMarV and incomplete RNA sequences deposited for ToANV in the NCBI database. Only after submission of our paper did additional information on ToANV become available (Turina et al., 2007). In this paper, ToANV is reported to also have three CPs of 38.4, 28 and 23.8 kDa, respectively. These correlate relatively well with the CP sizes of ToMarV (35, 26 and 24kDa, respectively). The most distinctive symptoms for ToANV were described as necrosis of growing points, resulting in a brown brittle dieback. For ToMarV, leaf necrosis starting at the base of the leaflet was the most distinctive symptom, and necrosis of growing points was never observed upon back-inoculation on tomato plants using Cs_2SO_4 gradient purified virus. ToANV has been reported to induce mild mosaic symptoms on *N. clevelandii* and *N. tabacum* cv. 'White Burley', while ToMarV causes systemic chlorosis in *N. clevelandii* and a latent systemic infection in *N. tabacum* cv. 'White Burley'.

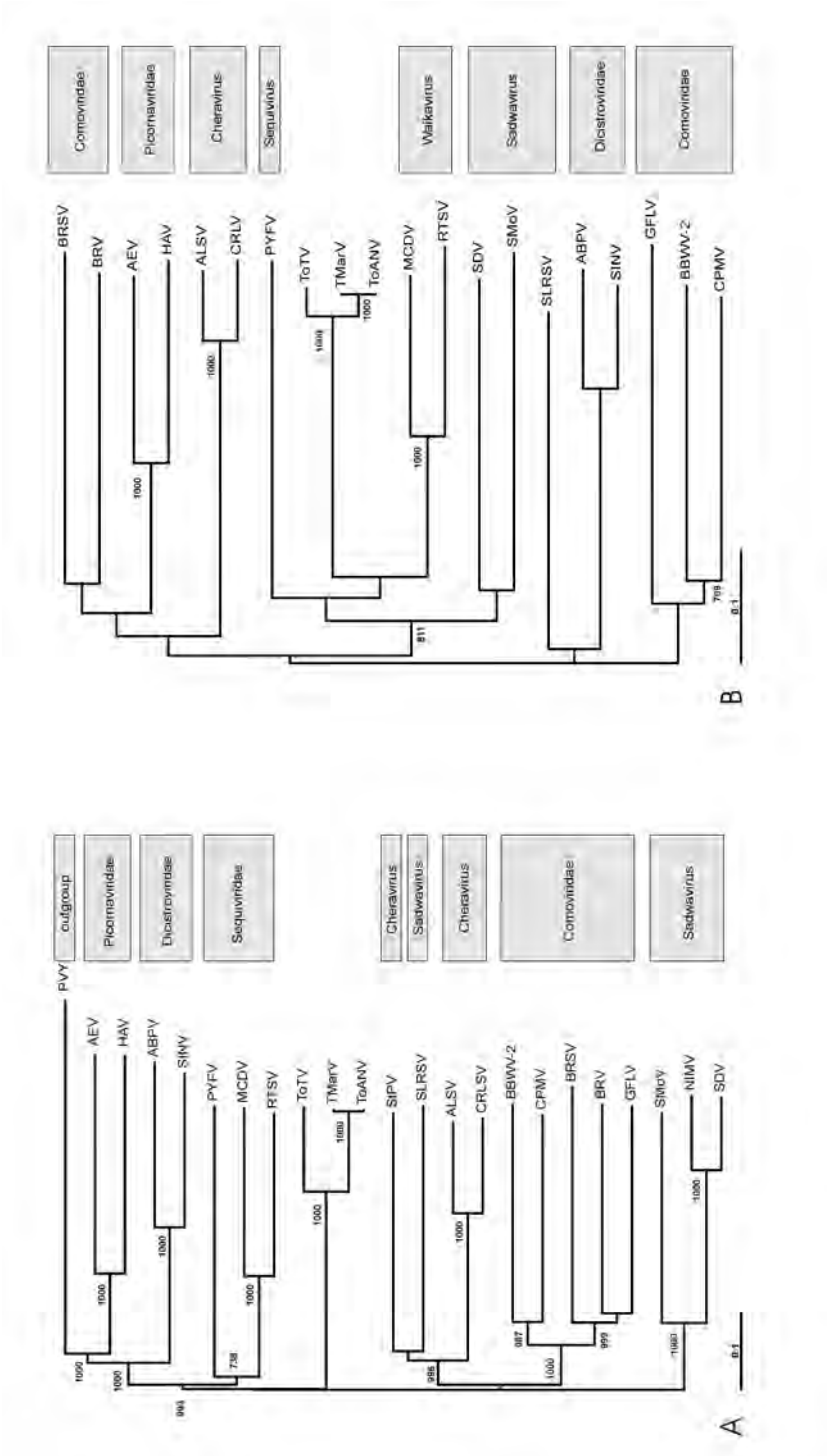
In addition, the relatively low levels of nucleotide sequence identity (less than 90%) between the two viruses in the 3'-UTRs of both RNA1 and RNA2 (88.5% and 85.6%, respectively) and in the largest putative CP (Vp35) (89%) suggest that the two viruses are not identical. They may be strains or isolates of the same virus species. Additional biological and molecular data on ToANV, especially the possible presence of ORF1 on RNA2, will be needed to determine its precise relationship to ToMarV.

Phylogenetic analysis based on the aa region between the CG protease motif (Bazan and Fletterick, 1988) and the GDD RdRp active site (Argos, 1988) in the RNA1–ORF1 were performed to determine the relationships between ToMarV, ToTV, ToANV and other viruses from the genera *Sadwavirus*,

Cheravirus and the families *Sequiviridae*, *Comoviridae*, *Dicistroviridae* and *Picornaviridae*. This region is proposed to be a good taxonomic predictor for classifying picorna-like viruses (Ikegami et al., 2002). The resulting dendrogram (Fig. 3.4A) shows that ToMarV clusters with ToTV and ToANV in a distinct branch. It also confirms the separate taxonomic position of these viruses from members of the genus *Cheravirus* (Le Gall et al., 2005; Verbeek et al., 2007), the only other related plant virus genus whose members have 3 CPs and 2 RNAs. A similar phylogenetic analysis on the basis of the helicase region between the motifs A and C (Gorbalenya et al., 1990) (aa 397–494) confirms the separate taxonomic position of ToMarV, ToTV and ToANV (Fig. 3.4B).

The data we present in this paper describe ToMarV as a new picorna-like plant virus, related to but distinct from ToTV (Verbeek et al., 2007). ToMarV and ToTV both share two ORFs on RNA2, which is a unique feature that clearly distinguishes them from other plant picorna-like viruses. These two viruses are likely to belong to the same new genus for which the name Torradovirus was recently proposed by the ICTV study group on plant picorna-like viruses.

Figure 3.4: Phylogenetic analysis of ToMarV and related viruses based on the alignment of (A) the region between the protease CG motif and the GDD RdRp motif (aa 1042–1499 of RNA1–ORF1) and (B) the helicase region between motifs A and C (aa 381–494 of RNA1–ORF1). Sequences included in the analysis are those of (with virus acronyms, genus and accession numbers in parentheses): acute bee paralysis virus (ABPV; unassigned species in the family *Dicistroviridae*; NP_066241), apple latent spherical virus (ALSV; *Cheravirus* NP_620568), avian encephalomyelitis virus (AEV; *Hepatovirus*; NP_653151), beet ringspot virus (BRSV; *Nepovirus*; NP_620112), blackcurrant reversion virus (BRV; *Nepovirus*; NP_612604), broad bean wilt virus-2 (BBWV-2; *Fabavirus*; AAK27841), cherry rasp leaf virus (CRLV; *Cheravirus*; YP_081444), cowpea mosaic virus (CPMV; *Comovirus*; NP_613283), grapevine fanleaf virus (GFLV; *Nepovirus*; NP_619689), hepatitis A virus (HAV; *Hepatovirus*; NP_041008), maize chlorotic dwarf virus (MCDV; *Waikavirus*; NP_619716), navel orange infectious mottling virus (NIMV; *Sadwavirus*; BAA74537), parsnip yellow fleck virus (PYFV; *Sequivirus*; NP_619734), rice tungro spherical virus (RTSV; *Waikavirus*; NP_042507), satsuma dwarf virus (SDV; *Sadwavirus*; NP_620566), Solenopsis invicta virus (SinV; unassigned species in the family *Dicistroviridae*; YP_164440), strawberry mottle virus (SMoV; *Sadwavirus*; NP_599086). strawberry latent ringspot virus (SLRSV; *Sadwavirus*; NC_006764), stocky prune virus (StPV; *Cheravirus*; DQ143874), tomato torrado virus (ToTV; ABD38934), tomato apex necrosis virus (ToANV; ABK33525). Potato virus Y (PVY; *Potyvirus*; ABA28320) was used as an outgroup sequence in the analyses. The numbers at each node are the bootstrap values for 1000 replicates (values below 700 are not shown). The scale bar represents the number of residue substitutions per site


Comoviridae
Picornaviridae
Cheravirus
Sequivirus
Waikavirus
Sadwavirus
Dicistroviridae
Comoviridae
BRSV
BRV
AEV
HAV
ALSV
CRLV
PYFV
ToTV
TMarV
ToANV
MCDV
RTSV
SDV
SMoV
SLRSV
ABPV
SINV
GFLV
BBWV-2
CPMV
B
outgroup
Picornaviridae
Dicistroymvirus
Sequiviridae
Cheravirus
Sadwavirus
Cheravirus
Comoviridae
Sadwavirus
PVY
AEV
HAV
ABPV
SINV
PYFV
MCDV
RTSV
ToTV
TMarV
ToANV
SlPV
SLRSV
ALSV
CRLSV
BBWV-2
CPMV
BRSV
BRV
GFLV
SMoV
NIMV
SDV
A

Chapter 3, section B

Tomato chocolàte virus: a new plant virus infecting tomato and a proposed member of the genus *Torradovirus*

M. Verbeek[1*], A. M. Dullemans[1*], J. F. J. M. van den Heuvel[2],

P. C. Maris[2], and R. A. A. van der Vlugt[1]

* Joint first authors

[1] Plant Research International, part of Wageningen UR, Wageningen, The Netherlands

[2] De Ruiter Seeds, currently known as Monsanto Vegetable Seeds Division, Bergschenhoek, The Netherlands

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Abstract

A new virus was isolated from a tomato plant from Guatemala showing necrotic spots on the bases of the leaves and chocolate-brown patches on the fruits. Structural and molecular analysis showed the virus to be clearly related to but distinct from the recently described *Tomato torrado virus* (ToTV) and *Tomato marchitez virus* (ToMarV), both members of the genus *Torradovirus*. The name tomato chocolàte virus is proposed for this new torradovirus.

Nucleotide sequence data reported are available in the GenBank database under accession numbers FJ560489 (RNA1) and FJ560490 (RNA2).

In 2007 tomato plants (*Solanum lycopersicum* L.) showing basal leaf necrosis and chocolate-brown spots on the fruits were sampled in the vicinity of Guatemala City, Guatemala. Locally the symptoms on the tomatoes were referred to as 'Chocolàte'. Electron microscopy on leaf material with typical symptoms showed the presence of spherical virus particles of 28–30 nm in diameter. The virus was mechanically transmissible to tomato cv. 'Moneymaker', *Physalis floridana* and several *Nicotiana* species including *N. hesperis* '67A' and *N. benthamiana*. Following a slightly modified purification protocol described earlier for *Tomato torrado virus* (ToTV) (Verbeek et al., 2007) the virus was purified from *N. hesperis* '67A' for further analysis.

Purified virus was inoculated to tomato plants cv. 'Moneymaker' in which it induced symptoms identical to those initially observed in tomato fields in Guatemala. The virus could be isolated from those back-inoculated plants, thus fulfilling Koch's postulates.

The isolate was tentatively designated tomato chocolàte virus isolate G01 (ToChV-G01) and deposited in the DSMZ Safe Deposit in Braunschweig, Germany, under accession number DSM 22139.

When purified RNA preparations of ToChV were analysed on a denaturing RNA gel (Verbeek et al., 2007), two RNA molecules with estimated sizes of 8 kb (RNA1) and 6 kb (RNA2) were observed (results not shown). Subjecting the purified virions to a denaturing polyacryl amide gel electrophoresis we observed three coat proteins of approximately 35, 26 and 24 kDa (Vp35, Vp26 and Vp24, respectively). Based on biological and structural data like symptomatology, particle size and morphology, number and molecular mass of coat proteins, and number of RNAs obtained for ToChV, a possible relationship with viruses from the genus Torradovirus (ToTV and *Tomato marchitez virus*; ToMarV (Verbeek et al., 2008) was suspected. Therefore, different upstream primers were derived from ToTV (DQ388879 and DQ388880) as well as ToMarV (EF681764, EF681765, EF063641 and EF063642) sequences and used in combination with a general oligo(dT) primer in a one-tube RT-PCR procedure (Superscript III One-Step RT-PCR system with platinum Taq High Fidelity, Invitrogen) on RNA isolated from

purified virus particles. This resulted in a limited number of PCR products. Sequence analyses of the amplicons indeed revealed low levels of identity with the ToTV and ToMarV RNAs. Based on the obtained sequence information, new ToChV-specific cDNA primers were generated and used to obtain additional sequence information in a 5'-RACE sequence-walking strategy. ToChV-specific primers were used for RT-PCR to confirm sequences of the two RNAs in both orientations.

Nucleotide and amino acid sequence data were analysed and assembled using the DNASTAR package V8 (Lasergene). Sequence comparisons with other viruses were performed with programs from the PHYLIP package. Multiple alignments and phylogenies were performed with the CLUSTALX program after bootstrapping in 1,000 replicates. Neighbour-joining consensus phylogenies were viewed using the NJplot program (Thompson et al., 1997) and printed using TreeView (Page, 1996).

RNA1 [7,474 nucleotides (nts), excluding the poly(A) tail] contains one open reading frame (RNA1-ORF1) of 6,465 nts encoding a predicted polyprotein of 2,155 amino acids (aa) with a molecular mass of 237 kDa. The 5'- and 3'-untranslated regions (UTR) of RNA1 are 135 and 871 nts in length, respectively. RNA2 of ToChV [5,695 nts, excluding the poly(A) tail] contains two open reading frames (RNA2-ORF1 and RNA2-ORF2) encoding predicted polyproteins of 189 and 1192 aa with molecular masses of 21 and 131 kDa, respectively. The 5'- and 3'-UTRs of RNA2 are 181 and 1,406 nts in length, respectively. The genomic organization is analogous to ToTV and ToMarV (Verbeek et al., 2007, 2008) with helicase, protease and RNA-dependent RNA polymerase (RdRp) located on RNA1, and a putative movement protein and three coat proteins (Vp35, Vp26 and Vp24) located on RNA2 (Fig. 3.5).

While writing this report the complete sequence of a virus designated tomato chocolate spot virus (ToChSV) became available through GenBank. Like ToChV, ToChSV was reported to be isolated from tomatoes grown in Guatemala. This virus has two ssRNA molecules of 7,492 nts (RNA1: GQ305131) and 5,109 nts (RNA2: GQ305132) with a similar genome

organization as the other three torradoviruses including the typical ORF1 on RNA2.

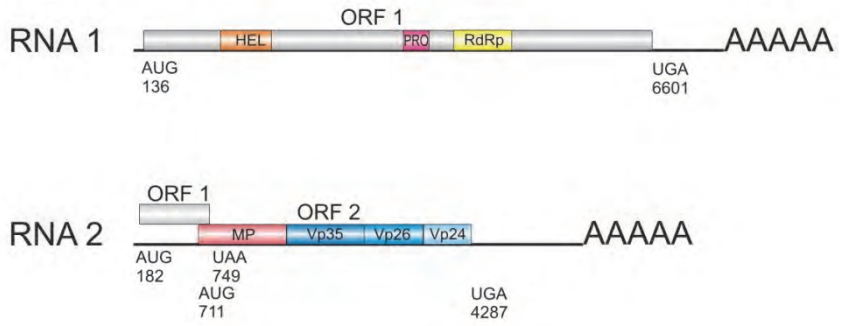


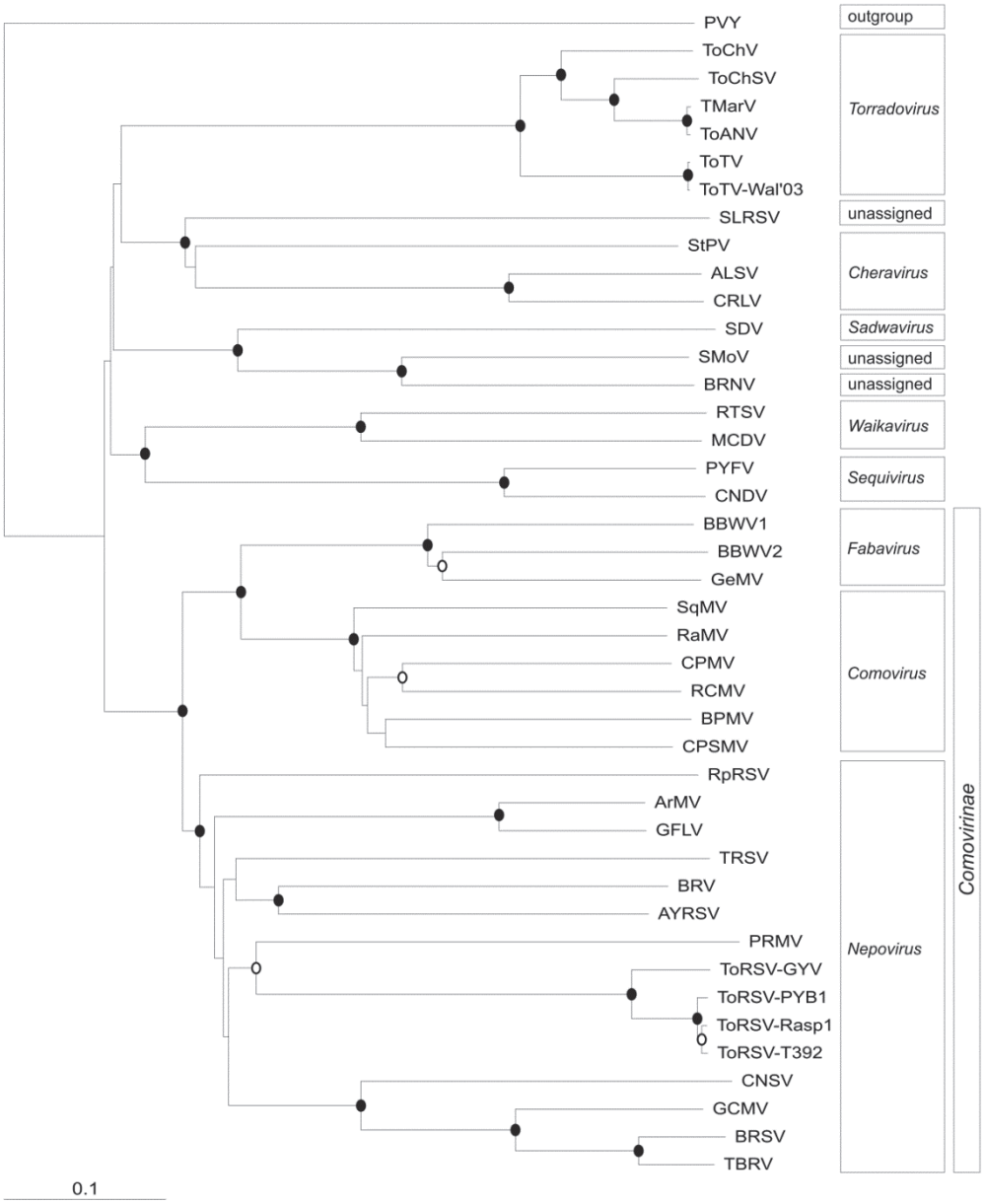
Figure 3.5: Genome organization of tomato chocolàte virus (ToChV). Positions of start and stop codons are indicated, as well as the relative positions of regions containing motifs of helicase (HEL), protease (PRO), and RNA-dependent RNA polymerase (RdRp) on RNA1 and of the three coat proteins (Vp35, Vp26 and Vp24) and putative movement protein (MP) on RNA2

In previous studies (Verbeek et al., 2007, 2008) phylogenetic comparisons based on the aa region between the CG protease motif (Bazan and Fletterick, 1988) and the GDD RdRp active site (Argos, 1988) in the RNA1-ORF1 were used to determine the relationships between torradoviruses and other viruses of the family *Secoviridae* (see for current taxonomy: http://www.ictvonline.org/virusTaxonomy.asp?). This region is proposed to be a good taxonomic predictor for classifying picorna-like viruses (Le Gall et al., 2008; Le Gall et al., 2007). A similar analysis was performed to determine the taxonomic position of ToChV and ToChSV. The resulting dendrogram (Fig. 3.6) shows that both ToChV and ToChSV cluster together with ToTV and ToMarV in the genus *Torradovirus*.

Sanfaçon et al. (2009) defined preliminary criteria for defining species within the genus *Torradovirus*. Torradoviruses are distinct species if they share less than 80% aa identity in the 3C-like Proteinase-RNA-dependent RNA polymerase (Prot-Pol) region of RNA1-ORF1 or less than 75% aa identity in the CP region of RNA2-ORF2.

Table 3.2 lists the percentages of identity between ToChV and other isolates of torradoviruses for the ORFs and regions of RNA1 and RNA2. Strictly based on the criterion set for the Prot-Pol region of RNA1 ToChV and ToChSV could be considered isolates of ToMarV. Comparisons of the complete CP sequences between the viruses also suggest that ToChV, ToMarV and ToChSV group as one species as they share between 82.0 and 87.1% aa identity.

Figure 3.6: Phylogenetic analysis of tomato chocolàte virus (ToChV) and members of the family *Secoviridae* based on the alignment of the region between the protease CG motif and GDD RdRp motif (aa 1,045–1,502 of RNA1-ORF1). Circles indicate nodes supported by bootstrap values above 80% (closed circles) or 60% (open circles); nodes without circles are not supported to these levels. The bar represents a p-distance of 0.1. The genera are delineated on the right. Sequences included in the analysis are those of (with virus acronyms, genus and accession numbers in parentheses): apple latent spherical virus (ALSV; *Cheravirus*; AB030940), arabis mosaic virus (ArMV; *Nepovirus*; AY303786), artichoke yellow ringspot virus (AYRSV; *Nepovirus*; AM087671), bean pod mottle virus (BPMV; *Comovirus*; U70866), beet ringspot virus (BRSV; *Nepovirus*; D00322), black raspberry necrosis virus (BRNV; unassigned species in the family *Secoviridae*; DQ344639), blackcurrant reversion virus (BRV; *Nepovirus*; AF368272), broad bean wilt virus 1 (BBWV1; *Fabavirus*; AB084450), broad bean wilt virus 2 (BBWV2; *Fabavirus*; AF225953), carrot necrotic dieback virus (CNDV; *Sequivirus*; EU980442), cherry rasp leaf virus (CRLV; *Cheravirus*; AJ621357), cowpea mosaic virus (CPMV; *Comovirus*; X00206), cowpea severe mosaic virus (CPSMV; *Comovirus*; M83830), cycas necrotic stunt virus (CNSV; *Nepovirus*; AB073147), gentian mosaic virus (GeMV; *Fabavirus*; AB084452), grapevine chrome mosaic virus (GCMV; *Nepovirus*; X15346), grapevine fanleaf virus (GFLV; *Nepovirus*; D00915), maize chlorotic dwarf virus (MCDV; *Waikavirus*; U67839), parsnip yellow fleck virus (PYFV; *Sequivirus*; D14066), peach rosette mosaic virus (PRMV; *Nepovirus*; AF016626), radish mosaic virus (RaMV; *Comovirus*; AB295643), raspberry ringspot virus (RpRSV; *Nepovirus*; AY303787), red clover mottle virus (RCMV; *Comovirus*; X64886), rice tungro spherical virus (RTSV; *Waikavirus*; M95497), satsuma dwarf virus (SDV; *Sadwavirus*; AB009958), squash mosaic virus (SqMV; *Comovirus*; AB054688), stocky prune virus (StPV; *Cheravirus*; DQ143874), strawberry latent ringspot virus (SLRSV; unassigned species in the family *Secoviridae*; AY860978), strawberry mottle virus (SMoV; unassigned species in the family *Secoviridae*; AJ311875), tobacco ringspot virus (TRSV; *Nepovirus*; U50869), tomato black ring virus (TBRV; *Nepovirus*; AY157993), tomato ringspot virus (ToRSV; *Nepovirus*; AF135410 (isolate T392), AF135409 (isolate Rasp1), AF 135408 (isolate PYB1) and AF135407 (isolate GYV), tomato torrado virus [ToTV; *Torradovirus*; DQ388879 (isolate PRI-ToTV0301) and EU563948 (ToTV-Wal'03)], tomato marchitez virus [ToMarV; *Torradovirus*; EF681764 (isolate PRI-TMarV0601) and EF063641 (ToANV)], tomato chocolate spot virus (ToChSV; *Torradovirus*; GQ305131). The sequence of Potato virus Y (PVY; *Potyvirus*; X12456) was used as an outgroup in the analyses


PVY
ToChV
ToChSV
TMarV
ToANV
ToTV
ToTV-Wal'03
SLRSV
StPV
ALSV
CRLV
SDV
SMoV
BRNV
RTSV
MCDV
PYFV
CNDV
BBWV1
BBWV2
GeMV
SqMV
RaMV
CPMV
RCMV
BPMV
CPSMV
RpRSV
ArMV
GFLV
TRSV
BRV
AYRSV
PRMV
ToRSV-GYV
ToRSV-PYB1
ToRSV-Rasp1
ToRSV-T392
CNSV
GCMV
BRSV
TBRV
0.1
outgroup
Torradovirus
unassigned
Cheravirus
Sadwavirus
unassigned
unassigned
Waikavirus
Sequivirus
Fabavirus
Comovirus
Nepovirus
Comovirinae

It should be noted, however, that also ToTV and ToMarV could be considered one species as they share 75.3% identity in this region. The criteria as set by Sanfaçon et al. (2009) are based on only a limited number of full sequences (2 isolates of ToTV (Budziszewska et al., 2008; Verbeek et al., 2007) and one of ToMarV (Verbeek et al., 2008) and an incomplete sequence of an isolate of ToMarV (tomato apex necrosis virus; ToANV (Turina et al., 2007)). As the authors indicate these preliminary criteria may need to be adjusted as more viruses become characterized.

To obtain a better understanding of the possible taxonomic relations between the four torradovirus sequences we performed more elaborate comparisons of the different coding and non-coding regions that have now become available. Overall levels of nt sequence identity between all viruses are well below 80% and mostly around or below 70% while for isolates of the same virus identity levels are significantly higher (ToTV and ToTV-Wal-'03: 99.0% for RNA1 and 99.1% for RNA2; ToMarV and ToANV: 95.3% for RNA1 and 79.9% for RNA2). However, since for ToANV only partial sequences are published (the 5'-ends of both RNAs are missing), these numbers may change if additional sequence information on ToANV becomes available. Overall sequence identity between ToChV and the other torradoviruses ranges from 62 to 71% (Table 3.2).

The 5'- and 3'-UTRs clearly differ between the four (tentative) species of the genus *Torradovirus*. The 5'–UTRs of both RNA1 and RNA2 of ToChV and ToChSV are fairly similar in length (135 vs. 138 for RNA1 and 181 vs. 171 nts for RNA2) but overall sequence identities are only 64 and 57% for RNA1 and RNA2, respectively (Table 3.2). The lengths of the 5'-UTRs of the RNAs1 and RNAs2 of ToTV and ToMarV are clearly different from ToChV with levels of overall identity varying between 44 and 76% (see Table 3.2).

Direct sequence comparisons between the total 3'-UTRs of RNA1 and RNA2 of the four torradovirus sequences are difficult because of large differences in their 3'-UTR lengths (Table 3.2). Identity levels in the 3'-UTR of RNA1 and 2 are generally lower than for the 5'-UTR and again lower in RNA2 in comparison to RNA1 (results not shown).

Overall sequence identity levels to the 3'-UTR of ToChV vary between 54% (ToTV) and 96% (ToChSV) for RNA1 and 44% (ToTV-Wal'03) and 78%

Table 3.2: Comparison of tomato chocolàte virus sequences with other members of the genus *Torradovirus*. Identities (%) for the amino acid sequences are given in bold and for the nucleotide sequences in roman, lengths (nts) in brackets. The question mark (?) indicates lack of sequence information on the tomato apex necrosis isolate of ToMarV.

Virus	Isolate	RNA1					RNA2								
		Overall (length)	*ORF1*		*UTR*		*Overall (length)*	*ORF1*	*ORF2*				*UTR*		
			Entire ORF	Prot-Pol	5'-UTR (length)	3'-UTR (length)			MP	CP region	Vp35	Vp26	Vp24	5'-UTR (length)	3'-UTR (length)
Tomato chocolate virus	ToChV-G01	- (7474)	-	-	- (135)	- (871)	- (5695)	-	-	-	-	-	-	- (181)	- (1406)
Tomato torrado virus	PRI-ToTV0301	63 (7808)	**64**	**79**	76 (106)	54 (1210)	62 (5403)	**60**	**65**	**74**	**67**	**83**	**74**	55 (181)	45 (1091)
Tomato torrado virus	ToTV-Wal03	63 (7829)	**64**	**79**	76 (107)	54 (1229)	62 (5404)	**61**	**65**	**74**	**68**	**83**	**74**	55 (181)	44 (1092)
Tomato marchitez virus	PRI-TMarV 0601	69 (7229)	**74**	**85**	61 (140)	74 (625)	69 (4906)	**64**	**74**	**83**	**80**	**87**	**83**	60 (138)	60 (652)
Tomato marchitez virus	ToANV	69 ? (6478)	**74**	**85**	? (?)	73 (627)	71 ? (3013)	?	?	**82**	**78**	**88**	**83**	? (?)	60 (647)
Tomato chocolate spot virus	-	71 (7492)	**73**	**82**	64 (138)	96 (867)	70 (5109)	**66**	**75**	**83**	**79**	**87**	**85**	57 (171)	78 (814)

(ToChSV) for RNA2 (Table 3.2). Remarkably in both 3'-UTRs identity levels for isolates of one virus are significantly higher, i.e. above 99% for ToTV and ToTV-Wal'03 and around 89% for ToMarV and the tomato apex necrosis isolate of ToMarV.

More detailed analyses of both 3'-UTRs of each torradovirus show that they share a unique species specific region (>99%) that is highly conserved between the 3'-terminal ends [directly preceding the poly(A) tail] of the two RNAs. Between species this region is clearly different in length but nearly identical between isolates of one virus; ToChV 787 nts, ToTV 989 nts, ToTV-Wal'03 990 nts, ToMarV 552 nts and ToANV 554 nts. Remarkably, ToChSV was the only virus lacking this conserved region at the end of the 3'-UTR of both RNAs. There is no obvious explanation for this but it should be noted that the 772 nts directly preceding the poly(A) tail of the 3'-UTR of RNA1 of this virus share 99.2% sequence identity with the last 772 nts of both RNA1 and RNA2 of ToChV. This suggests that the sequence of the 3'-UTR of RNA1 of ToChSV might be directly derived from ToChV.

With respect to the coding regions, we made comparisons of the RNA1-ORF1 (entire ORF, helicase region and Prot-Pol region), the RNA2-ORF1 and RNA2-ORF2 (MP and CP region) of ToChV to the other torradoviruses (Table 3.2). RNA1-ORF1 of ToChV shows aa sequence identities of 64% (ToTV) to 74% (ToMarV). The aa sequence identity for the helicase region, which is a highly conserved region, is much higher (95–96%, not shown in Table 3.2). The aa sequence identity in the Prot-Pol region varies between 79% (ToTV) and 85% (ToMarV).

RNA2-ORF1 of ToChV shows aa identities of 60% (ToTV) to 66% (ToChSV). In the second ORF of RNA2 the aa sequence identities in the MP region vary between 65% (ToTV) and 75% (ToChSV) and in the CP region between 74% (ToTV) and 83% (ToMarV and ToChSV). The different predicted CPs of ToChV share aa sequence identities of 67–88% with those of ToTV and ToMarV. Remarkably, the identity between the Vp26 analogues of the different viruses is significantly higher (over 83%) than for the other two CPs (67–80% and 74–85% for the Vp35 and Vp24, respectively) (Table 3.2).

In conclusion, there are very distinct sequence differences between the four (proposed) torradovirus species (ToTV, ToMarV, ToChV and ToChSV).

However, isolates of one virus species (as seen in the two isolates of ToTV and ToMarV) have high sequence similarities in the coding and non-coding regions, in which the (tentative) torradovirus species differ significantly. The RNA2-ORF1 is a region very similar in length between the currently known torradoviruses and unique for the genus *Torradovirus*. It displays a significant sequence variation between the four torradovirus sequences with levels of identities ranging from 60.4 to 73.9% and is nearly identical for the two isolates of ToTV (99.5%).

The 3'-UTRs of the torradoviruses clearly differ in length and identity between species but harbour a unique, nearly identical region in the two RNAs of one species. This virus-specific region differs significantly between different species both in length and in identity (between species <65% and for isolates of the same species from 85 to 99%).

Based on our observation we propose that aa identity levels below 75% in the RNA2-ORF1 and nt identity levels below 85% in the homologous region in the 3'-UTR of both RNAs of each torradovirus can serve as additional to the current tentative taxonomic criteria (Sanfaçon et al., 2009) which may need to be updated now that additional information has become available.

The analysis presented above and application of the newly proposed additional demarcation criteria leads us to conclude that tomato chocolàte virus is a new species in the genus *Torradovirus*.

Chapter 3, section C

Lettuce necrotic leaf curl virus, a new plant virus infecting lettuce and a proposed member of the genus *Torradovirus*

M. Verbeek[1*], A. M. Dullemans[1*], H.M.G. van Raaij[1],

J.Th.J. Verhoeven[2], and R. A. A. van der Vlugt[1]

* Joint first authors

[1] Plant Research International, part of Wageningen UR, Wageningen, The Netherlands

[2] National Plant Protection Organization, Wageningen, The Netherlands

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Abstract

A new virus was isolated from a lettuce plant grown in the open field in the Netherlands in 2011. This plant was showing conspicuous symptoms that consisted of necrosis and moderate leaf curling. The virus was mechanically transferred to indicator plants and a total RNA extract of one of these indicator plants was used for Next Generation Sequencing. Analysis of the obtained sequences and further biological studies showed that the virus was related to but clearly distinct from viruses belonging to the genus *Torradovirus*. The name lettuce necrotic leaf curl virus (LNLCV) is proposed for this new torradovirus.

Keywords

Next generation sequencing, *Secoviridae*, LNLCV

Nucleotide sequence data reported are available in the GenBank database under accession numbers KC855266 (RNA1) and KC855267 (RNA2).

In 2011 in the Netherlands, lettuce plants (*Lactuca sativa* L.) showing leaf necrosis at the basal parts of the leaves and leaf curling (Fig. 3.7a) were found in an open field of a lettuce grower. During this season approximately 30 lettuce plants with similar symptoms were found in this field, while in the next growing season (2012) these disease symptoms were not observed in a lettuce crop grown in the same field. A sample of such a symptomatic lettuce plant was mechanically inoculated to a plant of *Nicotiana benthamiana*, which was sent to the National Plant Protection Organization (NPPO) for diagnosis when young leaves expressed rugosity. Unfortunately, the original lettuce plant was lost. Homogenates of systemically infected leaves of this plant were sap inoculated to a series of indicator plants, consisting of *Nicotiana occidentalis* 'P1', *Chenopodium quinoa*, *Nicotiana glutinosa*, and *Nicotiana tabacum* cv. White Burley. Only plants of *N. occidentalis* 'P1' showed symptoms which consisted of local necrotic lesions, systemic necrosis, and stunting. Symptomatic plants of *N. occidentalis* 'P1' were used for Next Generation Sequencing (NGS) and further biological experiments. The isolate was propagated in *N. occidentalis* 'P1' and taken into the virus collection of the NPPO (isolate number: 5317015).

Total RNA was extracted from approximately 150 mg of leaf material of systemically infected *N. occidentalis* 'P1' using the RNeasy Plant Mini Kit of Qiagen (Hilden, Germany), following the manufacturer's protocol. DNA was removed from the sample by the On-Column DNase Digestion with the RNase-Free DNase Set (Qiagen) according to the manufacturer's protocol. RNA was eluted from the column with 50 µl nuclease-free water and the concentration was measured using the Quant-iT RiboGreen RNA Reagent and Kit (Invitrogen, Life Technologies, Paisley, UK). 750 ng of total RNA was used for the RNA library preparation suitable for Illumina HiSeq paired end sequencing. The RNA was precipitated on ice for 1 h using 1/10 volume 3 M NaAc, pH5.2, and 2 volumes EtOH (absolute) followed by centrifugation at 20,000 x g at 4 °C for 5 minutes. Precipitated RNA was washed twice with 500 µl EtOH 70%, air-dried and stored at -80 °C. The RNA pellet was dissolved in 19 µl fragmentation and priming buffer and processed further for end repair, adaptor ligation, first and second strand cDNA synthesis and final library amplification following the TruSeq RNA Sample preparation LT protocol (Illumina Inc, San Diego CA, USA). The final library was eluted in 30 µl elution buffer. Library quality was

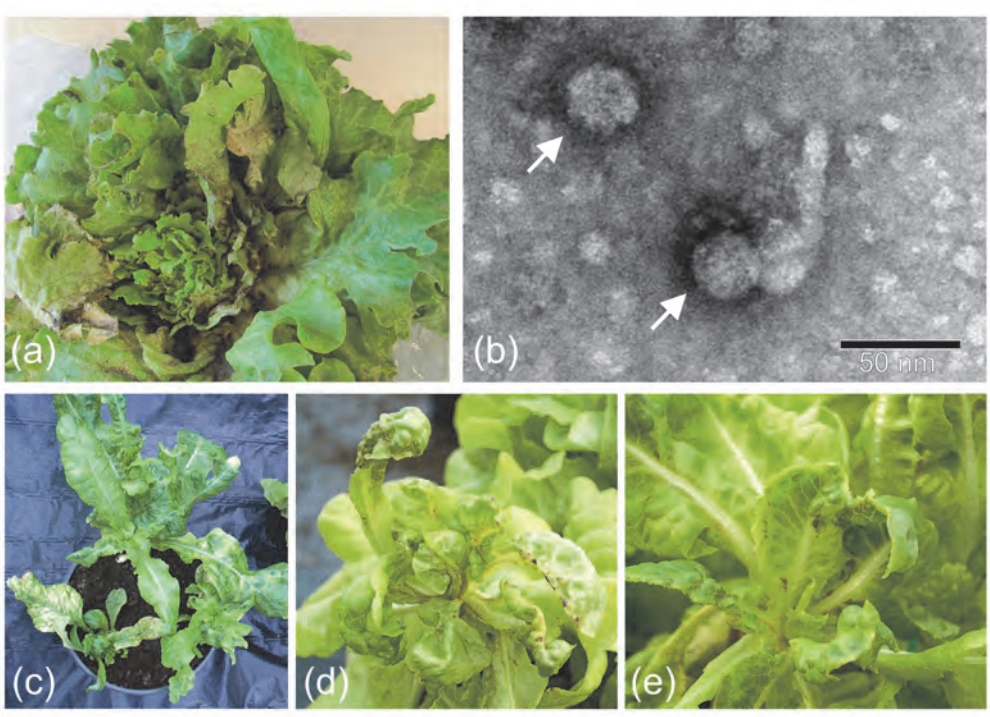


Figure 3.7: Symptoms of lettuce necrotic leaf curl virus in the original lettuce plant (a), and after mechanical inoculation to the lettuce cultivars 'Taiwan' (c), 'Patty' (d), and 'White Boston' (e). Electron micrograph of LNLCV virions (indicated by arrows) in a dip preparation of a *N. occidentalis* 'P1' leaf sample (b)

analysed using a Bioanalyzer 2100 DNA1000 chip (Agilent Technologies, Santa Clara CA, USA) and quantified using a Qubit quantitation platform using Quant-iT PicoGreen (Invitrogen, Life Technologies).

The Molecular Identifier (MID)-labelled library of our target virus and eight other indexed libraries were pooled in equimolar proportions and diluted to 6 pM for TruSeq Paired End v2 DNA clustering on a partial flow cell lane. Final sequencing was performed on a HiSeq 2000 platform using 101, 7, 101 flow cycles for sequencing paired end reads. All steps for clustering and subsequent sequencing were carried out according to the manufacturer's protocols. Reads were split per sample by corresponding MIDs using CASAVA 1.8 software (Illumina). The entire sequencing procedure was operated at the

services of Business Unit Bioscience, Plant Research International (Wageningen, the Netherlands).

A single run resulted in 34,911,214 MID labelled reads of the lettuce sample. Following removal of the MID sequences, the sequence reads were assembled using the CLC Genomics Workbench (CLC Bio, Aarhus, Denmark). This *de novo* assembly resulted in 3,887 sequence contigs. BLASTn (Zhang et al., 2000) and BLASTx (Altschul et al., 1997) searches with the contigs larger than 1 kb, and with a minimal count of 10,000 reads, were performed against the GenBank non-redundant protein and nucleotide databases. Most contigs showed identities with different *Nicotiana* sequences, while only two contigs were of viral origin. Analysis (BLASTx) of the amino acid sequence encoded by the first contig (6832 bp, assembled from 39.305 reads) resulted in a 41% identity with the polyprotein coded by the tomato marchitez virus (ToMarV) RNA1 (YP_001976147). A similar analysis on the amino acid sequence encoded by the second contig (5323 bp, assembled from 113.132 reads) resulted in a 39% identity with the tomato marchitez virus ORF2 polyprotein coded by RNA2 (YP_001976149). In comparison to the known torradovirus (family *Secoviridae*, genus *Torradovirus,* type species *Tomato torrado virus* (ToTV)) sequences, it was expected that both the contigs represented the two nearly complete torradovirus RNA segments. To obtain full length sequences, the 3'- and 5'-ends of both RNA segments were determined by linker mediated rapid amplification of cDNA ends (5'/3' RACE kit, version 10, Roche, Mannheim, Germany) according to the manufacturer's protocol, and subsequent direct sequencing of the amplicons. The assembled full length sequences were reconfirmed by aligning to all input reads. This resulted in two full length sequences (RNA1 and RNA2), representing the full genome of the lettuce-infecting virus.

RNA1 [7579 nucleotides (nts), excluding the poly(A) tail] contains one open reading frame (RNA1-ORF1) of 6669 nts. This RNA1-ORF1 encodes a predicted polyprotein of 2223 amino acids (aa) with a molecular mass of 246 kDa. The 5'- and 3'-untranslated regions (UTR) of RNA1 are 170 and 737 nts in length, respectively.

RNA2 [5290 nts, excluding the poly(A) tail] contains two open reading frames (RNA2-ORF1 and RNA2-ORF2). RNA2-ORF1 encodes a predicted protein of 211 aa (23 kDa) and RNA2-ORF2 encodes for a predicted

polyprotein of 1222 aa (134 kDa). The length of the RNA2 UTRs are 326 nts (5') and 766 nts (3').

The genomic organization is analogous to those of torradoviruses (Batuman et al., 2010; Sanfaçon et al., 2012; Verbeek et al., 2007, 2008, 2010a); one large polyprotein containing replication related motifs such as helicase, protease and RNA-dependent RNA polymerase (RdRp) located in RNA1, and in RNA2 one ORF with a still unknown function, and a second ORF, partially overlapping with RNA2-ORF1, coding for the putative movement protein and three coat proteins. The 3'-UTR regions of both RNAs are highly identical (96% in the final 734 nts). Both 5'-UTR regions, however, are very different in length, but are identical for the most 5'-located 11 nts. The 5'-UTR of RNA 2 seems to be unusually long in comparison with those of other torradoviruses. However, a sequence assembly artefact was ruled out by additional RT-PCRs and amplicon sequencing using several primer pairs on the originally obtained RNA. The reverse primers were chosen in the ORF2 region (around nts 1080 and 1220) and the forward primers around nts 20 and 200. The results from these experiments confirmed the nucleotide sequence found by NGS and 5'-RACE for the RNA2 5'-UTR region.

Different torradoviruses are considered distinct species if they share less than 80% aa identity in the 3C-like proteinase – RNA-dependent RNA polymerase (prot-pol) region of RNA1-ORF1 or less than 75% aa identity in the CP region of RNA2-ORF2 (Sanfaçon et al., 2012). Table 3.3 lists the - generally low - percentages of identity between the lettuce-infecting virus and other torradoviruses for the ORFs and regions of RNA1 and RNA2. Based on these results, we propose the name lettuce necrotic leaf curl virus (LNLCV) for this new virus and, according to the current demarcation criteria (Sanfaçon et al., 2012), to assign it as a new tentative species in the genus *Torradovirus.*

The presence of a torradovirus in the symptomatic plants of *N. occidentalis* 'P1' was confirmed by RT-PCR with generic primers designed for members of the genus *Torradovirus* (Verbeek et al., 2012). The use of both primer pairs, Torrado-1F/Torrado-1R and Torrado-2F/Torrado-2R, in RT-PCR resulted in fragments of the same length as expected for the use of these generic primers (371 and 515 bp, respectively). Direct sequencing on these PCR products confirmed the torradovirus-like origin of the sample and the

Table 3.3: Comparison of lettuce necrotic leaf curl virus amino acid sequences in the indicated regions with amino acid sequences of other members of the genus *Torradovirus* (ORF: open reading frame; prot-pol: 3C-like proteinase-RdRp region; MP: putative movement protein; CP: coat protein; Vp35, Vp26 and Vp23 represent the three distinct coat proteins)

Virus species	Acronym	GenBank Accession nrs (RNA1/RNA2)	% Identity at aa level									
			RNA1			RNA2						
			ORF1	helicase	prot-pol	ORF1	ORF2	MP	CP region	Vp35	Vp26	Vp23
Tomato torrado virus	ToTV	DQ388879/ DQ388880	34	55	55	25	32	23	39	35	43	42
Tomato marchitez virus	ToMarV	EF681764/ EF681765	35	54	58	26	33	25	40	33	45	43
Tomato chocolate spot virus	ToChSV	GQ305131/GQ305132	35	54	56	25	33	24	40	34	43	43
Tomato chocolate virus	ToChV	FJ560489/ FJ560490	34	54	57	24	31	23	38	32	45	42

amplicon sequence was 100% identical to the sequence of LNLCV that was found with NGS.

Electron microscopic analysis on dip preparations of systemically infected symptomatic *N. occidentalis* 'P1' leaves revealed the presence of spherical virus particles of approximately 30 nm in diameter (Fig. 3.7b). LNLCV was mechanically transmissible to the indicator plants *Physalis floridana*, *Physalis acutifolia* (synonym: *P. wrightii*), *N. benthamiana*, *N. occidentalis* and *N. hesperis* '67A', whereas no symptoms appeared on plants of *C. quinoa*, and *N. tabacum* cv. 'White Burley'. Surprisingly, this virus did not infect *N. glutinosa*, a good indicator plant for the other known torradoviruses (Batuman et al., 2010; Verbeek et al., 2007, 2008). The lack of infection in *N. glutinosa* was confirmed by back inoculation onto the susceptible indicator hosts *N. occidentalis* 'P1' and *N. hesperis* '67A', and by RT-PCR using the above mentioned generic primers.

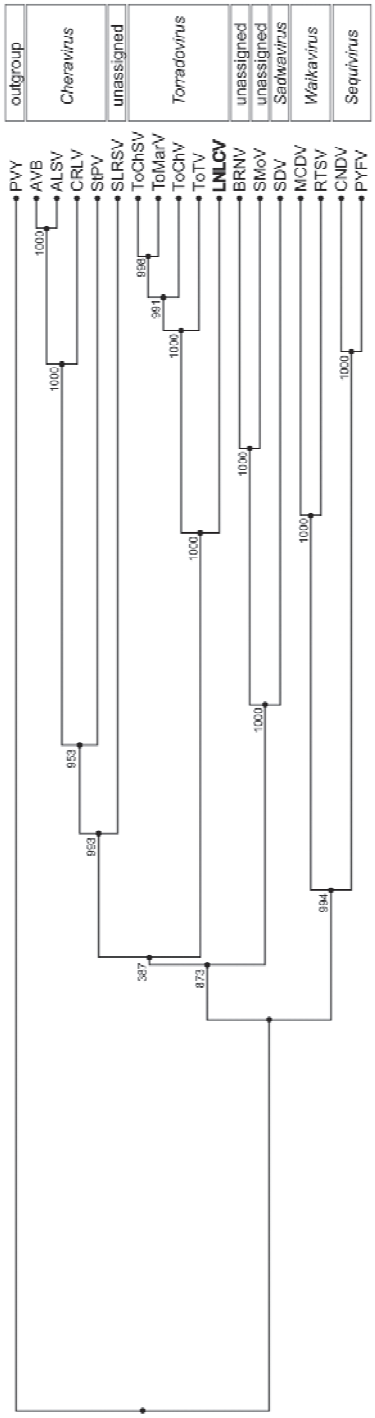
To study the symptomatology of this virus in lettuce, seedlings of the cultivars 'Taiwan', 'Patty', and 'White Boston' were mechanically inoculated in their three true leaf stage. This inoculation was performed by dusting the leaves with Carborundum and rubbing the leaves gently with a homogenate of systemically infected *N. occidentalis* 'P1' in 0.03M sodium/potassium phosphate buffer, pH 7.7. For each lettuce cultivar, twelve plants were inoculated and three plants were left untreated as negative control. The first symptoms appeared one week after inoculation and consisted of mottling and deformation of newly emerged leaves. After another week, the first necrotic symptoms appeared together with a characteristic curling of the young leaves (see Fig. 3.7c, d, e). In total 100% of the inoculated plants of the cultivars 'Taiwan' and 'White Boston' showed symptoms and 33% of cv. 'Patty'. The presence of LNLCV in the symptomatic lettuce plants was confirmed by RT-PCR using the Torrado-2F/Torrado-2R generic primers and direct sequencing of the amplicons.

In order to test that LNLCV is also biologically distinct from the currently known torradoviruses, which all infect certain cultivars of tomato, we mechanically inoculated the virus to eight plants of the tomato cultivar 'Realeza', which is very susceptible to ToTV, ToMarV and Tomato chocolàte

virus (ToChV) (results not shown). In the same experiment, as a positive control, three plants of lettuce 'White Boston' were inoculated. As a reciprocal test, we inoculated ToTV, ToMarV and ToChV to the lettuce cultivars 'Taiwan' and 'White Boston' (twelve plants per virus isolate) and to two plants of tomato cv. 'Realeza' as a positive control for each inoculation. These experiments showed that LNLCV did not infect tomato cv. 'Realeza' and that ToTV, ToMarV and ToChV could not infect lettuce. This was confirmed by testing the inoculated plants by inoculation onto *N. hesperis* '67A' and by RT-PCR, using the generic primers Torrado-2F/Torrado-2R (Verbeek et al., 2012). As ToTV is transmitted by whiteflies (Amari et al., 2008; Pospieszny et al., 2010), we performed some preliminary transmission experiments with the greenhouse whitefly *Trialeurodes vaporariorum* using the same acquisition access period and inoculation access period as described by Pospieszny (Pospieszny et al., 2010). However, none of these experiments led to transmission of LNLCV. More research is needed to investigate whether LNLCV can be vectored by whiteflies, but it is also possible that the isolate we obtained has lost whitefly transmissibility due to repeated mechanical transmission.

Both biological and molecular data support the characterization of LNLCV as a new torradovirus. This is further supported by phylogenetic analyses. In previous studies (Batuman et al., 2010; Verbeek et al., 2007, 2008, 2010a) phylogenetic comparisons based on the aa region between the CG protease motif (Bazan and Fletterick, 1988) and the GDD RdRp active site (Argos, 1988) in the RNA1-ORF1 were found to be good indicators of possible relationships between torradoviruses and other viruses in the family *Secoviridae* (Sanfaçon et al., 2012). This region is considered a useful taxonomic predictor for classifying plant-infecting picornavirales (Le Gall et al., 2008; Le Gall et al., 2007). A similar analysis was performed to examine the taxonomic position of LNLCV. Multiple alignments and phylogenies were performed with the CLC Genomics Workbench after bootstrapping in 1000 replicates. The resulting dendrogram based on the UPGMA algorithm (Fig. 3.8) shows that LNLCV clusters together with the known members of the genus *Torradovirus* and consequently, supports its assignment to this genus. The overall levels

Figure 3.8: Phylogenetic analysis of lettuce necrotic leaf curl virus (LNLCV) and members of the family *Secoviridae*, based on the alignment of the region between the protease CG motif and GDD RdRp motif (aa 1,065–1,526 of RNA1-ORF1). Bootstrap values are indicated at the nodes. The bar represents a p-distance of 0.4. Sequences included in the analysis are those of (with virus acronyms, genus and accession numbers in parentheses):
aracacha virus B (AVB; *Cheravirus*; JQ437415), apple latent spherical virus (ALSV; *Cheravirus*; AB030940), black raspberry necrosis virus (BRNV; unassigned species in the family *Secoviridae*; DQ344639), carrot necrotic dieback virus (CNDV; *Sequivirus*; EU980442), cherry rasp leaf virus (CRLV; *Cheravirus*; AJ621357), maize chlorotic dwarf virus (MCDV; *Waikavirus*; U67839), parsnip yellow fleck virus (PYFV; *Sequivirus*; D14066), rice tungro spherical virus (RTSV; *Waikavirus*; M95497), satsuma dwarf virus (SDV; *Sadwavirus*; AB009958), stocky prune virus (StPV; *Cheravirus*; DQ143874), strawberry latent ringspot virus (SLRSV; unassigned species in the family *Secoviridae*; AY860978), strawberry mottle virus (SMoV; unassigned species in the family *Secoviridae*; AJ311875), tomato torrado virus (ToTV; *Torradovirus*; DQ388879), tomato marchitez virus (ToMarV; *Torradovirus*; EF681764), tomato chocolate virus (ToChV; *Torradovirus*: J560489), tomato chocolate spot virus (ToChSV; *Torradovirus*; GQ305131). The sequence of Potato virus Y (PVY; *Potyvirus*; X12456) was used as an outgroup in the analyses


outgroup
Cheravirus
unassigned
Torradovirus
unassigned
unassigned
Sadwavirus
Waikavirus
Sequivirus
PVY
AVB
ALSV
CRLV
StPV
SLRSV
ToChSV
TolMarV
ToChV
ToTV
LNLCV
BRNV
SMoV
SDV
MCDV
RTSV
CNDV
PYFV
1000
998
991
1000
1000
1000
1000
1000
953
953
994
387
873
0.400

of identity between LNLCV and the other currently known tomato-infecting torradoviruses are remarkably low. As LNLCV represents the first torradovirus from a crop other than tomato, we can only speculate that this divergence may be due to co-evolution of this virus with plants from the *Asteraceae*, a plant family distinct from the family of *Solanaceae*. In conclusion, Lettuce necrotic leaf curl virus is a new virus and proposed to represent a new species in the genus *Torradovirus*. It will be of interest to know where this virus came from and whether it is infecting lettuce plants in other production areas.

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Chapter 4

Two generic PCR primer sets for the detection of members of the genus *Torradovirus*

M. Verbeek[1*], J. Tang[2*], and L.I. Ward[2]

* Joint first authors

[1] Plant Research International, part of Wageningen UR, Wageningen, The Netherlands

[2] Plant Health and Environment Laboratory, Ministry for Primary Industries, Auckland, New Zealand

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Abstract

Two degenerate primer pairs were designed for the universal detection of members of the genus *Torradovirus*. Primer pair Torrado-1F/Torrado-1R was designed based on the RNA-dependent RNA polymerase region located in RNA1 and primer pair Torrado-2F/Torrado-2R based on a region overlapping the two first coat proteins Vp35 and Vp26 in RNA2. The primers were used in two-step and one-step RT-PCR protocols. Both primer pairs were able to detect 14 out of 15 isolates belonging to the two torradovirus species *Tomato torrado virus* (ToTV) and *Tomato marchitez virus* (ToMarV) and the two tentative species Tomato chocolate spot virus (ToChSV) and Tomato chocolàte virus (ToChV). Due to poor sample quality, one isolate of ToTV was detected with primer pair Torrado-2F/Torrado-2R and not with primer pair Torrado-1F/Torrado-1R, suggesting that the latter primer pair was less sensitive. Nevertheless, both primer pairs proved to be suitable for the universal RT-PCR detection of torradoviruses and can be deployed for the detection of all currently known torradoviruses and possibly for the detection of new members of this group.

Introduction

Recently, the plant virus genus *Torradovirus* was created within the family *Secoviridae* to place two newly described virus species, *Tomato torrado virus* (ToTV) and *Tomato marchitez virus* (ToMarV) (Sanfaçon et al., 2012; Sanfaçon et al., 2009; Verbeek et al., 2007, 2008). In addition to ToTV and ToMarV, two tentative members of the genus *Torradovirus* have been described: Tomato chocolate spot virus (ToChSV) (Batuman et al., 2010) and Tomato chocolàte virus (ToChV) (Verbeek et al., 2010a). All torradoviruses described so far infect tomato (*Solanum lycopersicum* L.) in which they induce necrotic symptoms on leaves and, most of the time, on fruits. Torradoviruses have been reported from Australia, Colombia, France, Hungary, Italy, Panama, Poland, Spain (ToTV), Mexico (ToMarV and tomato apex necrosis virus, a member of the species Tomato marchitez virus) and Guatemala (ToChSV and ToChV) (Alfaro-Fernández et al., 2009; Alfaro-Fernández et al., 2007; Alfaro-Fernández et al., 2010a; Batuman et al., 2010; Davino et al., 2010; Gambley et al., 2010; Herrera-Vásquez et al., 2009; Pospieszny et al., 2007; Turina et al., 2007; Verbeek and Dullemans, 2012; Verbeek et al., 2007, 2008, 2010a; Verdin et al., 2009). ToTV, the type species of this genus, was discovered in tomato plants showing symptoms of "Torrado" disease. This disease was first observed in protected tomato crops in the Murcia province of Spain in spring 2001 (Alfaro-Fernández et al., 2010a). Local farmers called the disease "Torrado" because of the heavy necrotic symptoms they observed on the plants (torrado means burnt or roasted). It was shown that ToTV is transmitted by the whitefly species *Trialeurodes vaporariorum* (Pospieszny et al., 2010) and *Bemisia tabaci* (Amari et al., 2008). Under experimental conditions ToTV could be transmitted to other solanaceous species (e.g. pepper, eggplant and potato) and to a range of test plants (Amari et al., 2008; Pospieszny et al., 2010; Verbeek et al., 2007, 2008). In the field, ToTV has been found in several weed species which could act as reservoirs of the virus (Alfaro-Fernández et al., 2008). It has also been suggested that ToTV might be seed-transmitted (Gambley et al., 2010), but evidence is still lacking. Torradovirus particles are spherical with a diameter of approximately 28–30 nm. The torradovirus genome is bipartite and consists of single-stranded plus-sense RNA. The first RNA (RNA1) ranges from 7.2 kb for ToMarV to 7.8 kb for ToTV and has one open reading frame (ORF), which encodes replication

proteins including protease, helicase and RNA-dependent RNA polymerase (RdRp). The second RNA (RNA2) ranges from 4.9 kb for ToMarV to 5.7 kb for ToChV and has two ORFs. In comparison to other plant picorna-like viruses, the existence of the first ORF is unique for torradoviruses, but its function is still unclear. The second ORF has coding regions for a putative movement protein and the three coat proteins (35, 26 and 23 kDa) (Verbeek et al., 2007). Torradoviruses have remarkably long 3'-untranslated regions (UTRs) on both RNAs which range from approximately 652 nt (ToMarV) to over 1400 nt (ToChV). These 3'-UTRs possess unique species-specific regions which are highly conserved between the two RNAs of the same torradovirus species (Verbeek et al., 2010a).

Broad-spectrum or "universal" polymerase chain reaction (PCR) assays have been developed for a number of plant virus genera or even families, for example the genera *Ilarvirus* (Untiveros et al., 2010), *Nepovirus* (Wei and Clover, 2008), *Potexvirus* (Van der Vlugt et al., 1999) and *Tobravirus (Jones et al., 2008)*, and the families *Bromoviridae* (Untiveros et al., 2010), *Luteoviridae* (Chomič et al., 2010) and *Potyviridae* (Chen et al., 2001). These assays have been demonstrated to be an effective strategy for rapid and accurate virus detection in routine diagnosis and surveillance. Although species-specific PCR methods are available for all torradoviruses, there are no generic diagnostic methods for species belonging to the genus *Torradovirus*. Here we report on two primer pairs, designed within conserved regions of RNA1 and RNA2, respectively, which in reverse transcriptase (RT-) PCR prove capable of detecting all currently known torradoviruses.

Materials and methods

Virus isolates

Virus isolates used in this study and their origins are listed in Table 4.1. Torradovirus-infected plant tissue samples were kindly sent as dried or frozen leaf material from various locations. Most plant samples were from tomato, except for ToANV and ToChSV which were from *Nicotiana benthamiana*. The isolates were tested in two different laboratories; isolates nr. 1–11 were tested in the Plant Health and Environment Laboratory, Ministry of Primary Industries (MPI) in New Zealand, and isolates nr. 12–15 were tested at the

Table 4.1:Samples used in PCR tests and their origins

Sample No.	Species	Isolate	Origin	Provided by
1	**ToTV**	ToTV-FD2136	Australia	Cherie Gambley, DEEDI, Indooroopilly, Queensland, Australia
2	**ToTV**	ToTV-FD1883	Australia	Cherie Gambley, DEEDI, Indooroopilly, Queensland, Australia
3	**ToTV**	ToTV-FD2263	Australia	Cherie Gambley, DEEDI, Indooroopilly, Queensland, Australia
4	**ToTV**	ToTV-Can	Canary islands, Spain	Maria Carmen Cordoba-Selles, Universidad Politecnica de Valencia, Valencia, Spain
5	**ToTV**	ToTV-GNC06	Gran Canaria, Spain	Ana Alfaro-Fernández, Universidad Politecnica de Valencia, Valencia, Spain
6	**ToTV**	ToTV-H3	Hungary	Ana Alfaro-Fernández, Universidad Politecnica de Valencia, Valencia, Spain
7	**ToTV**	ToTV-MUR06	Spain	Ana Alfaro-Fernández, Universidad Politecnica de Valencia, Valencia, Spain
8	**ToTV**	ToTV-MUR09	Spain	Ana Alfaro-Fernández, Universidad Politecnica de Valencia, Valencia, Spain
9	**ToTV**	ToTV-TEN07	Tenerife, Spain	Ana Alfaro-Fernández, Universidad Politecnica de Valencia, Valencia, Spain
10	**ToMarV**	ToANV-VE434	Mexico	MassimoTurina, IVV, CNR, Torino, Italy
11	**ToChSV**	ToChSV	Guatemala	Ozgur Batuman, Department of Plant Pathology, University of California, Davis, USA
12*	**ToTV**	PRI-ToTV0301	Spain	Monsanto Vegetable Seeds Division, Bergschenhoek, The Netherlands
13*	**ToMarV**	PRI-TMarV0601	Mexico	Monsanto Vegetable Seeds Division, Bergschenhoek, The Netherlands
14*	**ToChV**	ToChV-G01	Guatemala	Monsanto Vegetable Seeds Division, Bergschenhoek, The Netherlands
15	**ToTV**	ToTV-T418	Colombia	Monsanto Vegetable Seeds Division, Bergschenhoek, The Netherlands

** Type isolate for this species*

virological laboratory of Plant Research International (PRI) in The Netherlands.

Nucleic acid extraction

Leaf samples (approximately 0.03 g of dry plant tissue per sample) were ground in an extraction bag (Bio-Rad, Hercules, CA, USA) containing 5 ml of lysis buffer P (Stratec Molecular GmbH, Berlin, Germany) with a Homex 6 tissue homogenizer (Bioreba AG, Reinach, Switzerland). RNA was then isolated using the InviMag® Plant DNA mini Kit/KFml (Stratec Molecular GmbH, Berlin, Germany) and a Kingfisher ml automated extraction instrument (Thermo Scientific, Waltham, MA, USA) as per the manufacturer's instructions. Nucleic acid was eluted into 100 µl of elution buffer and stored at −80 °C. For samples 12–15, 0.1 g of fresh plant tissue was frozen in liquid N_2, ground to a fine powder and total RNA was extracted using the Qiagen RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Nucleic acid was eluted into 50 µl of nuclease-free water and stored at −80 °C.

Primer design

Based on whole genome sequences of RNA1 and RNA2 published for ToTV, ToMarV, ToChSV and ToChV (GenBank ID: DQ388879, DQ388880, EF681764, EF681765, GQ305131, GQ305132, FJ560489, and FJ560490, respectively),

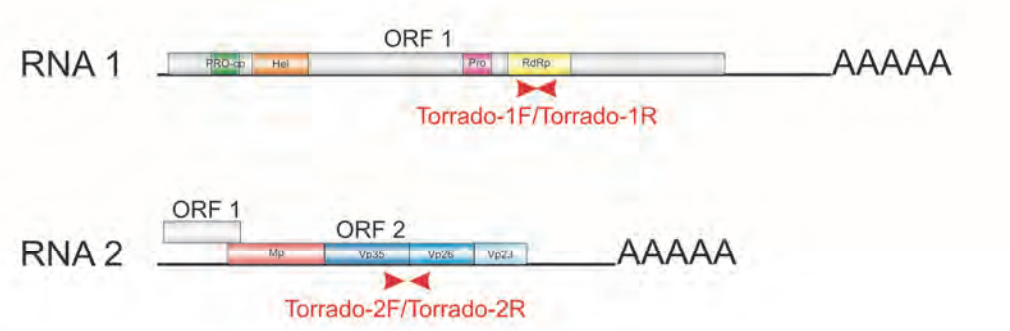

Figure 4.1: Genomic organization of members of the genus *Torradovirus*. Positions of primer pairs Torrado-1F/Torrado-1R and Torrado-2F/Torrado-2R are indicated.

two conserved regions were chosen to design degenerate primers for RT-PCR amplification. The first primer pair (Torrado-1F/Torrado-1R) was designed in the RdRpcoding region in RNA1 (Fig. 4.1) with a predicted amplicon of 371 bp. The second degenerate primer pair (Torrado-2F/Torrado-2R) was designed in a region overlapping the two first coat proteins Vp35 and Vp26 in RNA2 (Fig. 4.1). These primers had a predicted amplicon size of 515 bp. An overview of primer sequences, their positions on the different templates, and the expected amplicon sizes is given in Table 4.2.

PCR conditions

RT-PCR was performed in a two- or one-step format. For the two-step RT-PCR, complementary DNA (cDNA) was synthesized using Superscript™ III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) in a final volume of 20 µl. Total RNA (4 µl) was mixed with 4 µl of 5× RT buffer, 0.2 µl of 40 U/µl RNasin® Plus (Promega, Madison, WI, USA), 0.5 µl of 0.5 µg/µl random hexamer primers and nuclease free (DEPC-treated) water to give a reaction volume of 16 µl. This solution was heated for 10 min at 70 °C and immediately placed on ice for 1 min, then incubated with 0.5 µl each of water and 200 U/µl Superscript™ III reverse transcriptase, 2 µl of DTT and 1 µl of 10 mM dNTPs for 1 h at 50 °C. The PCR reaction was performed with GoTaq® Green Master Mix (Promega, Madison, WI, USA), using 2 µl of cDNA template, 10 µl of 2× GoTaq master mix, 1 µl each of 10 mM/µl forward primer and 10 mM/µl reverse primer and 6 µl of nuclease free water, making a total volume of 20 µl. The one-step RT-PCR was performed with the SuperScript® III One-Step RT-PCR system with Platinum® Taq polymerase (Invitrogen, Carlsbad, CA, USA) using 1 µl of RNA template, 1 µl each of 10 mM/µl forward primer and 10 mM/µl reverse primer, 1 µl of a 10 mg/ml BSA solution in water, 0.8 µl of SSIII RT/Platinum Taq mix and 0.2 µl of RNasin® Plus, and made up to a final volume of 20 µl with nuclease-free water. An alternative one-step RT-PCR was performed in the PRI laboratory using the Access RT-PCR System (Promega, Madison, WI, USA). For this RT-PCR, 1 µl of template was used in a mix of 5 µl of 5×AMV/Tfl buffer, 0.5 µl of 10 mM dNTPs, 1 µl of 25 mM $MgSO_4$, 1 µl each of 10 mM/µl forward primer and 10 mM/µl reverse primer, 0.5 µl of AMV reverse transcriptase, 0.5 µl of Tfl polymerase and 14.5 µl of nuclease free

Table 4.2: Primers, primer positions on the genomes of four torradoviruses, and expected amplicon sizes.

Primer	Primer sequence (5'-3')	Torradovirus species							
		ToTV (PRI-ToTV0301)		ToMarV (PRI-TMarV0601)		ToChSV		ToChV (ToChV-G01)	
		Position	Exp. size	Position	Exp.size	Position	Exp. size	Position	Exp. size
Torrado-1F	GCWGAYTAYTCMAGYTTTGATGG	RNA1 4247-4269	371	RNA1 4284-4306	371	RNA1 4288-4310	371	RNA1 4288-4310	371
Torrado-1R	GGWACWGCMACHAGRTTGTCATC	RNA1 4595-4617		RNA1 4631-4654		RNA1 4636-4658		RNA1 4636-4658	
Torrado-2F	TGGGATGARTGYAATGTKCT	RNA2 2589-2608	515	RNA2 2528-2547	515	RNA2 2561-2580	515	RNA2 2568-2587	515
Torrado-2R	CCWGTCCACCAYTTGCAATT	RNA2 3084-3103		RNA2 3023-3042		RNA2 3056-3075		RNA2 3063-3082	

water, making a total reaction volume of 25 µl. RT-PCR was performed on a GeneAmp® PCR system 9700 (Applied Biosystems, Foster City, CA, USA) at MPI, and on an Analytik Jena Alpha SC® (Analytik Jena AG, Jena, Germany) at PRI, using the cycling programs listed in Table 4.3.

An internal-control PCR was done for all samples listed in Table 4.1 prior to targeted PCR tests to ensure the PCR competency of the RNA extracts. The internal control PCR was performed using *nad*5-s and *nad5*-as primers which amplify a 181 bp part of the plant gene *nad*5 (NADHdehydrogenase subunit 5) (Menzel et al., 2002). The *nad*5 reaction was set-up as a one-step PCR using the master mix reagents described previously and using the same one-step cycling conditions shown for each laboratory in Table 4.3.

Table 4.3: PCR programs

Laboratory	MPI		PRI
Method	Two step RT-PCR[1]	One step RT-PCR[1]	One step RT-PCR
Thermocycler program	(Separate RT step)	30 min 48 °C	45 min 48 °C
	5 min 94 °C	5 min 94 °C	2 min 94 °C
	40 cycles:	40 cycles:	40 cycles:
	30 sec 94 °C	30 sec 94 °C	30 sec 94 °C
	30 sec 52 °C	30 sec 52 °C	60 sec 50 °C
	60 sec 72 °C	60 sec 72 °C	60 sec 68 °C
	5 min 72 °C	5 min 72 °C	6 min 68 °C

[1] Non-specific banding with primer pair Torrado-1F/Torrado-1R as observed in samples 9 and T could be prevented by lowering the extension temperature from 72 °C to 68 °C.

Agarose gel electrophoresis

A 5 µl aliquot from each reaction was mixed with 1 µl of Orange G loading buffer containing GelRed (Biotium, Hayward, CA, USA), loaded on a 1% agarose gel (Roche, Mannheim, Germany) in 0.5× TBE or 1× TAE buffer. The

sizes of PCR amplicons were estimated by comparison to a 100 bp DNA ladder or a 1 kb plus DNA ladder (Invitrogen, Carlsbad, CA, USA).

Cloning and sequencing

Products of the expected size were extracted using MicroSpin S300 HR Columns (GE Healthcare, Buckinghamshire, UK), and ligated into the pCR4-TOPO TA cloning vector system (Invitrogen, Carlsbad, CA, USA). All amplicons were cloned except for some PCR products obtained with the Torrado-2F/Torrado-2R primers (FD2136, 1883, 2263, ToTV-Can, GNC06, H3, MUR06 and MUR09) which were sequenced directly. A minimum of two clones per amplicon were sequenced using vector-specific primers on an ABI Avant 3100 Genetic analyzer using BigDye 3.2 chemistry by EcoGene®, Landcare Research (Auckland, New Zealand). Sequences were assembled and aligned using Geneious 5.0.4 software (Biomatters Ltd, Auckland, New Zealand). PCR amplicons of PRI-ToTV0301, PRI-TMarV0601, ToChV-G01 and ToTV-T418 were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and sequenced directly in a ABI prism 3730xl at the sequencing facilities of Greenomics™ (Wageningen, The Netherlands), using the same primers as used for the RT-PCR. The obtained sequences were aligned using CLC Main Workbench 6 software (CLC Bio, Aarhus, Denmark). All obtained sequences were compared to sequences held in the GenBank database using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1997).

Results

All nucleic acid tested with the internal-control RT-PCR for mRNA of the mitochondrial *nad*5 gene, resulted in a specific 181 bp product (data not shown) showing that the RNA extractions were of good quality and PCR competent. RT-PCR using primer pair Torrado-1F/Torrado-1R resulted in amplicons of the expected size (371 bp) for all tested torradovirus isolates (Fig. 4.2A and B), except for isolate TEN07 (sample nr. 9), which was probably due to a low virus concentration. These results did not differ in repeated experiments using both the one-step and two-step RT-PCR protocols. Faint bands, different in size from those expected for torradoviruses, were observed when healthy tomato leaf material (lane T) and isolate TEN07 were tested.

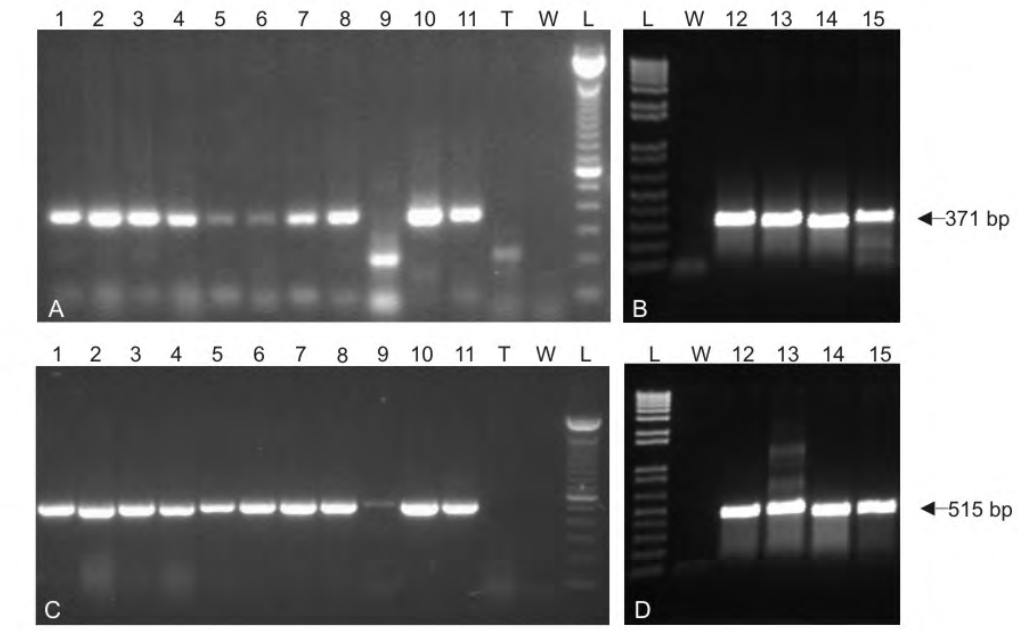

Figure 4.2: Results of RT-PCR on samples of torradoviruses using primer pairs Torrado-1F/Torrado-1R (A, B) and Torrado-2F/Torrado-2R (C and D). Taxonomy and origin of samples 1–15 are listed in Table 4.1. T = healthy tomato, W = water control, L = molecular markers. Experiments were carried out at MPI, New Zealand (A and C) or at PRI, The Netherlands (B and D). The expected amplicon sizes of each primer pair are indicated.

These non-specific bands were not observed when the extension temperature of the RT-PCR cycles was reduced from 72 ◦C to 68 ◦C. There were no non-specific bands observed when specific target sequences were present in the sample or in the water control (lane W). Using the second primer pair Torrado-2F/Torrado-2R, RT-PCR resulted in amplicons of the expected size (515 bp) (Fig. 4.2C and D). Also with isolate TEN07 a specific amplicon was obtained, however the signal was very low. For the second primer set no non-specific bands were observed in the healthy tomato leaf control or water control (lanes T and W, respectively). The nucleotide sequences obtained for all PCR amplicons confirmed the torradovirus origin of the viruses present in the tested samples. The nucleotide sequences also confirmed the species of all torradovirus isolates, however, additional sequences of ToChV were found in

the ToChSV sample (nr. 11), indicating a mixed infection of ToChSV and ToChV in this sample.

Discussion

In a time-span of just a few years, several viruses have been discovered belonging to the new genus *Torradovirus* (Batuman et al., 2010; Sanfaçon et al., 2009; Turina et al., 2007; Verbeek et al., 2007, 2008, 2010a). The diseases caused by these viruses have only been found in tomato crops, and are characterized by severe necrosis in leaves and fruits. These symptoms on tomatoes are typical and very similar for all of the torradoviruses. However, the nucleotide sequences for the torradovirus species differ substantially and it is therefore laborious to use different species-specific primers to determine which torradovirus may be causing the symptoms on suspected infected plant material. The use of generic primers in RT-PCR would be a useful tool to speed up torradovirus detection. The bi-partite genomes of viruses belonging to the genus *Torradovirus* contain some highly conserved regions. Two of these regions were selected to design two universal primer pairs, in particular the RdRp coding region in RNA1 and the coat protein region in RNA2, specifically overlapping the 3'-end of the Vp35-coding region and the 5'-end of the Vp26-coding region (Fig. 4.1). The two primer pairs, Torrado-1F/Torrado-1R and Torrado-2F/Torrado-2R were tested for their ability to detect 15 isolates belonging to four different torradovirus species (including two tentative species). Internal-control PCR amplicons were successfully obtained from all nucleic acid extracted from the torradovirus infected samples indicating that the RNA from all samples was PCR competent.

With the exception of isolate TEN07, which was not detected with primer pair Torrado-1F/Torrado-1R, all other torradovirus isolates could be detected using both primer pairs. Isolate TEN07 may have been in a low titer in the plant tissue, as this isolate was also negative when tested with ToTV-specific primers TR1F/1R and TR2F/2R (Pospieszny et al., 2007) in RT-PCR. This hypothesis was confirmed when isolate TEN07 was tested with primer pair Torrado-2F/Torrado-2R in RT-PCR and a small band was seen in the agarose gel. This result strongly suggests that primer pair Torrado-2F/Torrado-2R is more sensitive than primer pair Torrado-1F/Torrado-1R. The Torrado-2F/Torrado-2R primer pair is also more specific than the Torrado-

1F/Torrado-1R primers, as a faint band was observed with the latter primer pair when testing healthy tomato material. This non-specific band was also observed with isolate TEN07 (sample nr. 9), and is likely to have resulted because of the target virus being in a low titer. This faint band was not observed when the PCR extension temperature was reduced to 68 ^{o}C and it is very likely that the non-specific band was of plant origin. In the other torradovirus-positive samples this non-specific band was not observed. Both primer sets performed well in two different laboratories, even when different RNA extraction protocols, RT-PCR kits, cycling conditions and thermocyclers were used. Also no differences in results were noticed between one-step and two-step RT-PCR protocols. All sequences obtained from PCR amplicons matched the expected sequences in Genbank, except for the isolate of ToChSV, which contained sequences belonging to both ToChSV and ToChV. This result confirmed an earlier suspicion that the ToChSV isolate was co-infected with ToChV (Batuman et al., 2010; Verbeek et al., 2010a). Two universal primer pairs which can detect all currently known torradoviruses were developed in this study. The use of these universal primers might lead to an earlier detection and even the discovery of new viral species belonging to the genus *Torradovirus*.

Acknowledgements

We wish to thank all colleagues mentioned in Table 4.1 for sharing their torradovirus isolates for this study. We are grateful to G.R.G. Clover and R.A.A. van der Vlugt for their constructive comments on this manuscript.

Chapter 5

Torradoviruses are transmitted in a semi-persistent and stylet-borne manner by different whitefly vectors

M. Verbeek, P. J. van Bekkum, A.M. Dullemans and R.A.A. van der Vlugt

Plant Research International, part of Wageningen UR, P.O. Box 69, 6700 AB Wageningen, the Netherlands

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Summary

Members of the genus *Torradovirus* (family *Secoviridae*, type species *Tomato torrado virus*, ToTV) are spherical plant viruses which are transmitted by the whitefly species *Trialeurodes vaporariorum*, and *Bemisia tabaci*. Until today, knowledge on the mode of vector transmission was still lacking for torradoviruses. Here, the mode of transmission has been determined for *Tomato marchitez virus* (ToMarV). A minimal acquisition access period (AAP) and inoculation access period (IAP) of approximately 2 h each was required for vector transmission by *T. vaporariorum*, while optimal transmission required an AAP and IAP of at least 16 h respectively 8 h. Whiteflies could retain the virus under non-feeding conditions for at least 8 h without loss of transmission efficiency, but upon feeding on a non-host plant in between the AAP and IAP retained the virus for maximally 8 h. Similar conditions supported transmission of two other isolates, belonging to the species ToTV and Tomato chocolàte virus (ToChV), by *T. vaporariorum* and *B. tabaci.* Additionally, similar experiments revealed the identification of the banded-winged whitefly (*Trialeurodes abutilonea*) as a vector for these three virus species. The results are congruent with acquisition and retention periods generally measured for semi-persistent virus transmission. RT-PCR detection analysis of ToTV and ToMarV in the vector's body revealed their presence in the stylet, but not in the head where the pharynx of the foregut is located.

The results altogether indicate a semi-persistent stylet-borne mode of vector transmission for torradoviruses, which represent the first group of spherical viruses transmitted by at least three different species of whiteflies.

Keywords

Epidemiology, *Torradovirus*, *Trialeurodes abutilonea, Trialeurodes vaporariorum*, *Bemisia tabaci*

Introduction

The genus *Torradovirus* (family *Secoviridae*) currently harbors the two species *Tomato torrado virus* (ToTV) and *Tomato marchitez virus* (ToMarV). Additionally, two tentative species were assigned to this genus; Tomato chocolate spot virus (ToChSV) and Tomato chocolàte virus (ToChV) (Sanfaçon et al., 2012). All viruses mentioned infect tomato (*Solanum lycopersicum* L.) and are able to cause substantial economic losses in susceptible cultivars due to necrosis on the leaves and necrotic rings and patches on the fruits (Batuman et al., 2010; Verbeek et al., 2007, 2008, 2010a). A severe necrosis in leaves, stems and fruits may occur, giving the plant a burnt-like appearance. This burnt-like appearance was the origin of the name of torrado disease (torrado means burnt or roasted), given by the Spanish local farmers, who were facing this new disease in their tomato crops in the beginning of this century (Alfaro-Fernández et al., 2010a).

Members of the genus *Torradovirus* possess small spherical virions, approximately 30 nm in diameter, which are composed of three coat proteins with molecular masses of approximately 23, 26, and 35 kDa. The genome of torradoviruses is bi-partite and consists of single stranded positive sense RNAs with approximate sizes of 7.2-7.8 kb for RNA1 and 4.9-5.7 kb for RNA2. RNA1 contains one open reading frame (ORF) with motifs for protease, helicase and RNA-dependent RNA polymerase (RdRp). RNA2 contains two ORFs of which, after comparison to other plant picorna-like virus genomes, the most 5'-located ORF appears unique to torradoviruses. The function of this RNA2-ORF1-encoded protein is still unclear. The second ORF on RNA2 has coding regions for a putative movement protein and the three coat proteins (Verbeek et al., 2007). Torradovirus genomes have remarkably long 3'-untranslated regions (UTRs) on both RNAs which range from approximately 652 nt (ToMarV) to over 1400 nt (ToChV). These 3'UTRs possess unique species-specific regions which are highly conserved between the two RNAs of the same torradovirus species (Verbeek et al., 2010a).

The observation of large amounts of whiteflies (family *Aleyrodidae*) in greenhouses containing tomato crops infected with torrado disease pointed towards a whitefly-borne nature of the causal agent. Until 2005, all plant viruses transmitted by whiteflies belonged to either the genera *Begomovirus*, *Crinivirus*, *Ipomovirus* or *Carlavirus* (Jones, 2003; Navas-Castillo et al., 2011).

Virions from these viruses have either a geminate (begomoviruses) or filamentous (criniviruses, ipomoviruses and carlaviruses) morphology and are transmitted in a persistent and semi-persistent manner, respectively. However, for the whitefly transmitted carlavirus *Cowpea mild mottle virus* (CPMMV) both non-persistent and semi-persistent transmission was reported (Brown and Czosnek, 2002; Jeyanandarajah and Brunt, 1993; King et al., 2012; Navas-Castillo et al., 2011). Until then, no spherical viruses were reported to be whitefly transmitted. Early transmission experiments carried out by Pospieszny on a virus isolated from Polish greenhouse tomato crops, which only later was identified as an isolate of ToTV, showed that this virus was transmitted by the greenhouse whitefly *Trialeurodes vaporariorum* (Westwood) but not by the green peach aphid *Myzus persicae* (Pospieszny, 2005; Pospieszny et al., 2010). Later, Amari et al. (2008) showed that ToTV was also transmitted by the silverleaf whitefly *Bemisia tabaci* (Gennadius). Although these studies identified whiteflies as the insect vector of torrado disease, the mode of transmission remained unknown.

Unraveling the mode of transmission of torradovirus is an important factor in the understanding of the epidemiology of these viruses, which is needed for the development of disease management and control strategies (Jeger et al., 2004). Here, the length of time that whiteflies need to acquire these viruses (acquisition access period, AAP), the length of time needed to inoculate the virus into a new host (inoculation access period, IAP), and the length of time that the virus is retained and transmitted by the vector (retention period) has been determined for two isolates of ToMarV and the vector *T. vaporariorum*. Additional transmission studies, applying the optimal AAP and IAP as determined for ToMarV, were conducted with ToTV and ToChV using *T. vaporariorum* and *B. tabaci*.

As the banded-winged whitefly *T. abutilonea* (Haldeman) has been reported as a vector for four other plant viruses - amongst them are two viruses which are also transmitted by other whitefly species; *Sweet potato chlorotic stunt virus* (SPCSV) and *Tomato chlorosis virus* (ToCV) (Jones, 2003) -, also this whitefly was tested for its ability to transmit ToTV, ToMarV and ToChV.

The results indicate that torrado viruses are transmitted in a semi-persistent manner by the whitefly species *T. vaporariorum*, *T. abutilonea* and

B. tabaci. Initial localization studies of ToTV and ToMarV in the whitefly suggest that torradoviruses retain on the stylets and are thus transmitted in a stylet-borne manner.

Materials and methods

Virus isolates

Tomato torrado virus (ToTV), *Tomato marchitez virus* (ToMarV) and Tomato chocolàte virus (ToChV) were isolated from infected tomato leaves and propagated in *Physalis floridana*, *Nicotiana glutinosa* or *Nicotiana hesperis* '67A' as described before (Verbeek et al., 2007, 2008, 2010a). The origins of the virus isolates used in this study are listed in table 5.1. All virus isolates were maintained in alternative host plants in the greenhouse and stored as leaf samples in liquid nitrogen.

Table 5.1: Origin of virus isolates used in this study

Virus Species	Isolate	Origin
ToTV	PRI-ToTV0301	Spain
ToMarV	PRI-TMarV0601	Mexico
ToMarV	T592	Mexico
ToChV	ToChV-G01	Guatemala

Insect species

A colony of the greenhouse whitefly, *Trialeurodes vaporariorum* (Westwood), was a kind gift of J. Klapwijk, Koppert BV, Berkel en Rodenrijs, the Netherlands. This colony was propagated and maintained on *Nicotiana tabacum* cv. 'White Burley'. The silverleaf whitefly, *Bemisia tabaci* (Gennadius) (type B), was obtained from the Laboratory of Entomology, Wageningen University, Wageningen, The Netherlands. *B. tabaci* was also maintained on *N. tabacum* cv. 'White Burley'. Adults of the banded-winged whitefly, *Trialeurodes abutilonea* (Haldeman) were a kind gift of dr. W.M. Wintermantel, USDA, Salinas, Ca, USA. They were the start of a colony of *T. abutilonea* which was maintained on the host plant *Physalis acutifolia* (Miers) Sandwith (synonym: *P. wrightii).* A colony of the green peach aphid (*Myzus persicae*,

biotype Mp2) (Verbeek et al., 2010b) was maintained on Chinese cabbage (*Brassica rapa* var *pekinensis* L.). All colonies were kept in insect rearing cages (BugDorm, Taichung, Taiwan) in a climate chamber at 20 °C (± 1°C) and a day-night regime of 16 h / 8 h.

Transmission studies

After the evaluation of several test plants for their use in transmission studies, we decided to use *N. glutinosa* as source plant and *N. hesperis* '67A' as receptor plant. Both plants were accepted by the three whitefly species as a feeding source (*T. abutilonea* accepted *N. glutinosa* only for a period of 24 h), and were susceptible to all virus isolates used in the experiments. In order to prevent loss or decrease of transmission efficiency by repeated passages of mechanical inoculation, the *N. glutinosa* source plant was always mechanically inoculated with a homogenate from a torradovirus-infected *N. hesperis* '67A', which was inoculated by whiteflies previously. The first successful whitefly-mediated transmission was established following the procedure of Pospieszny et al (2010), using *T. vaporariorum* as vector.

Three time factors are indicative for the mode of transmission of plant viruses: a) the time that whiteflies need to acquire the virus from the source plant (acquisition access period, AAP), b) the length of time in which whiteflies can transmit the virus after acquisition (retention period), and c) the time that whiteflies need to inoculate the receptor plant (inoculation access period, IAP).

Determination of the minimal and optimal acquisition access period

The majority of the experiments were conducted with ToMarV and the whitefly species *T. vaporariorum*. In each experiment, approximately 275 adult whiteflies were caught in a 250 ml Erlenmeyer flask, using a simple aspirator device. We did not select for males or females or for a certain age, but caught the whiteflies randomly from the colony. When whiteflies were actively feeding and difficult to dislodge, they were left alone in order to avoid damaging them. The Erlenmeyer was covered with a piece of Parafilm® and the whiteflies were left for a starvation period of 2 h. Subsequently, the whiteflies were released in an insect rearing cage containing one *N. glutinosa* source plant. The whiteflies were left for probing and feeding on the source

plant for a certain AAP varying from 0.5 h to 48 h. When the whiteflies were given their AAP, 4 cohorts of 50 adults were caught in small Erlenmeyer flasks (50 ml) and the flasks were covered with Parafilm®. Each Erlenmeyer was placed near a *N. hesperis* '67A' test plant under a small cage. During this procedure the Parafilm® was removed from the opening of the Erlenmeyer, giving the whiteflies access to the test plant. For experiments with AAP's shorter than 24 h, the rest of the whiteflies remained on the source plant in order to fulfill an AAP of 24 h. Subsequently, 50 whiteflies were collected in the same way as described above and also placed under a cage containing a *N. hesperis* '67A' test plant. This control experiment was performed to check whether the source plant was in good condition to acquire virus from.

Following an IAP of 24 h, whiteflies were removed from the test plants using a small brush wetted in tap water containing a drop of detergent (hand soap). Removal of whiteflies with a small brush cannot be done without whiteflies escaping. Therefore, this procedure was carried out in a glove cabinet that was connected to a -40 freezer. Whiteflies that escaped from the plant were trapped in the top of the glove cabinet and were killed by frost after the test plants were removed from the glove cabinet. The plants were double checked for perchance remaining whiteflies and then transferred to the greenhouse and grown at 20 ^{o}C and 16 h light until evaluations for characteristic symptoms at two and three weeks post inoculation. The determination of the minimal AAP was conducted three times with ToMarV; two experiments with isolate T592 and one with isolate PRI-TMarV0601.

Determination of the retention period

Approximately 1400 adult *T. vaporariorum* were placed in a cage containing 4 source plants infected with ToMarV (isolate T592). After an AAP of 24 h, cohorts of 50 adults were caught in small 25 ml Erlenmeyer flasks. Four of the cohorts were transferred to test plants immediately (one cohort per test plant, no starvation period). Retention of the virus was tested by leaving the whiteflies in the other Erlenmeyers for a starvation period of 1, 2, 4, 6 or 8 h before transferring the whiteflies to the test plants for an IAP of 24 h. Per time point, 4 test plants were inoculated with one cohort of 50 whiteflies per test plant. A maximum of 8 h was used as starvation time, as longer periods will cause too many whiteflies to die. As this test only measures

the retention under non-feeding conditions, an alternative experiment was conducted where the whiteflies were allowed to feed for 1, 2, 4, 6, 8, 24 or 32 h in between the AAP and IAP on cotton (*Gossypium hirsutum* L.), which is a non-host for ToMarV. The determination of the maximal retention period on an intermediate host was measured three times; two experiments using isolate T592 and one using isolate PRI-TMarV0601.

Determination of the minimal and optimal inoculation access period

Experiments for the determination of the minimal IAP were conducted with *T. vaporariorum* and ToMarV, isolate PRI-TMarV0601. Whiteflies (approximately 700 adults) were given an AAP of 24 h on a source plant. After catching 12 cohorts of 50 whiteflies in small Erlenmeyers, the whiteflies were given a starvation period of 0.5 h in the same Erlenmeyer flasks. This was done to reduce the time until probing and acceptance of the test plant. Subsequently, each cohort of whiteflies was transferred to a small cage containing one *N. hesperis* '67A' plant for an IAP of different lengths (0.5, 1, 2, 4, 6, 8 and 24 h). Three experiments were performed, in each experiment two test plants were inoculated for each IAP (so in total 6 test plant were inoculated per time point, divided over three experimental dates). The whiteflies were removed from the plants (this was done within 5 minutes following the given IAP) as described above. By removing the insects by hand, one has more control on the IAP times (no 'slow' killing by chemicals with disturbed behavior of whiteflies). The plants were checked two more times within the next 10 min., assuring that no whiteflies remained on the plants before they were moved to the greenhouse. The plants were evaluated for symptoms two and three weeks post inoculation. The minimal IAP was also determined for isolate T592, but in this case in a single experiment four plants were inoculated at each of the time points 0.5, 1, 2, 4, 6, and 8 h.

Transmission of torradoviruses by different whitefly species

Transmission of ToTV, ToMarV, and ToChV by the three whitefly species *T. vaporariorum*, *T. abutilone*a, and *B. tabaci* was verified in 12 experiments, representing the 12 virus isolate – whitefly species combinations. In each experiment the whiteflies were given an AAP of 24 h on an infected *N. glutinosa* plant. Subsequently, the whiteflies were placed on a plant of *N.*

hesperis '67A' for an IAP of 24h. In each experiment 4 cohorts of 50 adults were used, each cohort to infect one test plant. Following the IAP, the whiteflies and plants were treated as described above.

RT-PCR

To confirm the infections in symptomatic test plants and the lack of infection in a-symptomatic test plants, reverse transcriptase polymerase chain reaction (RT-PCR) tests were performed on such plants using the universal torradovirus primer sets Torrado-1F/Torrado-1R and Torrado-2F/Torrado-2R, as described in Verbeek et al. (2012). The RT-PCR products were analyzed by mixing a 5 µl aliquot with 1 µl of Orange G (Sigma-Aldrich, St. Louis, MO, USA) loading buffer containing GelRed (Biotium, Hayward, CA, USA) and loading onto a 1% agarose gel (Roche, Mannheim, Germany) in 0.5×TBE buffer. After electrophoresis, the amplicon sizes were compared to a 1 kb plus DNA ladder (Invitrogen, Carlsbad, CA, USA).

Localization of the retention site of torradoviruses in their whitefly vector

Whiteflies were given an AAP of 20 h on a ToTV (PRI-ToTV0301)- or ToMarV (T592)-infected source plant. After virus acquisition, the whiteflies were caught in Erlenmeyer flasks and divided in three batches. The first batch was placed in a refrigerator (4^{o}C) for 0.5 h to calm down the whiteflies. The other two batches were left in the Erlenmeyer flasks for a starvation period of 4 or 7 h, respectively. Following the starvation period, the whiteflies were placed in a refrigerator for at least 0.5 h until further processing.

The whiteflies were then individually caught with the aid of a small brush and submerged in 70% alcohol in a small petri dish (or watch glass) and dissected by cutting the body at three places: a) the proboscis, as high as possible, most of the times between half way of the labium and just below the labrum, b) between head and thorax, and c) at the wasp-like waist between thorax and abdomen (Fig. 5.1). With this procedure four parts of the whitefly body were harvested: the proboscis (containing the stylets), the head, the thorax, and the abdomen.

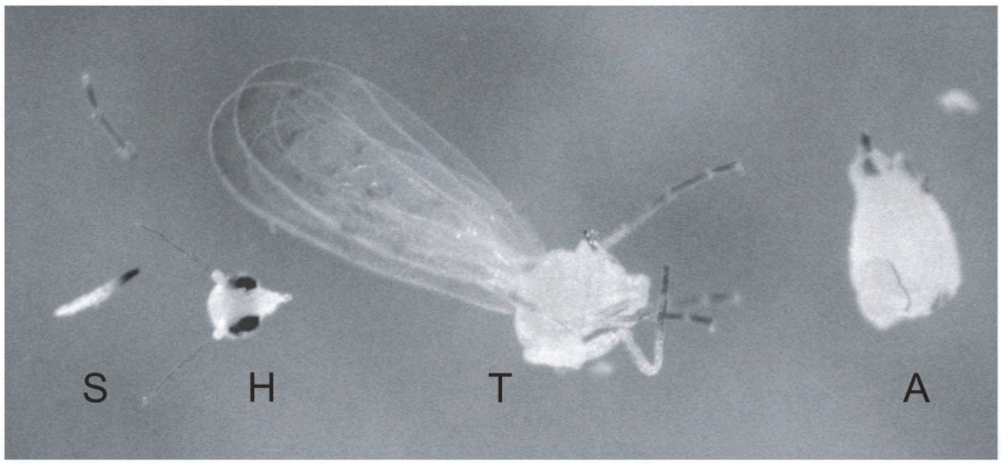


Figure 5.1: Example of a dissected *Trialeurodes vaporariorum*. From left to right the proboscis (containing the stylets) (S), head (H), thorax (T) and abdomen (A).

Dissection was carried out under binoculars with the aid of a micro scalpel (Feather MicroScalpel, angle 30°). After every dissection of an individual whitefly, the alcohol in the watch glass was replaced. The different dissected parts of in total 12 whiteflies were pooled in a 1.5 ml Eppendorf tube containing 25 µl Milli-Q water. As the dissected probosces were too small to take with forceps, they were taken up with the aid of a 10 µl pipet (set at a volume of 5 µl). The samples were stored in -20 °C until they were further processed. After slow thawing on ice, the samples were ground using a small Teflon pestle (tissue grinder for 1.5 eppendorf tubes). Subsequently, the samples were subjected to RNA extraction using the Qiagen RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), essentially following the manufacturers' protocol using 450 µl of RLT buffer, containing 4.5 µl β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA) to start the procedure and 30 µl RNase free water to elute the RNA sample. The extracted RNA was tested in an RT-PCR using the generic primer set Torrado-2F/Torrado-2R (Verbeek et al., 2012). PCR amplicons were analyzed as described above.

In a second experiment whiteflies of the species *T. vaporariorum*, *T abutilonea* and *B. tabaci* were allowed to acquire ToTV for an AAP of 20 h from an infected *N. glutinosa*. Simultaneously, *M. persicae* was allowed to acquire ToTV from an infected *N. occidentalis* 'P1', which was better accepted by this aphid than *N. glutinosa.* All insects were given a starvation period of 2 h at room temperature before placing them at 4 °C. Following the acquisition period, the insects were individually submerged in 70% alcohol in a watch glass under a binocular microscope and their proboscis, including the stylets, was removed. For the aphids we used micro-scissors to cut off the proboscis, for the whiteflies a micro scalpel was used. When the proboscis was cut off, a small amount of the alcohol containing the dissected proboscis was taken up with a 10 µl pipet (set at a volume of 5 µl). Per sample, the dissected probosces were pooled to a total of 15 in an Eppendorf tube containing 25 µl Milli-Q water. As a control, each time a proboscis was collected from the alcohol, also a small amount of this alcohol (or dissection fluid) was taken to test whether virus particles were released into the dissection fluid from eventually remaining virus in the guts. Also per insect species 5 whole bodies (except for the proboscis) were pooled in 25 µl Milli-Q water. After every dissection, the alcohol in the watch glass was replaced. The whole procedure was also performed with non-viruliferous adults for the three whitefly species as negative controls. The samples were stored at -20 °C until they were further processed as described above.

Results

Transmission experiments

Pilot experiments were conducted with different torradovirus isolates, whitefly numbers and test plants, following the conditions described by Pospieszny (2010). The results from these experiments (data not shown) were leading in the choice for the experimental set-up for the determination of the AAP, IAP and retention period.

Determination of the minimal and optimal acquisition access period

To determine the minimal and optimal AAP for the transmission of ToMarV by the vector *T. vaporariorum*, experiments with different AAPs were

performed. Transmission of the virus was observed, but only when the time that the whiteflies were allowed to probe and feed on the source plant was at least 2 h. Successful transmission was scored after appearance of characteristic symptoms, indicative for the presence of ToMarV, in the test plant *N hesperis* '67A'. These symptoms often started as necrotic local lesions at the feeding sites of the whiteflies, but only in small numbers (maximum 6 per plant). Systemic chlorosis, necrosis and distortion of the leaves were observed within 4-7 days post inoculation. However, this was not necessarily preceded by the occurrence of local lesions. Some plants reacted very slowly and did not show symptoms until 14 days post inoculation. For that reason, plants were evaluated two and three weeks post inoculation. To confirm that plants showing symptoms were indeed infected with torradovirus, and those that did not show symptoms were virus-free, six plants were analyzed by RT-PCR (three positive and three negative) for the presence of the virus. The RT-PCR results were congruent with the visual evaluation of the plants, based on which plants in following experiments were evaluated only visually. In one experiment, 100% infection (4 infected out of 4 inoculated plants) was obtained after whiteflies were given an AAP of 4 h, but during the two other experiments an AAP of 8 or 16 h was needed to get the same result (see Fig. 5.2A). In conclusion, the minimal AAP for ToMarV determined in these experiments was 2h, while an AAP of at least 16 h was needed for optimal transmission when both ToMarV isolates were considered.

Determination of the retention period of torradoviruses in whiteflies

Having determined the minimal AAP for acquisition of ToMarV, the retention period was determined. To this end *T. vaporariorum* that were given access to ToMarV-infected test plants, were next kept in an Erlenmeyer flask without feeding for increasing periods of time (i.e. the retention period) prior to transferring the whiteflies to test plants for virus inoculation. A period up to 8 h did not result in a lower transmission rate at all. When the whiteflies were given an intermediate host (cotton) in between AAP and IAP, the number of infected plants rapidly dropped with increasing retention periods and transmission was entirely lost between 8 and 24 h. (Fig. 5.2B) .

Determination of the minimal and optimal inoculation access period

To determine the inoculation access period of ToMarV, *T. vaporariorum* adults were given an AAP for 24 h and put on receptor plants during increasing periods of time to allow for probing and feeding. The minimal time that greenhouse whiteflies had to probe and feed on the receptor plant in order to inoculate ToMarV to ~20% of the receptor plants was 2 h but longer IAPs resulted in higher percentages of infected test plants. After an IAP of 8 h 100 % infection was achieved (see Fig. 5.2C).

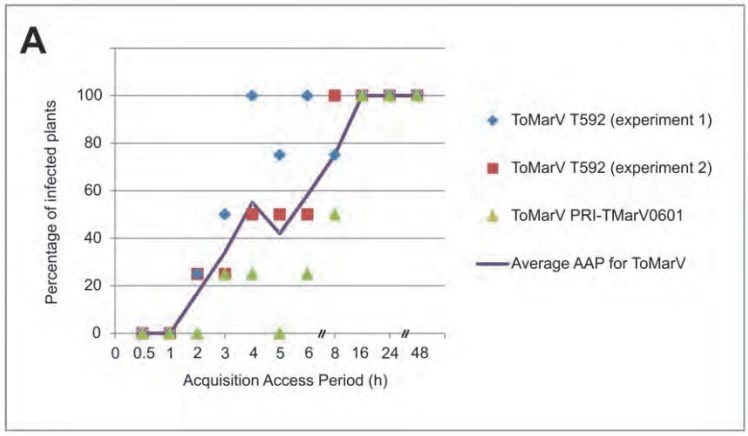
Transmission of torradoviruses by different whitefly species

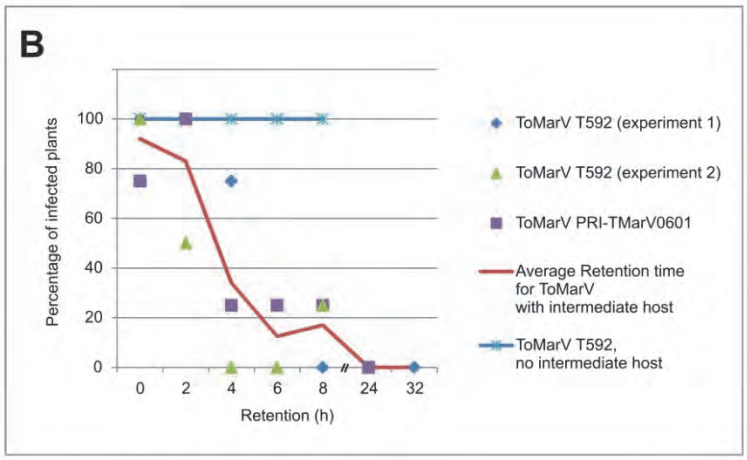
While *T. vaporariorum*, and *B. tabaci* were only reported to transmit an isolate of ToTV, we set out experiments to repeat this with another isolate of ToTV and to analyze whether these vectors were able to transmit isolates belonging to the torradoviruses ToMarV and ToChV. As ToTV was reported to be transmitted by two whitefly species, additional transmission experiments were conducted with the banded-winged whitefly (*T. abutilonea*), which has been reported as a vector of four plant viruses (Jones, 2003). Receptor plants were found positive for the presence of all torradoviruses in combination with each of the whitefly vectors (Table 5.2). Infections were confirmed in RT-PCR, and additionally the torradovirus species causing the infection was confirmed by direct sequencing of the obtained amplicons (Greenomics, Wageningen, the Netherlands).

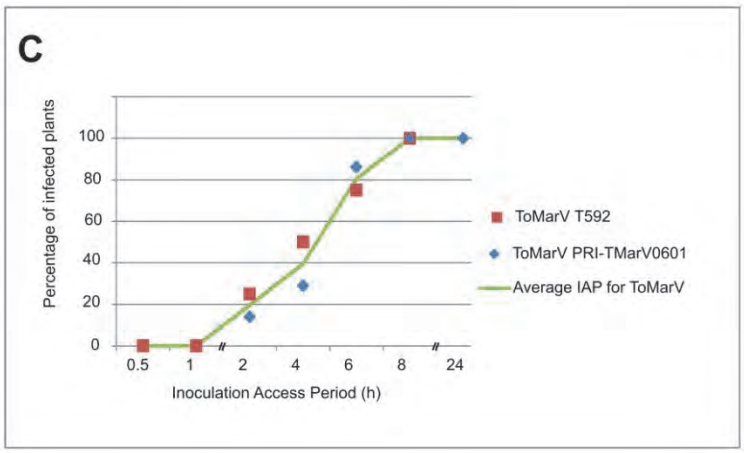
Table 5.2: Transmission of four torradovirus isolates by the whitefly species *Bemisia tabaci*, *Trialeurodes abutilonea*, and *T. vaporariorum* (nr of infected plants / nr of inoculated plants)

Virus Species	Isolate	*B. tabaci*	*T. abutilonea*	*T. vaporariorum*
ToTV	PRI-ToTV0301	2/4	2/4	2/4
ToMarV	PRI-TMarV0601	4/4	2/4	4/4
ToMarV	T592	4/4	4/4	4/4
ToChV	ToChV-G01	3/4	2/4	3/4

Figure 5.2: Determination of the (A) acquisition access period (AAP), (B) the retention time and (C) the inoculation access period (IAP) of the transmission of *Tomato marchitez virus* by *Trialeurodes vaporariorum*. The measured percentages of infected plants are indicated for each time point with different symbols for each experiment. The average AAP, retention period and IAP for each time point is represented by a continuous line.


A
Percentage of infected plants
100
80
60
40
20
0
0 0.5 1 2 3 4 5 6 8 16 24 48
Acquisition Access Period (h)
ToMarV T592 (experiment 1)
ToMarV T592 (experiment 2)
ToMarV PRI-TMarV0601
Average AAP for ToMarV


B
Percentage of infected plants
100
80
60
40
20
0
0 2 4 6 8 24 32
Retention (h)
ToMarV T592 (experiment 1)
ToMarV T592 (experiment 2)
ToMarV PRI-TMarV0601
Average Retention time for ToMarV with intermediate host
ToMarV T592, no intermediate host


C
Percentage of infected plants
100
80
60
40
20
0
0.5 1 2 4 6 8 24
Inoculation Access Period (h)
ToMarV T592
ToMarV PRI-TMarV0601
Average IAP for ToMarV

Localization of the retention site of torradoviruses in their whitefly vector

The results obtained from the transmission experiments indicated a semi-persistent mode of transmission and pointed towards the stylet/foregut as the site of retention. To test this hypothesis and further substantiate the indications for semi-persistent transmission, RT-PCR was employed to detect the presence of the virus in whole or dissected whitefly body extracts. Using the Torrado-2F/Torrado-2R primer set (Verbeek et al., 2012) a positive signal (amplicon of 515 bp) was obtained from whole body extracts of single whiteflies. However, similar tests on dissected probosces from single whiteflies failed, likely due to detection limits. To compensate for this, dissected probosces of 10-15 whiteflies were pooled into one sample. In a similar fashion, other parts of the body were tested.

When testing the probosces of whiteflies which had acquired ToTV, a clear positive signal was observed, even after a long time of starvation (see Fig. 5.3). In contrast, a clear positive signal was always absent from the head, while the signal from thorax and abdomen samples clearly decreased after several hours of starvation of the whiteflies. Similar results were obtained when whiteflies had acquired ToMarV, with one exception: the signals in thorax and abdomen were not lost after starvation for 8 h, even not when whiteflies were allowed to feed on cotton during 4 h following the AAP.

In analogy to the aforementioned approach, the presence of ToTV was assayed in various body parts prepared from *T. vaporariorum*, *B. tabaci* and *T. abutilonea* that acquired the virus. Again, ToTV could be detected in the stylets of all three species, and as well in the whole body homogenates (Fig. 5.4). However, no virus was detected in the dissection fluid, which was tested separately, indicating that the virus detected in the stylets was located in or at the stylets and not leaking from e.g. the intestinal tract of the insects. When the aphid *M. persicae* was tested as negative control, ToTV was not detected in the probosces, while the virus could be detected in the rest of the body, indicating that virus particles did not bind to stylets during passage towards the intestinal tract.

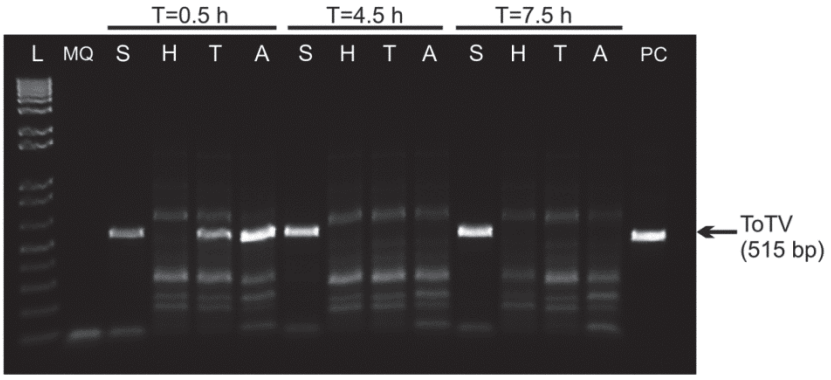


Figure 5.3: Detection of viral RNA (ToTV) by RT-PCR on dissected whiteflies using the Torrado-2F/Torrado-2R primers. The probosces with stylets (S), heads (H), thoraxes (T) and abdomens (A) of 12 adult whiteflies were pooled and tested 0.5 h, 4.5 h and 7.5 h after a 20 h acquisition access period on a ToTV infected plant. The whiteflies were starved for the indicated times (T) in a sealed erlenmeyer before dissection. A sample of ToTV-infected *Nicotiana hesperis* '67A' was used as a positive control (PC).

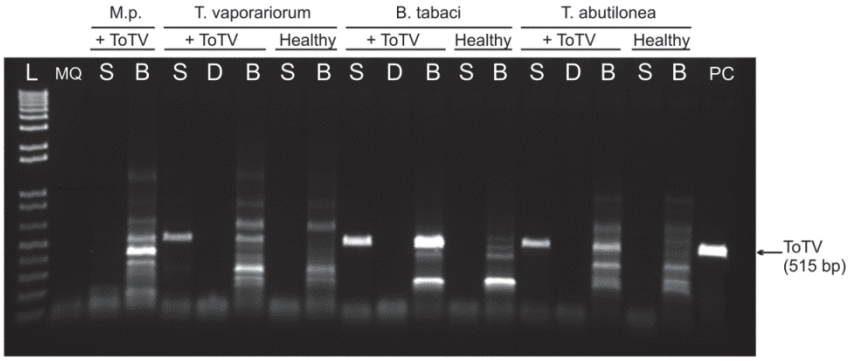


Figure 5.4: Detection of viral RNA (ToTV) by RT-PCR in the stylets of three whitefly species (*B. tabaci*, *T. vaporariorum* and *T. abutilonia*) and, as negative control, in the aphid *Myzus persicae* (M.p.) Pooled samples of 12 probosces with stylets (S), samples of dissection fluid (D), and rest bodies (B) were tested in RT-PCR using the Torrado-2F/Torrado-2R primers after the insects had acquired the virus for 20 h or had been placed on a healthy plant as negative control. A sample of ToTV-infected *Nicotiana hesperis* '67A' was used as a positive control (PC).

Discussion

As vectors interact with all three vertices of the epidemiological triangle (McNew, 1960), the pathogen, the host and the environment, they are of utmost importance to the epidemiology of infectious diseases. Understanding the way how vectors transmit pathogens will increase the knowledge about the epidemiology of a certain disease and will help to develop control measures. Homopteran insects, such as aphids and whiteflies, are efficient plant virus vectors. They have relatively small stylets which can operate intracellularly and pierce cells for probing without damaging the cell too much (Mitchell, 2004). So far only five whitefly species, i.e. *Bemisia tabaci*, *B. afer* sensu lato, *Trialeurodes vaporariorum*, *T. abutilonea* and *T. ricini* (Jones, 2003; Navas-Castillo et al., 2011), are reported to transmit plant viruses, and only few of those viruses are spread by more than one vector species. These present *Tomato chlorosis virus* (genus *Crinivirus*, ToCV) (transmitted by *B. tabaci*, *T. vaporariorum* and *T. abutilonea*) and *Sweet potato chlorotic stunt virus* (genus *Crinivirus*, SPCSV) (*B. tabaci, B. afer* sensu lato, and *T. abutilonea*) (Gamarra et al., 2010; Jones, 2003). There is one report that *Tomato yellow leaf curl virus* (genus *Begomovirus*, TYLCV) is transmitted not only by *B. tabaci* but also by *T. ricini* (Idriss et al., 1997), but this was not confirmed in later studies (Jones, 2003). *Cassava brown streak virus* (genus *Ipomovirus*, CBSV) may also be transmitted by *B. tabaci* and *B. afer*, but there is no solid evidence for *B. afer* being a vector for CBSV (Jones, 2003; Legg and Raya, 1998). Here we provide evidence that torradoviruses, *in casu* species ToTV, ToMarV and ToChV, are transmitted by three different whitefly species (*T. vaporariorum*, *B. tabaci* and *T. abutilonea)*, and that this involves a semi-persistent mode of transmission. This is supported by the required minimal periods measured for virus acquisition and virus inoculation (minimal 2 h), together with a retention period of the virus in the whitefly (up to 8 hours). Experiments to determine the AAP and IAP, as described in this paper, were initially performed with ToMarV, because of its higher virulence compared to ToTV and ToChV. Using this virus, 100% infections were obtained under optimal conditions, while results with ToTV and ToChV were more inconsistent. However, ToTV and ToChV were also efficiently transmitted under the conditions which were found optimal for ToMarV (AAP>16 h and IAP>8 h) by all three whitefly species tested. It is not unlikely that these viruses are

transmitted in a similar manner as ToMarV, as the mode of transmission of all viruses within a virus genus is similar and appears to be a stable evolutionary trait (Nault, 1997). Whether the three whitefly species transmit various torradoviruses with different efficiency, in analogy to the transmission of stylet-borne viruses by aphid vectors, e.g. *Potato virus Y* (Verbeek et al., 2010b), remains to be investigated.

Our data on the localization of the virus in the whitefly vector indicate that the virus retention site is likely to be found in the stylets. We also demonstrated that torradoviruses did not retain in the head sections of whiteflies at detectable levels. A head section contains the part of the feeding tract from labrum until the beginning of the esophagus, including the pharynx. Earlier it was theorized that in non-circular transmission, virions are held at internal vector retention sites from where they are inoculated to plants by egestion (Harris et al., 1996). An analysis of the homopteran feeding apparatus suggests that virions retained at sites beyond the true mouth in the pharynx of the foregut are non-egestible and, therefore, non-transmissible (Harris et al., 1996). As the foregut of whiteflies contain the pharynx (cibarium) and esophagus (Harris et al., 1996; Walker et al., 2010) it is likely that torradoviruses are not foregut-borne but stylet-borne. The positive signals in the thorax and abdomen for ToMarV are probably from virions which are attached to the walls and microvilli of the intestinal tract (esophagus, midgut and hindgut), and are more resistant to break down than virions of ToTV. It was generally assumed that non-persistent viruses are stylet-borne and that semi-persistent viruses are foregut-borne, but, as Ferreres and Moreno (2009) clearly state, such generalizations are inappropriate. A good example is the transmission of *Cauliflower mosaic virus* (CaMV) by aphids, which is transmitted in a semi-persistent manner, but clearly - with the aid of helper proteins - binds to the acrostyle, a structure in the common duct at the tip of the aphids' stylet (Uzest et al., 2010; Uzest et al., 2007).

This report describes the transmission parameters of torradoviruses, the first group of spherical viruses transmitted by whiteflies. Transmission is semi-persistent and involves retention of the virus at the stylets. Next to the aphid-transmitted CaMV, torradoviruses present the second example of

viruses that do not fit in the dogma of semi-persistent transmission in a foregut-borne manner.

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Chapter 6

General discussion

'Torrado', an emerging disease in tomato

An emerging disease is a new or previously known disease that has become of prominent economic or societal importance. The emergence of such a disease is often facilitated by a change (or changes) in some aspect(s) of the pathogen, pathogen vector, host or environment (Gilbertson and Batuman, 2013). In the early 2000s, in the Southeast of Spain an emerging disease was found in tomato crops (Alfaro-Fernández et al., 2010a). This disease caused heavy necrotic symptoms in tomato plants, indicated by local farmers as 'torrado' or 'torrao', and resulted in reduced tomato yield. Also the name 'cribado' (Spanish for 'sieve') has been used, referring to the 'shot-hole' symptoms (Fig. 6.1). These 'shot holes' are caused by necrotic spots in young leaves that turn into holes due to the growing of the surrounding tissue (Alfaro-Fernández et al., 2010b). The cause of this virus remained unclear until 2007, when a new virus was described and identified as the causal agent of this disease (Verbeek et al., 2007).

Figure 6.1: Example of 'cribado' or 'shot-hole' symptoms, here caused by *Tomato marchitez virus*

Tomato torrado virus

As already mentioned in the introduction of this thesis, the discovery and characterization of a new virus is an exciting event. The work described here has begun with such an event. However, the identification and characterization of *Tomato torrado virus* (ToTV) as the causal agent of the torrado disease in tomato did not only have scientific impact. As tomato growers in Spain, but also in other parts of the world (Fig. 6.2), were facing a new and devastating disease of which no one knew the cause, it was a relief to the parties involved that the causal agent had been identified. Also preliminary data became available on the epidemiology of this disease, as it was shown that whiteflies were transmitting the causal agent and spreading the disease. Tomato breeders were very keen on gaining further knowledge on the cause of this disease, because when a causal agent has been identified, they are able to screen their breeding lines for resistance. During the time, that the incidence of torrado disease was on the increase in the area of Murcia in the Southeast of Spain, breeding companies contacted research groups and supplied infected plant material for virus characterization. This was how the Plant Virus Section at Plant Research International started looking at some 'torrado' samples from tomato from Murcia, Spain. As all samples collected at that time in the area of Murcia, these samples were clearly infected with *Pepino mosaic virus* (genus *Potexvirus*, PepMV). At that time even the hypothesis was heard that the torrado disease could be caused by a new necrotic variant of PepMV. The first breakthrough was obtained when the samples were examined by electron microscopy (Chapter 2). It became clear that not only PepMV was present in the samples, which could be recognised as filamentous particles, but that also small spherical virus particles could be observed (Fig. 1.3). This prompted the research to find suitable 'filter' host plants for the separation of the spherical virus from the complex with PepMV, which was successful. Classical virological methods proved to be of great importance to separate and propagate this unknown virus, and to purify and characterize it. In this way it became possible to investigate intrinsic properties of the virus particles and to determine the full genomic sequence. Moreover, the isolated virus could be re-inoculated to tomato. When identical symptoms were observed as in the original material and the virus was re-

isolated from the inoculated plants, Koch's postulates were fulfilled, showing that the new virus was indeed the causal agent of the torrado disease. It became clear that this new spherical virus, which particles consisted of three coat proteins and harboured a bi-partite genome, could not be placed in any known virus genus (Chapter 2). This knowledge was an important step forward, as it would also redirect breeding efforts for resistance against the new virus in tomato varieties and accessions. To underscore the importance of this observation a worldwide patent on the use of ToTV for breeding activities was filed (Van den Heuvel et al., 2010).

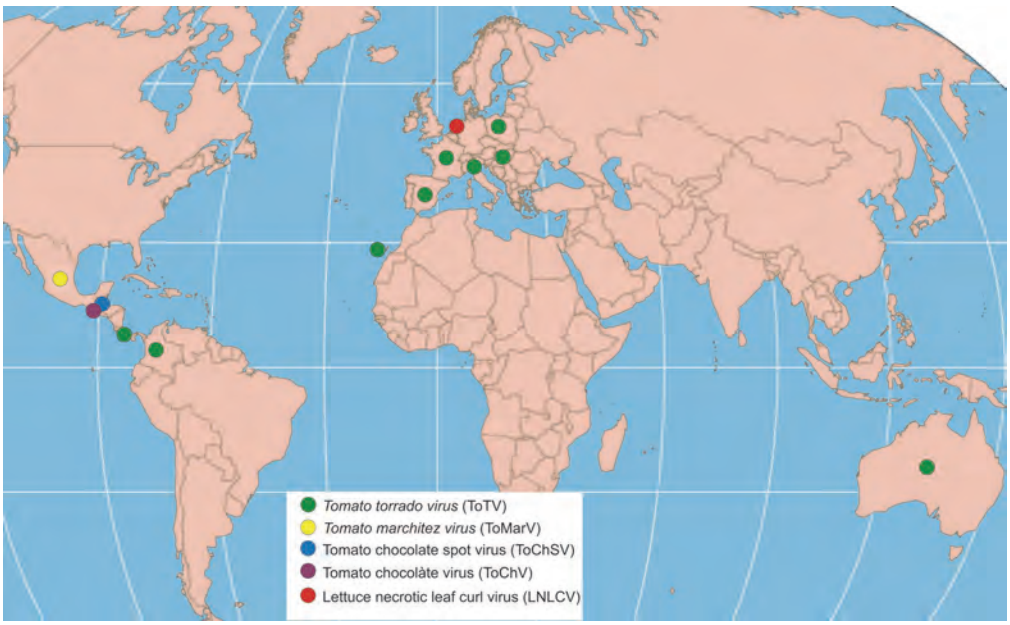

Figure 6.2: Geographical occurrence of torradoviruses, according to published reports

Expansion of the genus Torradovirus

Soon after ToTV was characterized, projects on the characterization of two other diseases started. These diseases were also found in tomato crops and caused symptoms similar to those of torrado disease. For both the causal agent(s) were unknown. The first one was marchitez disease, which was

occurring in Mexico. With the knowledge gained on ToTV it became possible to identify the causal agent of the marchitez disease as a virus that was clearly related to ToTV. However, biological properties and the full genomic sequence made it clear that this causal agent belonged to a new species. This new species, the causal agent of marchitez disease, was named *Tomato marchitez virus* (ToMarV) (Chapter 3a). When the characterization of ToMarV was published, the ICTV working group on plant picornavirales decided to propose a new genus to harbour these two new viruses. This genus was named *Torradovirus* and ToTV became the type species for this genus. Actually, about the same time ToMarV was studied; another research group investigated a similar virus from Mexico, which was also thought to be causing the marchitez disease. The Italian research group named this virus Tomato apex necrosis virus (ToANV). The partial sequence of this virus was published shortly ahead of the full sequence of ToMarV (Turina et al., 2007). The ICTV decided that because of the fact that the full sequence was available for ToMarV and that for ToANV the sequence was not available for some torradovirus-unique regions in the genome, the second species in the genus *Torradovirus* should be named *Tomato marchitez virus*. However, Tomato apex necrosis virus is not identical to ToMarV and it has to be investigated whether this virus represents a different strain within this species.

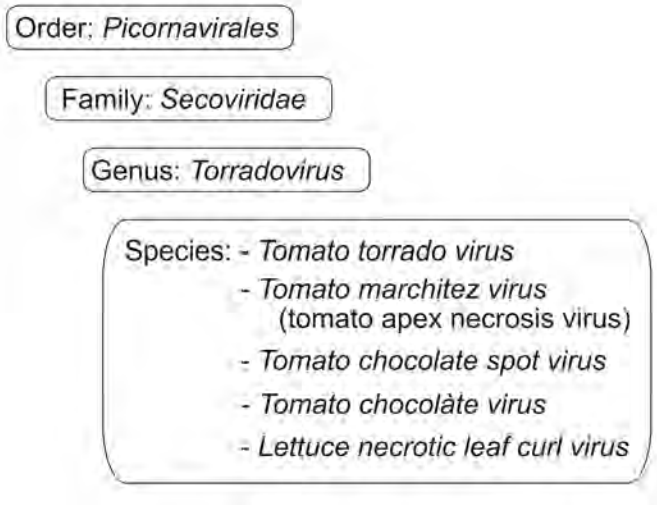

Figure 6.3: Currently proposed taxonomy of the genus *Torradovirus*

Another torrado-like disease was found in tomato crops in Guatemala. Referring to the chocolate brown spots on leaves and fruits, this disease was given the name chocolàte disease. Again, two groups were working at that time on samples from tomatoes showing this disease. One group, located in the USA, isolated and characterized a virus which they name Tomato chocolate spot virus (Batuman et al., 2010). In Wageningen work was done on a torradovirus, which was named Tomato chocolàte virus. Based on the full genome sequences of both viruses, and on additionally proposed species demarcation criteria (Verbeek et al., 2010a), a proposal was made to assign both viruses to a new species (Chapter 3b). In the current Ninth Report of the ICTV (King et al., 2012), both viruses are presented as tentative species within the genus *Torradovirus*. Remarkably, contrary to the other torradoviruses, where the 3' ends of the 3'-UTRs just preceding the poly(A) tails are nearly identical, this part of the RNA1 of ToChSV has a different sequence than the same region in RNA2. Moreover, the RNA1-3'-UTR of ToChSV is nearly identical (99.2%) to the same region in ToChV. This suggested that the original sample of ToChSV was co-infected with ToChV, an observation that was later confirmed when generic primers were tested on the original ToChSV sample (Chapter 4).

It is a remarkable feature that this new genus *Torradovirus*, which was created within the family of *Secoviridae* (Sanfaçon et al., 2009), now harbours four virus species all infecting tomato and causing similar diseases ('burning') in this crop. It took a while before the first torradovirus was found in a crop other than tomato. This virus was found in a lettuce plant, grown in the Netherlands, showing necrosis and leaf curling. This isolate was maintained for more than one year in test plants by repeated mechanical inoculation. Unfortunately, the original sample was lost, but the virus could be mechanically re-inoculated from the indicator plant *Nicotiana occidentalis* 'P1' to lettuce plants, enabling the study of the symptomatology of this virus. Analysis of the genome sequence, which was obtained by Next Generation Sequencing, revealed that the virus had a torradovirus-specific genome organization. The torradovirus from lettuce showed very low sequence identity levels with the tomato-infecting torradoviruses, but could still be detected using the generic primers sets Torrado-1F/Torrado-1R and Torrado-2F/Torrado-2R (which are described in Chapter 4). It was, however, not

possible to trace back the origin of the lettuce-derived virus, so it is not known whether the virus originates from lettuce or was introduced from another plant species. Nevertheless, this torradovirus induced clear symptoms in various lettuce cultivars following mechanical inoculation. It was therefore named after the symptoms it induces in these lettuce plants: Lettuce necrotic leaf curl virus, and proposed to represent a new species in the genus *Torradovirus* (Chapter 3c).

Properties of the torradovirus genome

The genomes of torradoviruses are bi-partite and consist of single stranded positive sense RNAs with approximate sizes of 7.2-7.8 kb for RNA1 and 4.9-5.7 kb for RNA2. RNA1 has a single open reading frame (ORF), which encodes replication proteins including protease, helicase and RNA-dependent RNA polymerase (RdRp). In the RNA2 two ORFs are located. The function of the RNA2-ORF1-encoded protein is still enigmatic. The second ORF on RNA2 has domains of a putative movement protein and encodes the three coat proteins.

Typical features of the Torradovirus genomes are the unusually long 3' un-translated regions (UTRs). They vary from approximately 652 nt (ToMarV) to over 1400 nt (ToChV), excluding the poly(A) tail. Currently, a specific function of these remarkably long 3'-UTRs is unknown. It has been reported that the 3' termini of positive strand RNA viruses (which can be a poly(A) tail, tRNA-like structure, or non-TLS heteropolymeric 3'-sequence) can have various functions in the regulation of RNA stability, translation and targeting of RNA to specific subcellular sites (Dreher, 1999). Current knowledge about functions of the entire 3'-UTR or RNA structures in the 3'-UTR is still fragmentary. One report on the function of such RNA structures of the 3'-UTR of dengue virus clearly shows that deletion of certain domains did not affect translation, but seriously compromised or abolished RNA synthesis (Alvarez et al., 2005). The large size of the 3'-UTRs of torradoviruses may be an indication of the existence of pivotal RNA structures, but this has to be investigated further, i.e. by mutational analysis.

Sequencing techniques

The full-length sequences of the ToTV, ToMarV and ToChV genomes were determined using automated Sanger sequencing. This method, invented in the 1970s, is based on the selective incorporation of chain-terminating dideoxyribonucleotides by DNA polymerase during in vitro DNA replication (Sanger et al., 1977). After the use of fluorescent dye terminators and high throughput capillary systems (Smith et al., 1986), it became the most widely used method for DNA sequencing during the last 25 years. In the 1990s, next generation sequencing (NGS) or pyro sequencing techniques were developed, allowing higher throughput and analysis of the whole genomes of higher organisms (Church, 2006). But also for the much smaller genomes of viruses, these NGS platforms were interesting, especially when unknown viruses were studied requiring a 'de novo' assembly of sequence reads (Adams et al., 2009; Barzon et al., 2013). When NGS became available and affordable to the PRI laboratory in Wageningen, pilot projects on the new platforms started to evaluate their performance on the sequencing of plant virus genomes and the possibilities for 'de novo' assembly. The use of the Illumina Solexa platform (Illumina Inc, San Diego CA, USA) on a sample of an unknown virus from lettuce resulted in the characterization of the new torradovirus LNLCV (Chapter 3C). The successful use of NGS in the characterization of a new virus clearly illustrates the power of these new sequencing techniques. However, it should be noted that besides the sequence information also biological and epidemiological data are essential in understanding the true identity and importance of new viruses.

***The RNA2-ORF1* of torradoviruses**

All torradoviruses described so-far have a unique feature in their genome organization in comparison to other picornavirales: the existence of a small ORF at the 5' end of the RNA2, partially overlapping the large ORF encoding for the putative movement protein and the three coat proteins. This small ORF (RNA2-ORF1) codes for a putative protein of approximately 20 kDa, of which the function is still unknown. One hypothesis for the function is that this protein might have a role in RNA silencing suppression (Ding, 2010). RNA silencing is a gene regulation system that is also used as an antiviral defence mechanism in plants and invertebrates. Viruses in turn often encode for

proteins that can attenuate or counteract this defence mechanism by interaction with certain components of the RNA silencing pathway. Many different viral suppressors of RNA silencing (VSRs), as these viral proteins are called, have been described. These proteins are remarkably diverse in structure and share no obvious sequence similarities (Burgyán and Havelda, 2011). These VSRs act at various steps in the silencing pathway (Burgyán, 2011). Some efficient plant virus VSRs are proteins with molecular masses in the same range as the torradovirus RNA2-ORF1 protein, like the tombusvirus protein P19, *Pothos latent virus* protein P14, or the closterovirus protein P21. Preliminary experiments with *Agrobacterium tumefaciens* transient transformation assays (ATTA), as described in Schnettler et al. (2010), in order to investigate whether the protein encoded by RNA2-ORF1 had the ability to suppress RNA silencing were inconclusive (D. de Ronde and M. Verbeek, unpublished results).

A second possibility for the function of the protein encoded by RNA2-ORF1 is a helper function for the transmission of torradoviruses by their whitefly vectors. Three whitefly species were identified as vector for at least the tomato-infecting torradoviruses ToTV, ToMarV and ToChV: *Trialeurodes vaporariorum*, *Bemisia tabaci* and *T. abutilonea* (Amari et al., 2008; Pospieszny et al., 2010, and this thesis). Torradoviruses are transmitted in a semi-persistent way and have their virus retention site in the stylets of the vector (Chapter 5). This is conflicting with the current dogma that semi-persistently transmitted viruses are foregut-borne. The only other virus for which such a system was described is *Cauliflower mosaic virus* (CaMV) (Uzest et al., 2007). In the transmission of CaMV by aphids, the P2 protein of CaMV acts as a helper component enabling the binding of the virus to the acrostyle, a specific area in the tip of the maxilary stylets of the aphid (Uzest et al., 2010). The P2 protein of CaMV is a polypeptide of 18 kDa (Armour et al., 1983; Moreno et al., 2005) and thus of similar size of the RNA2-ORF1 protein. At this moment we do not know yet if torradoviruses need a helper component for binding of the virus to the stylet or not. The existence of helper components in the transmission of caulimoviruses and potyviruses was shown when aphids were fed on artificial diets containing purified virus particles, which are free from the helper component (Pirone and Blanc, 1996). However, preliminary experiments with

purified ToTV in artificial diet for whiteflies were inconclusive due to the lability of purified ToTV virions.

Concluding remarks

This thesis comprises the work on torradoviruses from the first observation of a new spherical virus in samples of tomato with torrado disease from Murcia, Spain, to the knowledge of today. The torradoviruses are unique and important viruses to study. They are capable of inducing economically important diseases in crops, like in tomato, but also other crops are potential hosts, such as lettuce. Torradoviruses form a new genus within the family *Secoviridae*. They possess an additional ORF in the RNA2 that other picornavirales do not have. The function of the protein encoded by this extra ORF is still not known and deserves further investigation. Furthermore, torradoviruses are the first described spherical viruses, which are transmitted by whiteflies. They also are the first whitefly-vectored viruses that are semi-persistently transmitted and stylet-borne (for the whitefly-transmitted carlaviruses the situation is still unclear whether they are transmitted in a non-persistent or semi-persistent way (Brown and Czosnek, 2002; Jeyanandarajah and Brunt, 1993; King et al., 2012; Navas-Castillo et al., 2011). Whether a helper component is involved in the transmission of torradoviruses and, if so, where in the viral genome this helper protein is encoded remains to be investigated too. In conclusion, this thesis presents research on a new group of viruses which are the causal agents of economically important diseases in tomato and a disease in lettuce for which the agronomic importance is not known yet. It presents data on the characterization of four different torradovirus species and supplies tools for the specific and generic detection of torradoviruses. With the unravelling of the mechanism of the torradovirus transmission by their whitefly vectors, this thesis provides tools to better understand the epidemiology of this group of plant viruses. This knowledge can assist breeding companies, regulatory bodies and inspection services in the development of control measurements for torradoviruses. The current knowledge on torradoviruses is a starting point for further studies, such as how these viruses cause disease and what functions the typical genomic features represent. Ultimately such studies may lead to control or mitigation strategies via plant breeding.

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Summary

The research described in this thesis focuses on the molecular and biological characterization of a new group of plant viruses. At the start of this research only a 'torrado' disease in tomato was known, which is recognised by necrosis on leaves and fruits, that eventually turn into its typical and devastating burnt-like symptoms. Using a wide range of available (virological) tools the causal agent was identified as a so-far unknown spherical virus of approximately 28 nm in diameter. Further analysis revealed that the virus contained a bi-partite RNA genome of which the entire nucleotide sequence was elucidated. The virus was named after the Spanish name of the disease, hence *Tomato torrado virus* (ToTV). Although this virus has been found mainly in Europe, it is emerging and meanwhile found in Central America and Australia.

After the discovery of ToTV, another disease suspected to be caused by a torrado-like virus was analysed. It was shown that this disease, which was found in tomatoes grown in Mexico, was caused by a similar but distinct torradovirus named *Tomato marchitez virus* (ToMarV). Its molecular and biological features, as analysed and described in this thesis, supported the proposal of a new genus denoted *Torradovirus*, named after its first representative. This genus was recognized and classified by the International Committee on Taxonomy of Viruses (ICTV) into the family *Secoviridae* (order *Picornavirales*). Currently, the genus *Torradovirus* harbours, besides the recognised species ToTV and ToMarV, also the tentative species Tomato chocolàte virus (ToChV), Tomato chocolate spot virus (ToChSV, analysed by another research group), and Lettuce necrotic leaf curl virus (LNLCV).

Elucidation of torradovirus genome sequences revealed that their RNA1 contained one open reading frame (ORF) that contains motifs typical for proteins involved in replication, i.e. helicase, protease and RNA-dependent RNA polymerase (RdRp). RNA2, on the other hand, contained two ORFs (RNA2-ORF1 and RNA2-ORF2) that coded for a small (~20 kDa) protein of unknown function, the putative movement protein, and the three coat proteins, respectively.

Considering the economic importance of torradoviruses and their emerging character, attempts were made to develop a diagnostic tool to detect

the presence of these viruses. To this end generic PCR primer pairs (Torradovirus-1F/Torradovirus-1R and Torradovirus-2F/Torradovirus-2R) were developed against highly conserved regions in RNA1 and RNA2, respectively. Evaluation of these primer sets revealed that they supported detection of all currently known torradoviruses.

Limited information indicated that members of the genus *Torradovirus* were transmitted by whiteflies. By a detailed study using varying acquisition access periods and inoculation access periods, it was demonstrated that ToTV, ToMarV and ToChV are all transmitted by three whitefly species, namely *Trialeurodes vaporariorum*, *Trialeurodes abutilonea* and *Bemisia tabaci*. The mode of transmission was determined as semi-persistent, i.e. like viruses that enter and remain in the foregut until being transmitted to another plant host. However, localisation of the viral RNA within the whitefly vector confirmed the presence of virus in the stylets and thereby showed that torradoviruses represent the first spherical viruses transmitted by whiteflies in a semi-persistent and stylet-borne manner.

Samenvatting

Het onderzoek dat in dit proefschrift wordt beschreven richt zich op de moleculaire en biologische karakterisering van een nieuwe groep van plantenvirussen. Bij de aanvang van dit onderzoek kenden we alleen de 'torrado' ziekte in tomaat, herkenbaar aan necrose in bladeren en op vruchten. In een later stadium van de ziekte vertonen geïnfecteerde planten een ziektebeeld van ernstige necrose, alsof de planten zijn verbrand. Door gebruik te maken van verschillende virologische technieken werd de veroorzaker van deze ziekte geïdentificeerd. Het bleek te gaan om een nog onbekend bolvormig virus van ongeveer 28 nm in diameter. Dit virus heeft een tweeledig RNA genoom, waarvan de gehele nucleotidenvolgorde werd bepaald. Het virus werd genoemd naar de Spaanse naam van de ziekte die het veroorzaakt: *Tomato torrado virus* (ToTV). Het virus werd voornamelijk gevonden in Europa, maar heeft zich inmiddels uitgebreid naar Centraal Amerika en Australië.

Na de ontdekking van ToTV richtte het onderzoek zich op een nieuwe ziekte van tomaten in Mexico, mogelijk veroorzaakt door een torrado-achtig virus. Aangetoond kon worden dat die ziekte werd veroorzaakt door een nieuw virus dat verwant is aan ToTV. Dit virus heeft de naam *Tomato marchitez virus* (ToMarV) gekregen. Het onderzoek aan de moleculaire en biologische eigenschappen van dit virus, zoals beschreven in dit proefschrift, heeft ertoe bijgedragen dat werd voorgesteld om deze virussen taxonomisch onder te brengen in een nieuw genus (geslacht) met de naam *Torradovirus*, genoemd naar het eerste ontdekte virus, ToTV. Dit voorstel werd goedgekeurd door de Internationale Commissie voor Taxonomie van Virussen (ICTV) en het nieuwe genus werd ondergebracht in de familie van de *Secoviridae* in de orde van de *Picornavirales*. Tot het genus *Torradovirus* behoren vandaag de dag, naast ToTV en ToMarV, ook de voorlopige soorten Tomato chocolàte virus (ToChV), Tomato chocolate spot virus (ToChSV, beschreven door een andere onderzoeksgroep) en Lettuce necrotic leaf curl virus (LNLCV).

Aan de hand van de opheldering van totale genoomsequenties van torradovirussen kon worden vastgesteld dat het RNA1 één groot open leesraam (open reading frame, ORF) bevat. Dit RNA1-ORF1 bevat een aantal motieven die kenmerkend zijn voor eiwitten die bij de vermeerdering van het virus in de plantencel zijn betrokken (bijvoorbeeld helicase, protease en

polymerase). Op het RNA2 werden twee open leesramen gevonden. Van het eerste (RNA2-ORF1) is nog geen functie bekend. Op het tweede open leesraam werden de domeinen gevonden voor, respectievelijk, een mogelijk transport-eiwit en de drie manteleiwitten.

Gelet op het economische belang van ziekten die door torradovirussen worden veroorzaakt en de snelle opkomst van die ziekten, werd het belangrijk gevonden om een goede methode te ontwikkelen waarmee alle tot dan toe bekende torradovirussen konden worden opgespoord in plantmateriaal. Hiervoor werden twee paren van zogenaamde generieke primers ontwikkeld die in een PCR-toets kunnen worden gebruikt. Deze primers werden ontwikkeld om te binden aan zeer geconserveerde gebieden in het torradovirusgenoom. Het eerste primerpaar (Torradovirus-1F/Torradovirus-1R) bindt in het RdRP gebied in het RNA1 en het andere primerpaar (Torradovirus-2F/Torradovirus-2R) in het CP gebied van het RNA2. De primers werden getoetst op isolaten van alle tot dan toe bekende torradovirussen en bleken prima te voldoen.

Enkele studies hadden aangetoond dat virussen behorende tot het genus *Torradovirus* konden worden overgedragen door wittevliegen. In dit proefschrift worden enkele gedetailleerde studies beschreven waarin met variërende opname- en afgiftetijden werd nagegaan hoe torradovirussen door hun vector (de wittevlieg) worden overgedragen. Allereerst werd aangetoond dat maar liefst drie wittevliegsoorten, namelijk *Trialeurodes vaporariorum*, *Trialeurodes abutilonea* en *Bemisia tabaci*, de virussen ToTV, ToMarV en ToChV kunnen overbrengen. Ook de wijze van overdracht (persistent, semi-persistent of non-persistent) werd met behulp van overdrachtsexperimenten vastgesteld. Torradovirussen worden op een semi-persistente wijze overgedragen door hun vector. Normaal gesproken worden semi-persistent overgedragen virussen na hun opname door het insect gelokaliseerd in de zogenaamde voordarm, waarvandaan ze weer worden losgelaten in een eventuele nieuwe plant. Echter, door het lokaliseren van viraal RNA in de wittevlieg kon worden vastgesteld dat torradovirussen juist binden aan de styletten en niet in de voordarm. Torradovirussen vormen zo de eerste groep door wittevliegen overgedragen bolvormige plantenvirussen die op een semi-persistente en stylet-gerelateerde wijze worden verspreid.

Abbreviations

aa	amino acid(s)
AAP	acquisition access period
ABPV	*Acute bee paralysis virus*
AEV	*Avian encephalomyelitis virus*
ALSV	*Apple latent spherical virus*
ArMV	*Arabis mosaic virus*
ATTA	*Agrobacterium tumefaciens* transient transformation assay
AVB	Aracacha virus B
AYRSV	*Artichoke yellow ringspot virus*
BBWV	*Broad bean wilt virus*
BLAST	basic local alignment search tool
BPMV	*Bean pod mottle virus*
BRNV	*Black raspberry necrosis virus*
BRSV	*Beet ringspot virus*
BRV	*Blackcurrant reversion virus*
CaMV	*Cauliflower mosaic virus*
CBSV	*Cassava brown streak virus*
cDNA	complementary DNA
CNDV	*Carrot necrotic dieback virus*
CNSV	*Cycas necrotic stunt virus*
CP	coat protein
CPMV	*Cowpea mosaic virus*
CPMMV	*Cowpea mild mottle virus*
CPSMV	*Cowpea severe mosaic virus*
CRLV	*Cherry rasp leaf virus*
DAS	double antibody sandwich
DNA	deoxyribonucleic acid
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
ELISA	enzyme-linked immunosorbent assay
GCMV	*Grapevine chrome mosaic virus*
GeMV	*Gentian mosaic virus*
GFLV	*Grapevine fanleaf virus*
HAV	*Hepatitis A virus*
hel	helicase
HPeV	*Human parechovirus*
IAP	inoculation access period
ICTV	International Committee on Taxonomy of Viruses
kb	kilo base
kDa	kilo Dalton

LNLCV	Lettuce necrotic leaf curl virus
MCDV	*Maize chlorotic dwarf virus*
MID	molecular identifier
MP	movement protein
MPI	Ministery of Primary Industries, New Zealand
MS/MS	tandem mass spectrometry
NCBI	National Center for Biotechnology Information
NGS	next generation sequencing
NIMV	Navel orange infectious mottling virus
nt	nucleotide(s)
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PatMMV	Patchouli mild mosaic virus
PEG	polyethylene glycol
PepMV	*Pepino mosaic virus*
pol	polymerase
PRI	Plant Research International, Wageningen
PRMV	*Peach rosette mosaic virus*
pro-co	protease cofactor
prot	protease
PVY	*Potato virus Y*
PYFV	*Parsnip yellow fleck virus*
RACE	rapid amplification of cDNA ends
RaMV	*Radish mosaic virus*
RCMV	*Red clover mottle virus*
RdRp	RNA-dependent RNA polymerase
RNA	ribonucleic acid
RpRSV	*Raspberry ringspot virus*
RT	reverse transcriptase
RTSV	*Rice tungro spherical virus*
SDV	*Satsuma dwarf virus*
SinV	*Solenopsis invicta virus*
SLRSV	*Strawberry latent ringspot virus*
SMoV	*Strawberry mottle virus*
SPCSV	*Sweet potato chlorotic stunt virus*
SqMV	*Squash mosaic virus*
StPV	*Stocky prune virus*
TBRV	*Tomato black ring virus*
ToANV	Tomato apex necrosis virus

ToChSV	Tomato chocolate spot virus
ToChV	Tomato chocolàte virus
ToCV	*Tomato chlorosis virus*
ToMarV	*Tomato marchitez virus*
ToRSV	*Tomato ringspot virus*
ToTV	*Tomato torrado virus*
TRSV	*Tobacco ringspot virus*
UTR	un-translated region
Vp	viral protein
VSR	viral suppressor of RNA silencing

Nawoord

Aan het einde van dit boekje is er natuurlijk plaats voor het bedanken van een aantal mensen. Mensen die belangrijk waren voor de totstandkoming van dit proefschrift en in de (lange) periode daarnaartoe.

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En de collega's van de leerstoelgroep virologie onder leiding van Just en Monique. Ik had de laatste tijd de bevoorrechte positie om zowel bij PRI te werken als op de leerstoelgroep en wil jullie bedanken voor de plezierige contacten. Nu we met de virologie-groep van PRI-BIOINT zijn verhuisd en werkelijk naast elkaar zijn komen te zitten, zie ik uit naar een nog intensievere samenwerking!

Curriculum vitae

Marinus (Martin) Verbeek was born on the 26th of January 1965 in Andel, the Netherlands. After primary school, he went to the Oude Hoven lyceum in Gorinchem. In 1989 he got his BSc in horticulture at the "Hogere Tuinbouwschool", a part of the "Hogere Agrarische Scholen van de KNBTB" in 's-Hertogenbosch. During this course, Martin did an internship at the Institute for Plant Protection (Instituut voor Plantenziektenkundig Onderzoek, IPO) in Wageningen, where he worked with viruses of *Allium spp.* under the supervision of the late dr. Lute Bos and ir. Paul van Dijk. After graduation, he was employed at IPO for two months, still on the subject of *Allium* viruses, and then military service had to be fulfilled. During 16 months Martin was trained and employed as a sergeant medical troops. Fortunately, immediately after military service, a job for a technician came available in the virology section of IPO. Together with dr. ir. Hans van den Heuvel he worked for approximately 10 years on – besides other small projects – the transmission of *Potato leafroll virus* by aphids and on the characterization of Lettuce big-vein disease.

In 2000 he became a senior technician virology. Two years later the opportunity was given to become a junior scientist, which he gladly accepted. In the meantime IPO was merged with two other institutes into Plant Research International. At PRI, Martin worked on and lead several projects on viruses of vegetables, ornamentals and potatoes. In 2003 he was asked to lead a project on a new disease that was found in tomato crops in the Southeast of Spain. Soon it became clear that this disease was caused by a new virus, which later was named *Tomato torrado virus*. That discovery was the start of the research conducted by the plant virology section of PRI on torradoviruses and served as a prelude to this thesis. At the end of 2010, Martin was admitted to the PhD program at Wageningen University and to the Graduate school Experimental Plant Sciences.

(Publications marked with an asterisk are part of the research described in this thesis)

* M. Verbeek, P.J. van Bekkum, A.M. Dullemans and R.A.A. van der Vlugt (2013) Torradoviruses are transmitted in a semi-persistent and stylet-borne manner by different whitefly vectors. *Virus Research, submitted*

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* M. Verbeek, J. Tang and L.I. Ward (2012) Two generic PCR primer sets for the detection of members of the genus *Torradovirus*. *Journal of Virological Methods, 185: 184-188*

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M. Verbeek, P.G.M. Piron, A.M. Dullemans, C. Cuperus, G.W. van den Bovenkamp and R.A.A. van der Vlugt (2010) Determination of aphid transmission efficiencies for N, NTN and Wilga strains of *Potato virus Y*. *Bioforsk Fokus 5(5): 44*

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Patents: 1) WO2006085749 A1 / PCT/NL2006/000009
2) EP2170933 A1 / EP20080712602

Education Statement of the Graduate School
Experimental Plant Sciences

Issued to: **Martin Verbeek**
Date: **19 September 2013**
Group: **Business Unit Biointeractions and Plant Health & Laboratory of Virology**
Wageningen University & Research Centre

1) Start-up phase	*date*
► **First presentation of your project**	
Characterization and epidemiology of Torradoviruses	Dec 13, 2010
► **Writing or rewriting a project proposal**	
Characterization and epidemiology of Torradoviruses	Nov 2010
► **Writing a review or book chapter**	
M. Verbeek, A. Dullemans & R. van der Vlugt. Torradoviruses, in: Vector-Mediated Transmission of Plant Pathogens. Ed: Judith K. Brown, Univ. of Arizona, Tuscon, USA. American Phytopathological Society Press, St. Paul, MN	Mar 2011
► **MSc courses**	
Laboratory use of isotopes	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*7.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS ExPectationS day	Nov 19, 2010
EPS ExPectationS day	Nov 18, 2011
► **EPS theme symposia**	
EPS Theme 1 symposium "Development biology of plants", Wageningen	Jan 19, 2012
EPS Theme 2 symposium "Interactions between Plants and Biotic Agents", Wageningen	Feb 10, 2012
► **NWO Lunteren days and other National Platforms**	
NWO-ALW meeting Experimental Plant Sciences, Lunteren	Apr 05, 2011
DAVS meeting KNAW, Amsterdam	Mar 18, 2011
NWO-ALW meeting Experimental Plant Sciences, Lunteren	Apr 02-03, 2012
► **Seminars (series), workshops and symposia**	
Invited Seminars (David Baulcombe (UK), Richard Elliott (UK), Bryony Bonning (USA))	Sep 2010-Oct 2011
Invited WEES Seminar Michael Strand (USA)	Apr 26, 2012
Rob Goldbach Memorial Lecture David Baulcombe (UK)	Oct 10, 2012
Workshop PrimeDiagnostics Luminex Training	Oct 23-24, 2012
KNPV - Plantum - EPS Symposium 'Intraspecific Pathogen Variation - Implications and Opportunities'	Jan 22, 2013
► **Seminar plus**	
► **International symposia and congresses**	
Bioforsk-konferansen,Sandefjord, Norway	Feb 04-05, 2009
Advances in Virology, Harrogate, UK	Apr 01-03, 2009
PVYwide second meeting, Paris, France	Jun 08-09, 2009
AAB potato meeting, Edinburgh, UK	Sep 16, 2009
11th International Plant Virus Epidemiology Symposium and 3rd Workshop of the Plant Virus Ecology Network,	Jun 20-24, 2010
14th Triennial meeting of the Virology Section of the European Association for Potato Research (EAPR), Hamar,	Jul 04-09, 2010
International Advances in Plant Virology, Joint meeting of the AAB and NKP, Arnhem, NL	Sep 05-07, 2010
GM Viruses as Medicine, Panacea or Pandora's Box, Amsterdam, NL	Jan 20, 2011
IWGLVV meeting, Antequera, Spain	May 17-20, 2011
PVYwide fourth meeting, Changins, Switzerland	May 30-31, 2011
AAB advances in Virology, Dublin, Ireland	Mar 28-30, 2012
PVYWide, fifth meeting, Edinburgh, Scotland	May 31-Jun 01, 2012
EFPP 10th congress, 'IPM 2.0', Wageningen	Oct 01-05, 2012
12th Internation Plant Virus Epidemiology Symposium, Arusha, Tanzania	Jan 28-Feb 01, 2013

Continued on next page

►	**Presentations**	
	Aphid transmission of Potato virus Y (oral, invited), Bioforsk Konferansen, Sandefjord, Norway	Feb 05, 2009
	Tomato torrado virus and tomato marchitez virus, new plant picorna like viruses infecting tomato (oral) AAB,	Apr 01, 2009
	The control of PVY in Dutch seed potato culture (oral) AAB, Harrogate, UK	Apr 02, 2009
	Determination of aphids' Relative Efficiency Factors for the N, NTN and Wilga strains of Potato virus Y (oral) PVYwide,	Jun 08, 2009
	Determination of aphid transmission efficiencies for N, NTN and Wilga strains of PVY (oral) Edinburgh, Scotland	Sep 16, 2009
	Het genus Torradovirus, een nieuw geslacht van plantenvirussen (oral) KNPV spring symposium, Wageningen, NL	Jun 16, 2010
	The genus Torradovirus, a new plant virus genus (oral) IPVE, Ithaca, USA	Jun 21, 2010
	Determination of aphid transmission efficiencies for N, NTN and Wilga strains of Potato virus Y (oral) IPVE, Ithaca, USA	Jun 23, 2010
	Determination of aphid transmission efficiencies for N, NTN and Wilga strains of Potato virus Y (oral) EAPR, Hamar,	Jul 05, 2010
	Aardappelvirus Y (oral, invited) to symposium for potato breeders, Wageningen, NL	Jul 14, 2010
	The genus Torradovirus, a new plant virus genus (oral) Joint meeting AAB and NKP, Arnhem, NL	Sep 06, 2010
	Determination of aphid transmission efficiencies for N, NTN and Wilga strains of PVY (poster) Joint meeting AAB-NKP,	Sep 05-07, 2010
	Resistance tests for virus diseases (oral, invited) 5th workshop Phytopathology, Roelofarendsveen, NL	Feb 02, 2012
	Lettuce big-vein virus is the causal agent of a syndrome of necrotic rings and spots in lettuce (oral) AAB Dublin, Ireland	Mar 29, 2012
	Two generic primer sets for the detection of members of the genus Torradovirus (poster), AAB Dublin and NWO-ALW	Apr 02-03, 2012
	Lettuce big-vein virus is the causal agent of a syndrome of necrotic rings and spots in lettuce (poster) IPVE, Arusha,	Jan 28, 2013
	Transmission of torradoviruses by their whitefly vectors (oral), IPVE, Arusha, Tanzania	Jan 29, 2013
►	**IAB interview**	Nov 14, 2012
►	**Excursions**	
	Subtotal Scientific Exposure	*34.4 credits**

3) In-Depth Studies		*date*
►	**EPS courses or other PhD courses**	
	Statistics: Toegepaste statistiek	Jan 26-Mar 30, 2010
	Bioinformatics, a user's approach	Mar 04-08, 2013
►	**Journal club**	
►	**Individual research training**	
	Subtotal In-Depth Studies	*3.9 credits**

4) Personal development		*date*
►	**Skill training courses**	
	Project and Time Management	Sep 27-Nov 08, 2011
	Scientific writing	Apr 24-Jun 19, 2012
	Commercial discussion techniques	Feb 20-21, Apr 03, 2013
►	**Organisation of PhD students day, course or conference**	
	Organisation of the 12th International Conference on Virus Diseases of Ornamental Plants, Haarlem, NL and Member editorial board proceedings	Apr 2008-Apr 2010
	Organisation of the 10th EFPP congres 'IPM 2.0', Wageningen	Oct 2011-Oct 2012
►	**Membership of Board, Committee or PhD council**	
	Member of the Management Team COST action FA0807 'Integrated Management of Phytoplasma Diseases'	May 2009 - 2013
	Subtotal Personal Development	*8.7 credits**

TOTAL NUMBER OF CREDIT POINTS*	**54.5**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

** A credit represents a normative study load of 28 hours of study.*

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Cover:	Fruit symptoms in tomato cv. 'Realeza' caused by *Tomato marchitez virus* (ToMarV). Insert: electron micrograph of ToMarV particles
Cover design:	Martin Verbeek
Thesis layout:	Martin Verbeek
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