Title

Investigation of viruses causing recent outbreaks of tomato necrotic dwarf and squash vein yellowing in California reveals different means of emergence and role of recombination

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Publication Date

2022

Peer reviewed|Thesis/dissertation

Investigation of viruses causing recent outbreaks of tomato necrotic dwarf and squash vein yellowing in California reveals different means of emergence and role of recombination

By

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR IN PHYLOSOPHY

in

PLANT PATHOLOGY

in the

OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA

DAVIS

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2022

Acknowledgements

First, I would like to thank my family for their unconditional support, none of this would have been possible without them.

I would like to express my gratitude to Distinguished Professor Robert L. Gilbertson for giving me the opportunity to do my PhD and all the advice and mentorship throughout these years. Your example has inspired me to be a better scientist and do my best in every endeavor to come.

I would also like to thank my committee members, Dr. Gitta Coaker and Dr. Savithrama Dinesh-Kumar, thank you for your guidance and support. I have learned so much from both of you and will continue to apply these lessons throughout my career.

I would like to thank all the members of the Gilbertson laboratory. It was great to get to share this process with such excellent professionals and scientists.

I would like to thank the graduate group at the Plant Pathology department, it was great to meet such great people, I wish you all the best in your careers, you really deserve it.

Finally, to my friends, so many people to thank, especially Ariadna, Anna, Pao and Annie. The laughs and tears we shared are priceless and I will cherish them forever.

Dissertation abstract

In this dissertation I investigated aspects of two plant virus species, *tomato necrotic dwarf virus* (ToNDV) and *Squash vein yellowing virus* (SqVYV), which have emerged in different ways in California. In Chapter Two, I further characterized a new isolate of ToNDV (genus Torradovirus) associated with a necrosis disease outbreak in Kern County, CA in 2015. This involved obtaining a pure isolate (ToNDV-K15) and generating full-length infectious RNA 1 and RNA 2 clones. Using these clones, I determined the complete genome sequence of this isolate and used agroinoculation to fulfill Koch's postulates for this disease and show that the host range of the virus was limited to solanaceous species, and that virus derived from the infectious clones was whitefly-transmissible. Finally, pairwise comparisons and phylogenetic analyses seemed to support species status for ToNDV. The infectious clones allowed me to investigate the role and function of movement-associated genes/proteins encoded by RNA 2. In Chapter Two, I used mutational analyses to investigate the role of ORF1 and MP in infection and identify important motifs in involved in cell-to-cell movement. Modeling was then used to infer how selected mutations may have impaired structure and function. Subcellular localization with GFP-tagged fusion proteins showed that ORF1 and MP were targeted to the cell membrane and plasmodesmata (PD); however, the interaction of ORF1GFP was transient and involved a network and motile vesicles, whereas MP accumulated in PD and co-localized with the TMV-MP PD marker. ORF1 binds, consistent with the hypothesis the vesicles are involved in trasport. Interestingly, phylogenetic analyses of the MP revealed evidence of recombination and an unexpected relationship with MPs of polerovirus (family *Sobemoviridae*). Together, these results provide further insight into the function these RNA-2 encoded proteins in torradovirus movement and evidence for a two-MP type strategy.

In Chapter Three, I report the completion and analysis of the complete genome sequence of an isolate of SqVYV involved in a yellowing disease outbreak in cucurbits in Imperial Valley in 2014. The genome of this isolate (SqVYV-CA) has the typical organization of the SqVYV-type ipomoviruses, including lack of a HC-Pro gene/protein and a P1a and P1b genes/proteins at the 5' end. Notably, my research revealed a recombination event in the P1a gene of SqVYV-CA, involving a portion of the P1 gene of an uncharacterized potyvirus and is predicted to generate a hybrid P1/P1a protein. Finally, using the divergent P1 sequence in the hybrid P1/P1a gene of SqVYV-CA, I developed a primer pair that allows for the specific RT-PCR detection of SqVYV-CA. I validated the specificity of the test, i.e., it did not detect SqVYV-FL or amplify non-specific fragments from the negative control. In 2021, this SqVYV-CA RT-PCR test allowed for the detection of the virus two of the four samples with yellowing symptoms from Fresno Co. This is the first report of SqVYV in the Fresno area and is a potential threat to cucurbit production, especially watermelon.

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Chapter I

Introduction

Plant viruses and the evaluation of plants for resistance to viruses

Plant pathogens such as bacteria, fungi and viruses cause at least 20% of losses to agricultural production worldwide, according to the FAO ("Pathogens, precipitation and produce prices," 2021). Viruses continue to pose problems for farmers, and these include existing as well as emerging viruses. Furthermore, these diseases pose a threat to the world food security, which requires appropriate management and treatment (Lapidot et al., 2014; Patil et al., 2015).

Insects vector about 75% of plant viruses (Hogenhout et al. 2008) and often are a key target for designing management strategies. Most of these insects have piercing-sucking mouthparts but in very different types of modes of transmission, i.e., non-persistent, semi-persistent, persistent and persistent propagative (Blanc et al., 2014). Further know the mode of transmission will impact management approach. There are some instances different viruses can have the same strategies that may allow for management of multiple viruses, e.g., *Cucumber mosaic virus* (CMV) and potyviruses by aphid species. One of these examples is viruses that have a common insect vector, that is the spreading method for several viruses. In some cases, many viruses are vectored by a single insect vector, e.g., Begomovirus. One example of very different types of viruses transmitted by the same vector is torradoviruses and potyviruses, which are both transmitted by the invasive whitefly, a supervector, Bemisia tabaci MEAM1 species, which is polyphagous and has developed insecticide resistance (De Barro et al. 2011; Gilbertson et al. 2015). An example of two different viruses that are transmitted by *B. tabaci* whiteflies are torradoviruses and potyviruses (Gilbertson et. al., 2015). Losses due to plant virus increases the pressure on farmers to supply the market. Management of viruses can be achieved by several methods, dealing by a combination of strategies. However, managing of an insect vector, whether is arthropods, mites, fungi, humans, etc. play a central role in an IPM program (Batuman et al. 2020). The most preferred option for

management is planting resistant varieties, which possess resistance genes introduced by breeding. These varieties resist infection or become infected but tolerate the viral infection without losses in yield. Some varieties can also be resistant to transmission of viruses because the vectors are not able to transmit the virus due to structural constrains (Groen et al. 2017). Resistant varieties with these characteristics are one of the main tools for effective and sustainable management. Furthermore, there is a constant need for research to identify new sources of resistance and to develop new varieties by adding new resistance genes by conventional breeding or some of the new methods available (Fuchs, 2017). Resistant varieties are the end-product of years of breeding and evaluation that require multiple factors including (i) sources of resistance that can be introgressed into the commercial varieties and (ii) a disease screening method that is efficient and can be developed into a high throughput method. This will often require inoculation the plants with the virus either naturally or artificially. Plants are then evaluated including symptom development, pathogen levels determined as yields taken. In the case of some bacteria and fungi, the plants can be inoculated directly with cultures or suspensions of the pathogen. Because viruses are obligate pathogens, other methods must be used, and the method will depend on the biology of the virus.

For mosaic type of virus, e.g., potyviruses, tospoviruses and torradoviruses, inoculation can be done by mechanical (sap) means in which epidermal or mesophyll cells are wounded with an abrasive during rubbing of plant sap allowing for infection of these cells via virions in sap and the subsequent healthy cells. However, this type of transmission does not work phloem-limited viruses that cause yellows and leaf curl diseases (Hull, 2009). In the case of phloem-limited viruses, where mechanical inoculation is not possible, vector transmission or grafting were used. However, biotechnology has allowed for development of vector independent inoculation of stems,

e.g., agroinoculation. This refers to the inoculation of plants with infectious clones of viruses using *Agrobacterium tumefaciens* as a 'vector' to deliver the virus via transient transformation of wounded plant cells, e.g., via needle puncture inoculation (Hou et al., 1998). Thus, the complete viral genome is cloned into a binary vector that will be transformed into *A. tumefaciens*. The *A. tumefaciens* bacteria harboring the binary vector with the viral construct can be infiltrated into the plants. The viral DNA (DNA viruses) of cDNA (RNA viruses) will be inserted into the plant genome via the type IV secretion system. For the infection to be successful, the construct that harbors the viral genome imitates the normal viral infection and allow for replicational release (DNA viruses) or transcriptional release via promoters/regulatory sequences that will release the infectious nucleic acid that will via infection cause an eventual systemic infection. When the virus infects the plant, viral proteins will be produced, and a normal infection occurs. After 10-14 days, the viral symptoms can typically be observed. These constructs are thus referred to as infectious clones of the virus. Infectious clones are critical tools for fulfilling Koch's postulates for viruses, conducting genetic analyses and high throughput screening for resistance (Brewer et al., 2018). In the latter case, the agroinoculation method is also a very efficient way of screening for resistance, as it does not require maintaining infected plants, allows long-term storage, it is easily revivable and can be highly efficient. Agroinoculation of viral infectious clones has been demonstrated for a variety of crops and are commonly used for research in crops such as tomatoes and cucurbits (Mori et al., 2021; Zheng et al., 2015).

Tomato and cucurbits are vegetable crops that have been improved by resistance breeding. In particular, viral diseases are targeted because in tomato the can cause up to 40% losses in the field (Gilbertson et al. 2017). Both these crops present an important source of income for farmers and enrich the diet of people in the US and worldwide. In California, the crops account for 1.2

billion and 103.5 million dollars in California, respectively (CDFA production statistics). Resistance genes for numerous viruses have been introgressed into commercial tomato varieties including, tomato mosaic virus, tobacco mosaic virus, tomato spotted wilt virus and tomato yellow leaf curl virus. In the case of cucurbits, several virus resistant varieties are sold commercially, e.g. CMV resistant cultivars.

Viruses have high mutation rates, which facilitates the breaking or overcoming resistance, sometimes relatively quickly for single dominant genes. Therefore, continuous research is necessary to identify and introgress new resistance genes into vegetable crops to increase durability of the resistance (Fuchs, 2017). An example of this resistance-breaking phenomenon is the breaking of TSWV-Sw5 resistance in varieties of tomato. A single point mutation in the movement protein of the virus (NSm) rendered the protein unrecognizable, and the virus was able to infect freely in the host (Almási et al., 2015; Batuman et al., 2016; de Ronde et al., 2014; Roggero et al., 2002). Plant virus movement proteins are attractive targets for engineering resistance. A virus that lacks the capacity for local or systemic movement, will not cause substantial losses to yield and will be less transmissible, if at all, by insect vectors due to the low quantity or absence of virions (Galvez et al., 2014).

Cell-to-cell and long-distance movement of plant viruses

Plant virus cell-to-cell movement involves two mechanisms, first, cell-to-cell movement where viruses move to the adjacent cells to establish new infections and second, systemic or long-distance movement, in which the virus moves through the vascular tissue reaching newly emerging tissues throughout the plant (Rojas et al., 2001; Rojas et al. 2016). Both types of movement increase viral presence in the plant and impeding movement will result in restricted infection and

transmission. Plant viruses are well equipped for moving cell-to-cell or long-distance. The viral movement is an attractive target for developing resistance strategies.

Cell-to-cell movement of viruses is enabled by dynamic plasma membrane lined nano channels called plasmodesmata (PD), that are commonly 50-60 nm in diameter and contain proteinaceous material. PD provide a simplasmic communication gateway for exchange of trafficking molecules between adjacent cells (Lucas et al. 2009). PDs are highly regulated and dynamic, and the size exclusion limit (SEL) dictates the size of molecules that will be able to traffic or diffuse through them cell-to-cell. Callose, a β-1,3-glucan polysaccharide, controls the aperture of PD at the neck region (Vatén et al., 2011) and thus that dictates the SEL. SEL dynamics are dependent on biotic and abiotic factors and callose deposition; closure of SEL, can be used as a defense mechanism controlling the permeability of PD. The balance between β-1,3-glucanase and β-1.3-synthase enzymes activity determines the amount of callose deposited in the neck of the PD, as those enzymes degrade and synthesize callose, respectively (Bucher et al., 2001). High levels of callose can reduce or even stop the movement of molecules through PD (Wu et al., 2018). The PD aperture mechanism is used by both the plant and pathogens, e.g., viruses use PDs to move through the tissue and cause infection in the adjacent cells (Rojas et al., 2016).

Pathogens have developed strategies to bypass the SEL constrains. An instrumental protein for plant virus infection is the MP, because it can interact with host or viral proteins to increase the SEL or modify the PD to allow the passage of virions or ribonucleoprotein complexes from cell-to-cell to mediate virus spread in a process often referred to as ‘gating’ (Kumar & Dasgupta, 2021). According to their cell-to-cell movement, viruses can be classified in different groups (i) ssDNA and ssRNA viruses that slightly modify the PD and move cell-to-cell as a vRNA-MP or a

vRNA-MP-CP complex or (ii) the second group which includes viruses that dramatically modify PD using MP lined tubules, through which, the intact virions move cell-to-cell (Rojas et al., 2016). This group includes dsDNA and negative sense RNA viruses.

The movement system of TMV falls into the first category as the MP binds viral RNA (vRNA) and this complex transits through gated PD. The MP of TMV, also called P30, targets to the PD, possibly facilitated by an ER-lumen-localized chaperone, calreticulin (Jia et al., 2009). At the PD, P30 may recruit specific β-1,3-glucanases to induce hydrolysis of callose and increase SEL (Lee & Lu, 2011). *Potyvirus* (*Potyviridae*) also modify the PD but in a different manner than TMV. During infection, one of the potyviral proteins, PIPO interacts with host factors to localize to the plasma membrane and the PD (Wei et al., 2010). HC-Pro, another potyviral protein, increases SEL size (Rojas et al., 1997) and the cylindrical inclusion protein (CI) forms conical structures that are visible with a light microscope. CI threads can traverse PD and connect two CI inclusions across the cell wall (Rodríguez-Cerezo et al., 1997).

Members of the Secoviridae family, such as comovirus or nepovirus, use a strategy that falls into the second category. The MP of *Cowpea mosaic virus* (CPMV) is the only protein necessary to form tubules that transverse the PD and allow the CPMV intact virions to move cell-to-cell (Pouwels et al., 2003; Wellink et al., 1993). Tomato infecting (TI) Torradoviruses (*Secoviridae*), have a MP similar to the MP of Umbraviruses (*Tombusviridae*) (van der Vlugt et al., 2015) . The umbravirus MP forms tubules on the surface of protoplasts and binds ssRNA *in vitro* (Nurkiyanova et al., 2001). In the case of NTI torradoviruses, the MP is more closely related to members of the *Bromoviridae* family, e.g. MP of CMV (van der Vlugt et al., 2015), which also form tubules when expressed in protoplasts (Canto & Palukaitis, 2007).

Long distance movement of plant virus also involves the PDs symplasmic continuity. The sieve elements (SE) of the vascular system will function as conduits through which virions or viral nucleic acid protein complexes (vNAPC) can move from the original site of infection (sources), into the newly developing tissues (sinks). To reach the SE, viruses must move through the bundle sheath, vascular parenchyma, and companion cells until they reach the phloem. The PDs in the companion cells (CC) that are adjacent and connecting CC to the SE, have a unique deltoid shape and larger SELs, thereby allowing for the passage cell-to-cell of the virus into the vascular tissue (Rojas et al., 2016; Seo & Kim, 2016).

Family Secoviridae

Members of the Secoviridae family of plant viruses share common features like a non-enveloped icosahedral virion of approximately 25-30 nm diameter and are in the order Picornavirales (Le Gall et al., 2008). Within this family are all picorna-like plant viruses, and these are classified into 8 genera: *Sequivirus*, *Waikavirus*, *Torradovirus*, *Cheravirus* and *Sadwavirus* and, within the Comovirinae subfamily, the *Comovirus*, *Fabavirus*, *Nepovirus* genera. These viruses have a mono- or bipartite positive-sense single-stranded RNA genome that ranges from 9.0 to 13.7 kb (Sanfaçon et al., 2020). Viruses with a bipartite genome, have their genomic RNAs independently encapsidated. The 5’ end of the genomic RNAs is covalently linked to a small genome-linked VPg (2-4 kDa) which protects the 5’ end of the virus and aids in (Rajamäki & Valkonen, 2002) (Fig. 1). (Sanfaçon et al., 2020; Thompson et al., 2014) and the 3’ end has a poly-A tract.

In most cases, each RNA encodes for a single polyprotein that is cleaved into the mature proteins needed for viral infection and follow the common Helicase-Protease-Polymerase spatial

protein organization before cleavage. Viruses in this family have a long history of economic importance, from the oldest one reported to be economically important *Grapevine fanleaf virus* dating back to 1865 (Martelli & Boudon-Padieu, 2006) to new and emerging members like those in the genus Torradovirus.

Torradovirus

Torradovirus characteristics and geographical distribution

Torradoviruses are an emerging group of plant viruses in the family Secoviridae. The genus Torradovirus was erected in 2009 based on distinctive aspects of the genome organization and squences (Sanfaçon et al., 2009). The first species to be described was *Tomato torrado virus* (ToTV). This virus was recognized in tomato plants that showed unusual disease symptoms including a striking burning of the leaves and shoots (Verbeek et al., 2007). These symptoms first appeared in the early 2000s in the region of Murcia in Spain, and farmers described it as "torrado", which means burnt or scorched. Studies performed with the suspected causal virus revealed it is mechanically transmissible and infects mostly Solanaceous species (Amari et al., 2008; Verbeek et al., 2008; Verbeek et al., 2010). Consistent with the association with whiteflies, the virus was also transmissible by whiteflies, *Bemisia tabaci, Trialeurodes vaporiarorum* and *T. abutilonea* (Verbeek et al., 2014). Seed transmission of ToTV is low with a 0.38% of overall vertical transmission (Pospieszny et al., 2019) resulting from experiments made with several tomato varieties.

This virus was purified and spherical virions measuring ~25nm, were associated with the disease (Verbeek et al., 2007). Subsequently, infectious clones of the viral RNAs were generated

and, following delivery by agroinoculation developed torrado disease symptoms and fulfilled Koch's postulates for ToTV (Wieczorek et al. 2014) and torrado disease.

In 2003 a similar disease appeared in Sinaloa, Mexico, and it was described as "marchitez", which means wilt. Although initially it appeared to be unusually severe symptoms of tomato spotted wilt, tests for this virus were negative (Verbeek et al., 2008). Characterization of the virus revealed torradovirus-like properties including similar-sized and shaped virions. This virus was shown to be a new species of Torradovirus based on sequence comparisons and virions properties and, was named *Tomato marchitez virus* (ToMarV) (Verbeek et al., 2008). Similar results were reported in another study but the virus was named Tomato apex necrosis virus (Turina et al., 2007).

Since then, torradoviruses have been reported infecting tomatoes in Guatemala (tomato chocolate spot virus (ToChSV) and tomato chocolate virus (ToChV) (Batuman et al., 2010; Verbeek et al., 2010), Colombia (Leiva et al., 2022), Panama, Hungary (José Angel Herrera-Vásquez et al., 2015), Poland (Pospieszny et al., 2010), Italy (Ferriol et al., 2018), France, Australia (José Angel Herrera-Vásquez et al., 2015) and the United States (Wintermantel et al., 2018) (Table 1). In all cases, a burn-like symptom was observed at the bases of leaves and stems that eventually will cover the leaf completely. Infected plants can be stunted, and severe necrosis may develop in fruits. Thus, this local evolution of multiple torrado species in distinct geographical areas to cause the same symptoms in the susceptible tomato host.

At first, torradoviruses were described only from tomato with the torrado-like disease symptoms. Several non-tomato infecting (NTI) torradoviruses were discovered, mainly through the use of HTS analysis. These species include *Lettuce necrotic leaf curl virus* (LNLCV) from The

Netherlands (Verbeek et al. 2014) *Motherwort yellow mottle virus* (MYMoV) from South Korea (Seo et al., 2014), *Carrot torradovirus 1* (CTV-1) from the UK (Rozado-Aguirre et al., 2016), *Squash chlorotic leaf spot virus* (SCLSV) from Sudan (Lecoq et al., 2016) and Cassava torrado-like virus (CsTLV) from Colombia (Leiva et al., 2022) (Table 1). To date, the NTI (non-tomato infecting) torradoviruses have not been associated with economically important diseases, but they have revealed that much more genetic diversity existed in the genus.

Torradovirus genome and proteins

With the complete genomes of 5 tomato-infecting torradoviruses available, evidence of a conserved genome organization emerged. The torradovirus genome (Fig. 1) consists of two positive-sense, single-stranded RNAs that are independently encapsidated in icosahedral virions of ~30 nm in diameter (Verbeek et al., 2007). The RNA1 is ~7 kb and encodes for the proteins involved in replication and protein processing (Fig. 1). Depending on the torradovirus, RNA 2 ranges from 3-5 kb and encodes for the proteins involved in movement and encapsidation (Fig. 1) (van der Vlugt et al., 2015). The RNA 2 of all torradoviruses has a unique open reading frame (ORF1) at the 5' end, which was associated with long-distance movement (Ferriol et al., 2017).

Extensive studies of whitefly transmission of torradoviruses have been conducted with ToTV and ToNDV. The virus is transmitted in a semi-persistent manner by three species of whiteflies, *Bemisia tabaci*, *Trialeurodes abutilonea* and *T. vaporiarorum* (Amari et al. 2008; Verbeek et al. 2014; Wintermantel et al. 2018). Usually, fields with high incidence of torradovirus diseases are associated with high populations of whiteflies.

The curious case of tomato necrotic dwarf disease: The first recognized torradoviral disease in tomato

The case of tomato necrotic dwarf disease (ToNDD) and the subsequent identification of tomato necrotic dwarf virus (ToNDV) revealed that a torradovirus disease was affecting tomato in the US, well before the disease was observed in Spain. ToNDD was first observed in the 1980s in tomato fields in Imperial Valley, Southern California (Larsen, 1984). It was associated with severe necrosis in leaves and stems and high whitefly populations. This disease was one factor that was partially responsible for the displacement of processing tomato production from the Imperial Valley. However, the etiology of this disease remained unclear until evidence of whitefly transmission was provided and viral genome sequencing revealed that the causal agent was a torradovirus (Wintermantel et al. 2018) . ToNDV has the typical torradovirus genome organization and nucleotide sequence analyses indicate it represents a distinct species that is most closely related to ToMarV.

The role of the proteins encoded by the torradovirus genome during infection has been predicted based on *in silico* analysis, but limited experimental data is available (van der Vlugt et al., 2015). RNA 1 encodes for a polyprotein that gets processed into the replicase, VpG, protease and protease cofactor (Fig. 1). The RNA-dependent RNA-polymerase (replicase) mediate replication, the VPg protein is covalently linked to the 5' end of the genomic segments, although this has not been experimentally determined for any torradoviruses. The protease and the protease co-factor are predicted to function in the cleavage of the polyproteins into mature and functional proteins.

RNA 2 encodes a polyprotein that is processed into the movement protein and three capsid proteins (Vp35, Vp26 and Vp24). As previously noted, RNA 2 has a novel ORF, which is predicted to encode a ~23 kDa protein. In ToANV, ORF1 has been associated with phloem loading and deemed essential for systemic infection (Ferrioli et al. 2016). The putative MP of torradoviruses belongs to the 3A movement protein family of plant viruses (Carrasco et al., 2018; van der Vlugt et al., 2015). It was determined that a phenylalanine (F) residue at position 210 in the predicted MP in ToTV, plays a role in development of symptoms and infectivity of the virus in *N. benthamiana* and *Solanum lycopersicum cv*. Beta Lux (Wieczorek & Obrepalska-Steplowska, 2016). One of ToTV capsid proteins, Vp26, was also associated with the development of necrotic symptoms in *S. lycopersicum cv*. Beta Lux when delivered into a PVX-based vector expression system (Wieczorek et al., 2019). In general, very little experimental information is available on torradovirus protein function and their interactions with host factors. A better understanding of the pathogen biology should provide insight into gene function and facilitate the design of strategies for engineering torradovirus resistant plants.

Management of TI-torradoviruses

The main form of preventing and treating viral diseases transmitted by whiteflies is deployment of resistant varieties, therefore efforts to screen for resistance in available material, as the ones described later, is a valuable effort for torradovirus management. Although torradoviruses do not cause major diseases in the field, ToTV was placed in the European and Mediterranean Plant Protection Organization in 2013, but it has been removed since. ToNDV caused major problems in the Imperial Valley area in the 1980s, but with planting of less tomatoes, the virus was greatly reduced. Since then, ToNDV has only been found in late-planted tomatoes, when the

whitefly populations are high in the southern California area. The virus has been found in weeds in Kern County, north of IV. Therefore, ToNDV is still in present but proper management of tomato plantings in the region have greatly reduced any damages. Management programs already in place for control of tomato viruses transmitted by whiteflies, can also be applied in prevention and management of torradovirus infections.

Vector management and a host-free period serve the purpose of reducing the vector and inoculum sources for that vector. Pesticides against whiteflies can be applied when needed, however, insecticides are often unsuccessful due to the indiscriminate use and the development of resistance by the vector populations (Gilbertson et al., 2015; Gilbertson et al., 2017). Removal of infected plants and weeds can help reduce incidence of the disease in the field if it is present, like in the case of southern California, where ToNDV has been continuously reported since the 1980s in weeds, mainly belonging to the Solanaceous family.

Family Potyviridae

General characteristics

In contrast with torradoviruses, some members of the *Potyviridae* family have been extensively studied. This family is one of the largest in terms of species among positive-strand RNA viruses. The family is comprised of 232 species that are divided in 12 genera (Wylie et al., 2017). The genera of the family are *Arepavirus, Bevemovirus, Brambyvirus, Bymovirus, Celavirus, Macluravirus, Poacevirus, Potyvirus, Roymovirus, Rymovirus, Tritimovirus* and *Ipomovirus*. The genus Potyvirus has the most species with 158 members (Valli et al. 2015) and includes many economically important species.

Genome and proteins

The virion of all members of the family is formed by a single capsid protein that forms a non-enveloped flexuous and filamentous virion that can be 650-950 nm long and 11-20 nm in diameter (Revers & García, 2015). The genome is a single-stranded positive-sense RNA that ranges from 8.2-11.5 kb (Fig 2). Most of the viruses in the family are monopartite, except for those in the genus *Bymovirus*, which are bipartite (Wylie et al., 2017). These viruses typically induce mosaic symptoms, and most can be transmitted mechanically. In nature, transmission is via insect vectors. The vectors that transmit the members of the Potiviridae family include whiteflies (*Ipomoviurus*), aphids (*Potyvirus* and *Macluravirus*), mites (*Rymovirus*, *Tritimovirus* and *Poacevirus*) and plasmodiophorids (*Bymovirus*). To date, vectors have not been identified for members of the genera *Brambyvirus*, *Bevemovirus*, *Arepavirus*, *Celavirus* and *Roymovirus*, do not have a defined vector so far (Revers & García, 2015; Sangeetha & Jebasingh, 2021).

The potyviridae genomic RNA encodes for a single large polyprotein that is processed into the functional mature proteins, the presence of these proteins varies by genus (Fig. 2). The N-terminal proteins in the polyprotein include P1 and HC-Pro. Both proteins undergo self-cleaving in their C-terminus after translation. The P1 protein is involved in RNA binding, pathogenicity, and suppression of gene silencing (Pasin et al. 2014). The P1 protein has protease function and cleaves the polyprotein at the junction of the C-terminus and the HC-Pro (Fig. 2). Furthermore, the P1 protein varies in size among the genera in the family (Valli et al., 2008). In the Potyvirus genus, the P1 is the first protease and it contributes to RNA silencing (Pasin et al., 2014).

The next protein present in the polyprotein 5' end is HC-Pro for most of the potyviridae family. This is a multifunctional protein that has roles in aphid transmission, modulations of protease activity, genome amplification, cell-to-cell and long-distance movement (Valli et al., 2018), silencing suppression (Kasschau et al., 2003) and as a proteinase that cleaves at the junction of P1 and HC-Pro. It is important to note that HC-Pro is not present in all genera, e.g. members of the *Ipomovirus* genus have the P1a and P1b proteins but not a HC-Pro (Li et al, 2008), in the case of *Bymovirus* the proteins are named P2-1 and P2-2 serve this role (Revers & García, 2015; Yang et al., 2021).

The N-terminus of the P3 protein and a 'pretty interesting potyviral open reading frame' (PIPO) are the proteins of the polyprotein that follow HC-Pro or P1b. The P3 protein had not been associated with a specific function until PIPO was discovered. It was determined a functional fusion protein of P3 and PIPO is created via ribosomal frameshift or transcriptional slippage (Chung et al., 2008). PIPO protein has been associated with the coordination of the conical structure formed in PD for cell-to-cell movement (Wei et al., 2010). The interaction between P3 and P3N-PIPO is essential for the recruitment of CI to cytoplasmatic structures that contain 6K2 and for the association of those structures with the PD localized CI protein (Chai et al., 2020).

The 6K1 and 6K2 proteins that flank the CI protein need to be studied further, but 6K1 mutations abolish infectivity (Cui & Wang, 2016). The 6K1 protein localizes to the viral replication complex, and it is likely to be associated with the membrane and involved in RNA translation (Taiyun Wei, Huang, et al., 2010). The 6K2 protein is an integral membrane protein that induces ER proliferation to form viral replication complexes (VRCs) (Wei & Wang, 2008). The cylindrical inclusion (CI) protein, the largest protein encoded by all members of the family,

forms the characteristic cylindrical inclusions. The CI protein has several domains, including the N-termin, C-termini and two helicases. The N-terminal region is essential for cell-to-cell movement, whereas the C-terminus and helicase regions are involved in viral replication (Deng et al., 2015).

Located in the 3' end of the polyprotein, the NIa protein will be partially processes into VPg and a Protease. The VPg will be covalently linked to the 5' end of the genome, and the protease further mediates proper cleavage of the polyprotein. The NIb protein is the viral RNA-dependent RNA polymerase, which mediates viral replication (Shen et al., 2020). The final protein in the 3' end of the polyprotein is the CP, which is involved in vector transmission via the 'DAG' motif, and encapsidation (Martínez-Turiño & García, 2020).

Ipomovirus

Species

The genus Ipomovirus has only 7 viral species, *Coccinia mottle virus* (CocMoV), *Cucumber vein yellowing virus* (CVTV), *Squash vein yellowing virus* (SqVYV), *Tomato mild mottle virus* (TomMMoV), *Ugandan cassava brown streak virus* (UCBSV) and the type species *Sweet potato mild mottle virus* (SPMMV). Members of this genus cause substantial economic losses worldwide. SPMMV causes important losses in sweet potato (*Ipomoea batatas*) in Uganda, Kenya, Tanzania and Rwanda (Tugume et al., 2010). UCBSV causes brown streak disease of cassava (*Manihot esculenta*) in Africa, and it's a threat to the major staple foods in Africa (Patil et al., 2015). CVYV causes disease in cucumber and watermelon in countries of North Africa, West Asia and Southern Europe (Galipiensoet al., 2012). SqVYV causes watermelon vine decline, an

important disease of watermelons in Florida (Adkins et al., 2007) and Israel (Reingold et al., 2016). In pumpkins and squash, this virus induces vein yellowing and mosaic. TomMMoV causes losses in tomato and other solanaceous crops, like eggplant (Dombrovsky et al 2014).

Genome and proteins

In general, ipomovirus has not been as extensively studied as members of the genus *Potyvirus*. However, the complete genome of eight species has been determined and revealed a genome size ranging from 9069-10818 nt. The genome encodes a polyprotein of 2902-3011 amino acids, depending on the species, and PIPO, a small open reading frame (Dombrovsky et al 2014; Ferriol et al. 2020; Revers and García 2015). Notably, Ipomoviruses do not encode for an HC-Pro protein, but instead possess two PI proteins, PI-a and PI-b (Fig 2.) (Li et al., 2008).

SqVYV

SqVYV is an ipomovirus that was first discovered infecting squashes in Florida, USA in 2007 (Adkins et al., 2007). This was the first ipomovirus to be described from the New World. The virus has been detected in Indiana (Egel & Adkins, 2007), Puerto Rico (Acevedo et al., 2013), and California (Batuman et al. 2015). The host range of SqVYV is limited to members of the Cucurbitaceae. Although SqVYV was discovered infecting squash and causes a mosaic type disease, the virus causes more severe disease in watermelon, referred to as watermelon vine decline (WVD). Plants in the *Citrullus* genus (watermelon), develop vein yellowing, chlorotic lesions, systemic wilting, and plant death within 7-10 dpi. Older plants in the greenhouse present systemic wilt and fruit symptoms, like discoloration and rind necrosis, after 14 dpi (Adkins et al., 2007). The rapid vine-decline results in plant death and unmarketable fruit. In the case of other cucurbits,

such as *Cucurbita* and *Luffa* (pumpkin, tropical pumpkin, and squash) the symptoms of SqVYV infection are mainly vein yellowing of leaves. Cantaloupe and cucumber (*Cucumis*) develop transient symptoms that are detectable by back inoculation or RT-PCR, but only in symptomatic leaves; after the symptoms disappear, the virus is no longer detectable (Adkins et al., 2007).

SqVYV virions are long flexous rod-shaped filamentous particles of ~700nm and encapsidate the ~10 kb genome. The polyprotein is 364 kDa, which is processed into mature functional proteins by viral proteases (Adkins et al., 2007). The genome has a VPg protein covalently linked to the 5' end, but instead has two P1 proteins, P1a and P1b, and a poly-A tract at the 3' end. As for other ipomoviruses, SqVYV does not encode for a HC-Pro protein (Li et al., 2008). This virus is transmitted in a semi-persistent manner by whiteflies (*Bemisia tabaci*) with infectivity lost after 4 to 6 h (Adkins et al., 2007).

Figures

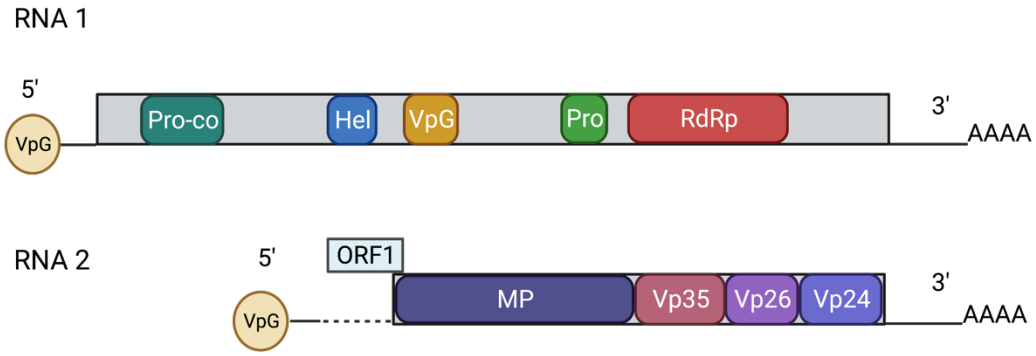

Figure 1. Diagram of the general genome organization of a torradovirus. The 5' and 3' end UTRs of RNA 1 and RNA 2 are depicted with a solid black line. The 5' ends are covalently linked to the VPg protein where the 3' end UTR has a poly-A tract. The polyprotein encoded by each RNA is represented by a gray box. Colored boxes represent the approximate location of the mature proteins: protease cofactor (Pro-co), Helicase (Hel), VPg, Protease (Pro) and the RNA-dependent-RNA-polymerase (RdRp). RNA 2 encodes for ORF1 and the second ORF encodes the polyprotein. The MP and the CPs, Vp35, Vp26 and Vp24, are represented by different colored boxes.

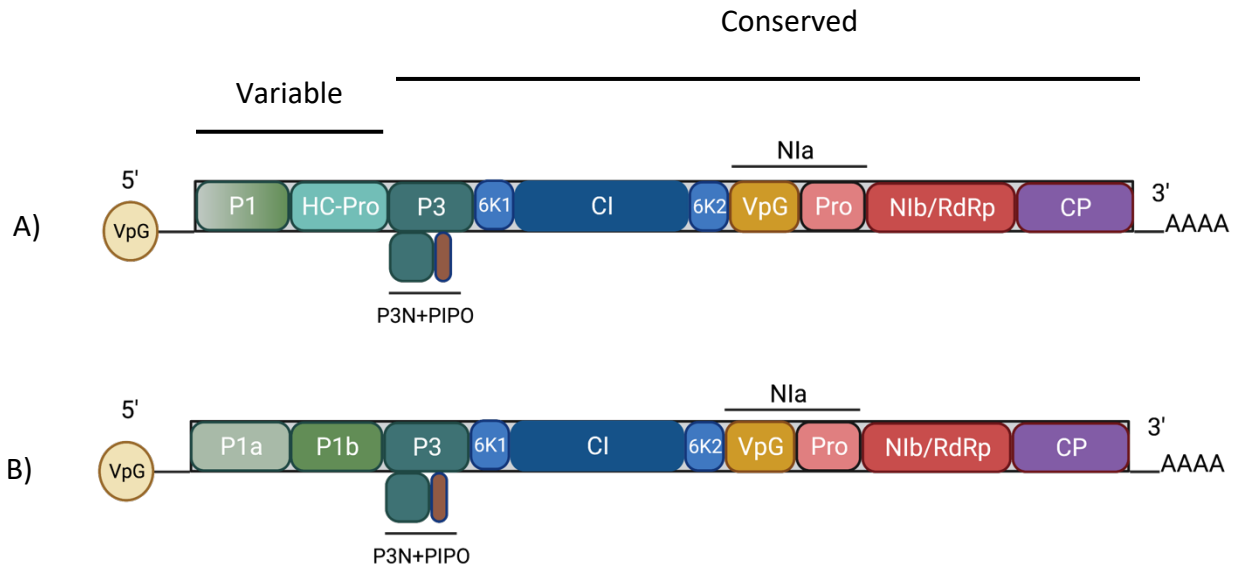

Figure 2. Maps of the general genomes of members of the genera *Potyvirus* (A) and *Ipomovirus* (B). The maps have a 5' linked VPg protein is shown attached to the 5' end. 5'UTR and 3'UTR are indicated by solid black lines and the poly A tract is indicated by consecutive As. ORFs encoding polyproteins are shown by gray boxes with the final mature proteins highlighted by different colored boxes. NIa protein is indicate on top of its products, VPg and Pro. The *pipo* ORF is shown below the genome, including the N terminus of the P3 protein that form P3N+PIPO.

Tables

Table 1. Countries and dates of detection of Torradovirus infection.

Symptoms were first observed in:	Virus	Location	Crop	Citation
1984	ToNDV-R	Imperial Valley, CA, USA	Tomato	(Larsen, 1984)
1999	ToChSV	Guatemala	Tomato	(O. Batuman et al., 2010)
2001	ToTV	Italy	Tomato	(Ferriol et al., 2018)
2003	ToTV	Spain	Tomato	(M. Verbeek et al., 2007)
2003	ToTV	Poland	Tomato	(Pospieszny et al., 2010)
2005	ToANV	Mexico	Tomato	(Turina et al., 2007b)
2005	ToTV	Australia	Tomato	(Gambley et al., 2010)
2007	ToChV	Mexico	Tomato	(Martin Verbeek et al., 2010)
2007-2008	ToTV	Hungary	Tomato	(Alfaro-Fernández et al., 2009)
2008	ToTV	France	Tomato	(Verdin et al., 2009)
2008	ToTV	Panama	Tomato	(Herrera-Vásquez et al., 2009)
2008	ToTV	Colombia	Tomato	(M Verbeek & Dullemans, 2012)
2015	ToTV	South Africa	Tomato	(Moodley et al., 2015)
2015	ToNDV-K15	Kern Country, CA, USA	Tomato	Present study
2015	ToTV	Morocco	Tomato	(Afechtal, 2020)
~2006	CaTV	UK	**Carrot**	(Rozado-Aguirre et al., 2016)
2011	LNLCV	Netherlands	**Lettuce**	(Verbeek et al., 2014)
2012	SCLSV	Sudan	**Squash**	(Lecoq et al., 2016)
2012	MYMoV	South Korea	**Motherwort**	(Seo et al., 2014)
2020	CsTLV	Colombia	**Cassava**	(Leiva et al., 2022)

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Chapter II

A late-season outbreak of a necrosis disease of tomato associated with high whitefly populations was caused by the reemergence of a *Tomato necrotic dwarf virus* (ToNDV) in Kern County, California, and application of an infectious clone for evaluation of resistance in tomato.

A late-season outbreak of a necrosis disease of tomato associated with high whitefly populations was caused by the reemergence of a *Tomato necrotic dwarf virus* (ToNDV) in Kern County, California, and application of an infectious clone for evaluation of resistance in tomato.

Abstract

Tomatoes in a late-planted tomato field in Kern County, California were affected by a necrosis inducing disease. Samples taken from symptomatic plants tested negative for infection by tomato spotted wilt virus. The samples were tested for torradoviruses, a necrosis-inducing virus in tomato and it was confirmed that a torradovirus was present in the samples and sequencing results showed the presence of *Tomato necrotic dwarf virus* (ToNDV). Because the plants were also infected by *Tomato mosaic virus* (ToMV), whitefly transmission was used to obtain plants with the single infection of the causal agent and mechanical transmission was used to infect *Nicotiana benthamiana* plants. An infectious clone of the new isolate, ToNDV-K15, was constructed and analyzed. The sequence identity confirmed a bipartite genome with RNA 1 of 7.2 kb and an RNA 2 of 4.9kb. The genome organization was consistent with torradoviruses, an emergent group of plant viruses. Nucleotide sequence comparisons revealed 94.3% identity with ToNDV-R for RNA 1 and 94.8% for RNA 2. The new isolate of ToNDV showed identities of 74-39.5% with RNA 1 of other torradoviruses and 72.8-35.7% with RNA 2 with other members of the genus. Whitefly transmissibility experiments showed ToNDV-K15 is transmissible by whiteflies, *Trialeurodes vaporarorium* and *T. abutilonea*. The infectious clone developed also allowed for the analysis of a partial host range which showed that infection by ToNDV-K15 isolate was restricted to the Solanaceae family. Finally, the infectious clone allowed for the screening of resistance in tomato cultivars, which showed high incidence of infection but also possible resistance.

Introduction

The genus *Torradovirus* (family *Secoviridae)* was relatively recently described to include emerging viruses associated with outbreaks of a burning or scorching of tomatoes leaves and shoots in locations all over the world (Gilbertson et al., 2015; van der Vlugt et al., 2015). One of the first outbreaks of this new burning disease of tomato occurred in Spain in 2001 and the symptoms included burn-like necrotic spots on the leaves and stems of affected plants. In some cases fruits show necrotic etching and rings (Gilbertson et al., 2017). A new type of plus sense ssRNA was associated with the symptoms, which was given the species name *Tomato torrado virus* (ToTV) (Verbeek et al. 2007). In addition to ToTV from the Old World, additional species of this type of virus were being described associated with these same burning symptoms of tomato plants in countries of the New World, Tomato marchitez virus (ToMarV) in Mexico (Verbeek et al., 2008) and in Guatemala, tomato chocolate spot virus (ToChSV) (Batuman et al., 2010). These viruses were subsequently placed into a new genus, Torradovirus. Members of the genus are most closely related to plus sense ssRNA viruses in Secoviridae family (Thompson et al., 2017).

The torradovirus genome is comprised in two segments, RNA 1 and RNA 2 of approximately 7 kb and 5 kb, respectively. Each RNA is individually encapsidated by the three capsid proteins (CPs), Vp35, Vp26 and Vp24, to form an icosahedral virion of 30 nm in diameter (Verbeek et al. 2007). Both RNAs have a VpG linked to the 5' end, and a 3' poly(A) tail. RNA 1 encodes for a polyprotein that is processed into a RNA-dependent RNA polymerase (RdRp), protease, protease-cofactor, VpG and helicase proteins. RNA 2 is translated into a polyprotein that is cleaved into a putative movement protein (MP) and the three capsid proteins (CPs). In addition, the torradovirus RNA 2 has an open reading frame (ORF1) that is located upstream of the start codon of the polyprotein (van der Vlugt et al., 2015). ORF1 encodes a protein of approximately

20 kDa that has been associated with systemic movement of tomato apex necrosis virus (ToANV) (Ferrioli et al. 2018).

In addition to the tomato-infecting (TI) torradoviruses, divergent torradoviruses infecting other plant species have also been identified (Lecoq et al., 2016; Rozado-Aguirre et al., 2016; Seo et al., 2014; Verbeek et al., 2014). The first of these non-tomato infecting (NTI) torradoviruses was *Lettuce necrotic leaf curl virus* (LNLCV) infecting lettuce in The Netherlands in 2011 (Verbeek et al., 2014). The NTI torradoviruses discovered to date have not been associated with outbreaks of diseases of economic importance. Through the application of high throughput sequencing (HTS), these NTI torradoviruses have been discovered worldwide in plants including motherwort, carrot, lettuce, squash (Lecoq et al. 2016; Verbeek, et al. 2014; Rozado-Aguirre et al. 2016; Seo et al. 2014; Turina et al. 2007; M. Verbeek et al. 2008). Phylogenetic analyses have revealed that TI and NIT torradoviruses comprise distinct lineages.

In nature, TI torradoviruses are vectored in a semipersistent manner by several species of whiteflies, including *Bemisia tabaci, Trialeurodes vaporiarorum* and *Trialeurodes abutilonea* (Verbeek et al. 2014; Wintermantel et al. 2018). In particular, the worldwide emergence of the *B. tabaci* supervector may have driven the emergence of TI torradoviruses (Gilbertson et al., 2015). Thus outbreaks of TI torradoviruses infecting tomato have been reported in 13 countries from four continents (Larsen 1984; Verdin et al. 2009; Afechtal 2020; Batuman et al. 2010; Verbeek et al. 2010; Verbeek and Dullemans 2012; Verbeek et al. 2007; van der Vlugt et al., 2015). This suggests a combination of local emergence and long-distance spread.

In 2015, samples were received from a late-planted experimental tomato plot in Kern County, CA was infected with *B. tabaci* whiteflies and showed severe virus-like disease symptoms, including stunting and distorted growth and curled, crumpled and extensive necrosis of leaves. These

symptoms were similar to those induced by *Tomato spotted wilt virus* (TSWV), but immunostrips tests for TSWV were negative. Having ruled out other viruses, the possibility of a torradovirus was considered. Indeed, RT-PCR tests with degenerate torradovirus primer pairs, revealed the expected-size fragments consistent with torradovirus infection. Furthermore, comparisons of sequences of these PCR-amplified fragments suggest this was an outbreak of tomato necrotic dwarf disease (ToNDD), which occurred in the Imperial Valley, Southern California in the 1970s-80s (Larsen, 1984).

Here I provide evidence that this, late season disease outbreak in tomatoes was caused by ToNDV, the first report of this disease since 1984. Furthermore, I characterized an isolate associated with this outbreak, ToNDV-K15 including developing infectious RNA 1 and RNA 2 clones. An agroinoculation system was developed and used to determine the host range, whitefly transmission and screening of tomato for resistance.

Materials and methods

Sample collection and mechanical transmission

In October 2015, severe virus-like symptoms were observed in leaves and stems of tomato plants in a late-season experimental plot in Kern County, California, USA. Samples with representative symptoms were collected by Farm advisor Joe Nuñez and sent to UC Davis. These samples were used in lateral flow tests (Agdia, Elkhart IN) and samples were stored at -80 C for further analysis. Mechanical (sap) inoculation was performed by grinding symptomatic tomato leaves in 0.01M potassium phosphate buffer pH 7.2. The resulting sap was rubbed onto leaves previously sprinkled with celite. Plants were maintained in a controlled environment

chamber (Conviron PGR15; 250 μmol·m^{-2}·s^{-1}, 16-h photoperiod, 22C day/night, RH 60%). Fourteen days after inoculation the plants were visually assessed for symptoms.

Nucleic acid extraction and virus detection with RT-PCR

Total RNA was extracted from infected leaves with RNAeasy extraction kit (Qiagen) following manufacturer's instructions and cDNA was generated with 6ul RNA and SSII enzyme according to manufacturer instructions (Thermo Fisher). The cDNA was used in PCR with primer pairs for selected infecting viruses. The results were analyzed by agarose gel electrophoresis in 0.8% gels. Samples were analyzed by RT-PCR with torradovirus specific primers for RNA 1 (ToR1F, ToR1R) and RNA 2 (ToR2F, ToR2R) and later with ToNDV specific primers for RNA 1 (ToNDVR1F, ToNDVR1R) and RNA 2 (ToNDVR2F, ToNDVR2R). Primers sequences are presented in Table 3.

Construction of the infectious cDNA clone of ToNDV

To generate infectious clones, leaf issue from newly emergent leaves of ToNDV-infected *N. benthamiana* plants was collected 14 dpi, and total RNA was extracted with the Qiagen RNAeasy extraction kit according to manufacturer's recommendations. The RNA 1 and RNA 2 cDNAs were synthesized with Superscript IV (Invitrogen) and polyT primers. Reactions were incubated for 1.5 h at 50 C. This cDNA was then used as a template for the PCR reactions.

The Gibson assembly strategy was used to generate full-length RNA 1 and RNA 2 clones. Here, the 5' and 3' primer pairs, designed to direct the amplification of RNA 1 and RNA 2 were designed with overlapping ends that are complementary to the sequences of the pJL89 binary vector for assembly (primer sequences are presented in Table 3).

The cDNA was amplified by touchdown PCR, with primers designed based on the pJL89 vector to generate overlapping sequences for assembly in the Gibson reaction (Table 3). The touchdown PCR amplification was performed with Phusion High Fidelity polymerase (NEB). The touchdown phase of the PCR for both RNAs was 10 cycles of 98 C for 2 min, 60 C for 30 sec (this temperature was reduced by one degree each cycle) followed by an extension of 72C for 10 min. The next stage of the PCR continued with 25 cycles of 98 C for 10 sec, 50 C for 30 sec and 72 C for 10 min with a final extension of 72 C for 5 min. The PCR products were visualized in 0.8% agarose gels, excised, recovered with the Zymo Gel DNA recovery kit (Zymo research) according to manufacturer's recommendations. One Gibson assembly reaction (NEB) was made for RNA 1 and RNA 2 with 200 ng of RNA 1 or RNA 2 and 200 ng of pJL89 binary vector were used in a 20 ul final volume. The assembly was performed at 52 C for 1.5 h. Then 5 ul of the assembly was transformed into *E. coli* DH10B (Invitrogen) cells by electroporation, plated in LB Kanamycin plates and grown overnight at 28 C. Colonies were selected and grown overnight with shaking in liquid LB media with kanamycin at 28 C. Plasmid minipreparations were made with selected cultures with the Zymo miniprep Kit according to manufacturer's recommendations. Plasmid DNAs were analyzed by agarose gel electrophoresis in a 0.8% agarose gels and RNA 1 and RNA 2 inserts were identified based on size. Selected plasmids having the inserts of the correct were sent for sequencing with the 35S-F primer and Rbz reverse primer. The clones with insert of RNA 1 and RNA 2 were sequenced completely and transformed into *A. tumefaciens* C58 cells by electroporation.

Agroinoculation

Liquid cultures of *A. tumefaciens* strains carrying the full-length RNA 1 and RNA 2 of ToNDV-K15 were grown in LB media with kanamycin overnight at 28 C. The cells were concentrated by centrifugation and resuspended in 1 mM $MgCl_2$ to an OD of 1.0. The RNA 1 and RNA 2 agroclones were co-inoculated into plants by stem puncture beneath the shoot apex. Five plants of each variety or species were agroinoculated and one plant was agroinoculated with the empty pJL89 vector as a negative control. Plants were maintained in a growth chamber as previously described. By 14 dpi, plants were examined for symptoms and samples of the newly emerged leaves taken for RT-PCR tests with ToNDV RNA 1 and RNA 2 specific primer pairs (Table 3).

Sequencing of the infectious ToNDV RNA 1 and RNA 2 cDNA clone

Sequencing was done by primer walking by Macrogen USA. The primer sequences used are shown in Table 3. The trimming and assembly of the RNA 1 and RNA 2 sequences was performed in Geneious.

Host range

Initial agroinoculations experiments were performed with 3-4 weeks old *N. benthamiana* and tomato plants (varieties NUN6394 and NUN638). Following the establishment of infectivity of the ToNDV RNA 1 and RNA 2 clones, a partial host range for ToNDV was determined by agroinoculating 5 plants of the following crop and non-crop species: (i) *Nicotiana benthamiana,* (ii) *N. glutinosa,* (iii) *Chenopodium quinoa,* (iv) *C. amaranthicolor,* (v) *Datura stramonium,* (vi) common bean (*Phaseolus vulgaris)* cv. Topcrop, (vii) eggplant (*Solanum melongena*), (viii)

butternut squash *(Cucurbita moschata)* and the tomato cvs: Glamour, NUN6394, NUN6385 and TY-1. By 14 dpi, the plants were visually evaluated for symptoms and samples from the newly emergent leaves were collected for virus detection by RT-PCR. Total RNA was extracted from leaf samples of each of the inoculated plants and the empty vector control plants by the Qiagen RNeasy kit, and cDNA was synthesized with SuperScript II (Thermo Fisher) and random primers. RT-PCR with a specific primer pair for ToNDV VP35 CP gene in RNA2 was used. A plant of each species/varieties were agroinoculated with the vector alone as a negative control.

Whitefly transmission

Whitefly transmission experiments were performed in the laboratory of collaborator William Wintermantel by following the method described in (Wintermantel et al., 2018). Briefly, adult *T. abutilonea* or *T. vaporariorum* whiteflies were given a 48-h acquisition access period with free feeding on infected pepper leaves, and were collected and placed in clip cages (20-40 per cage), after which clip cages were attached to the underside of test plants at the two true-leaf growth stage for a 48-h inoculation access period (IAP). Following the IAP, clip cages were removed and plants were placed in mesh cages that were placed in growth chambers. Inoculated leaves were removed at 1week post-inoculation to prevent maturation of whitefly nymphs, and plants were treated with systemic insecticides. Plants were evaluated visually for the presence of viral symptoms at 3 weeks post-inoculation and infection with ToNDV was verified by RT-PCR.

Sequence comparisons and phylogenetic analyses

Sequence comparisons were performed in Geneious. Phylogenetic analyses were performed with the RNA 1 and RNA 2 sequences of the infectious clones. For these analyses nucleotide and amino acid sequences were obtained from NCBI for both RNAs, ORF1 and both UTRs for ToNDV-R (KC999058.1, NC_027927.1), ToTV (NC_009013.1, NC_009032.1), ToMarV (NC_010987.1, NC_010988.1), ToChSV (NC_013075.1, GQ305132.1), Tomato chocolate virus (ToChV) (FJ560489.1,FJ560490.1), LNLCV(NC_035214.1,NC_035219.1), Motherwort yellow mosaic virus (MYMoV) (NC_035218.1,NC_035220.1), Carrot torradovirus like virus (CaTV1)(LC436363.1, NC_025480.2), Squash chlorotic leaf spot virus (SCLSV) (NC_035221.1, NC_035215.1) and *Cherry raspleaf virus* (CRLV) (Genus *Cheravirus*, family Secoviridae) (NC_006271.1, NC_006272.1) was used as an outgroup. Sequences were aligned with the relevant ToNDV-K15 sequences in Geneious with the MAFFT algorithm. The phylogenetic trees were generated with a GTR model for nt and Poisson model for amino acids in MrBayes in Geneious prime 2022.2.1 (Biomatters) with 100,000 bootstraps and a burn of 10,000. The trees were also visualized in Geneious prime.

Tomato varieties evaluation

To screen for resistance to ToNDV-K15, 37 tomato varieties were tested. For each variety, two groups of 19 plants were agroinoculated with the ToNDV-K15 as described. In addition, plants of two susceptible controls (cvs. N6394 and N6428) and negative (LA-3474-R tomato variety) were also included. The plants were agroinoculated at the two true leaves stage as previously described (REF). Two weeks after the first inoculation, the plants were inoculated again by the same method and symptoms were evaluated four weeks after the first inoculation. Rating system

consisted of presence or absence of necrotic symptoms in the leaves, with comments on degree of severity of symptoms.

Results

Characterization of ToNDV-K15 virus

Samples of tomato branches from the late-planted field with severe virus-like symptoms were received from Kern County in October 2015. All of the branches showed distorted growth; leaves were curled, crumpled and necrotic; and stems and petioles were also necrotic (Fig 1). It was also noted that this plot was infested with *B. tabaci* whiteflies. These symptoms most closely resembled those of spotted wilt disease but immunostrip and RT-PCR tests were negative for TSWV infection. Furthermore, RT-PCR tests for Alfalfa mosaic virus and Tomato necrotic spot virus, two other viruses that induce necrosis symptoms in tomato in California also were negative (data not shown).

However, it was noted that the pattern of necrosis of leaves and stems bore resemblance to symptoms of torradovirus infection of tomato. Therefore, an RT-PCR test was performed with RNA extracts of representative leaves and a general torradovirus primer pairs for RNA 1 (ToNDVR1F-ToNDVR1R) and RNA 2 (ToNDVR2F-ToNDVR2R) that directs the amplification of an ~500 bp fragment from RNA1 and RNA2. RT-PCR test with both torradovirus primer pairs the expected size ~500bp fragment was amplified from 7/10 of the samples tested. This suggested that a torradovirus was involved in the disease and that it may be transmitted by *B. tabaci* whiteflies. A BLAST search with sequences of these putative torradovirus RNA 1 and RNA 2 fragments revealed a ~94% identity with RNA 1 and RNA 2 sequences of ToNDV-R (NC_027926.1, NC_027927.1). These results indicated that the disease symptoms in those late-

planted processing tomato field were due, at least in part, to infection with this torradovirus. Moreover, these symptoms also bore resemblance to those of the ‘tomato necrotic dwarf disease.

Mechanical transmission of ToNDV-K15

Nicotiana benthamiana plants mechanically inoculated with sap prepared from representative leaves with symptoms of this torradovirus-associated disease from Kern County developed systemic necrosis by 14 dpi, which is indicative of tobamovirus infection. The plants were tested by RT-PCR and showed a mixed infection by ToNDV and *Tomato mosaic virus* (ToMV) with specific primers. Efforts to eliminate ToMV with resistant tobacco varieties were not successful because the ToMV was not confined to inoculated leaves based on detection of ToMV in upper newly emerged leaves, therefore whitefly transmission was used. Here, these experiments yielded *D. stramonium* plants infected only with ToNDV when tested by RT-PCR. The *D. stramonium* plants, positive by RT-PCR for only ToNDV, were used as an inoculum source to extract nucleic acids and amplify the complete genomes of ToNDV RNA1 and RNA2 for the generation of the infectious clone.

Whitefly transmissibility of ToNDV-K15

Whitefly transmissibility experiments using ToNDV-K15, via agroinoculation of pepper as source plants demonstrated that the virus was transmitted effectively by whiteflies of the *T. abutilonea* and *T. vaporiarorum* species.

Generation of the infectious clone of ToNDV-K15

We obtained one clone for RNA 1 in plasmid 7 and two clones for RNA 2, RNA 2.1 and RNA 2.2 in plasmids 32 and 54 (numbers don't match; 3 RNA2 but only two plasmids) of the expected sizes after the analysis of plasmids from 70 putative *E. coli* colonies. The 5' and 3' ends of the inserts in these 3 recombinant plasmids showed 100% similarity with the ToNDV-R isolate and confirmed the ToNDV sequence. One *A. tumefaciens* strain, C58, was selected for transformation of each of the RNA 1 and RNA 2. Recombinant plasmids (referred to as agroclones) were grown, mixed and inoculated into *N. benthamiana* and *S. lycopersicum* N6394, N6485 plants. By five dpi necrotic spots were observed on the base portion of the newly emerged leaves of inoculated plants (Fig 3). By 14 dpi, tomato leaves had strong necrosis in the basal part of leaves. *N. benthamiana* plants presented downward curling and light discoloration (Fig 3). RT-PCR tests of *N. benthamiana* and *S. lycopersicum* leaves amplified the expected size fragment from all symptomatic leaves that were tested. Therefore, the clones of RNA 1 and RNA 2.1 are infectious and induced symptoms in both *N. benthamiana* and tomato plants.

Sequence analysis of ToNDV-K15

Having established that RNA 1 and RNA 2.1 clones are infectious in tomato and *N. benthamiana* plants, I next determined the complete sequence of the cloned RNA 1 and RNA 2. Analyses of the RNA 1 and RNA 2 sequences and corresponding ORFs revealed that ToNDV-K15 had a genome organization typical of a TI torradovirus (Fig 2).

The 5'UTRs of ToNDV-K15 is 150 bp for RNA 1 and 136 bp for RNA 2. Nucleotide identity of the 5' UTR sequences of RNA1 of ToNDV-R and ToNDV-K15 is 96.7% and, 95.6% in the RNA2. The 3'UTR size of ToNDV-K15 is 633 bp for RNA 1 and 632 bp for RNA 2 and

they are 94.9% identical with ToNDV-R. Thus, both viruses show a short 5'UTR and a longer 3'UTR, as it has been reported for members of the torradovirus genus (Ferriol et al., 2018; Rozado-Aguirre et al., 2016; Seo et al., 2015; Verbeek et al., 2007).

RNA 1of ToNDV-K15 is 7.2 kb long and encodes for one open reading frame which is cleaved into proteins involved in replication i.e., a helicase, protease, protease co-factor and a RdRp. The complete nucleotide sequence of RNA 1 of ToNDV-K15 had the highest identity (94%) with that of ToNDV-R, whereas the next highest identity was with ToMarV (76%). The amino acid sequence of the polyprotein encoded by RNA-1 of ToNDV-K15 was 98.42% identical to that of ToNDV-R followed by 83.4% with that of ToMarV (QCI56101.1). The RdRp regions of ToNDV-R and ToNDV-K15 are 97% identical, with the next highest identity (77%) with ToMarV. Comparisons between sequences of NTI and TI torradoviruses showed identities <40% in the propol region and in the 3CPs region (Table 4).

Comparison between RNA1 of ToNDV-K15 with ToNDV-R showed differences in region between 520 to 941 amino acid in which there are twelve amino acid differences. These changes occur after the helicase and before the protease motifs. At amino acid 1193, there is another change close to the N-terminus of the RdRp. Other differences locate between amino acids 1657 to 2125 with 19 aa changes, this region occurs after the putative RdRp. In summary, RNA1 polyprotein sequence differs by 32 amino acids between ToNDV-R and ToNDV-K15.

The RNA 2 of ToNDV-K15 is 4912 bp. The complete nucleotide sequence of the ToNDV-K15 RNA-2 is 94.8% identical to that of ToNDV-R, 78.2% identical with ToMarV, and 57.3% identical with ToTV. The K15 RNA 2 has the typical two open reading frames of TI torradoviruses. The first ORF (ORF1) starts at nucleotide 137 and encodes for a protein of 20.8 kDa. The second open reading frame (ORF2) encodes for a polyprotein that, after cleavage, will generate the

putative movement protein (MP, 52.8 kDa) and three CPs (Vp35, Vp26 and Vp24). The aa sequence of ORF1 of RNA2 of ToNDV-K15 is 99% identical to that of ToNDV-R (Table 4) with the next closest (82.1%) with ToMarV. The aa sequence of the ORF2 of RNA2 of ToNDV-K15 shows a 98.8% amino acid identity with those of ToNDV-R, with the next closest identity (91.0%) with that of ToMarV (YP_001976149.1).

In ORF2, amino acid differences in ToNDV-K15 and ToNDV-R are concentrated in region of aa 300-530, specifically at positions 317, 350, 426, 437, 461, 464, 465, 472, 511, 530. First eight changes occur in the C-terminus of the movement protein and the last two in the N-terminus of the Vp35 CP. Furthermore, there are amino acid changes in positions 751, 821, 1034, 1101. Thus, there are a total of 14 aa differences in the RNA 2 encoded proteins MP and Vp35.

In summary, the nucleotide changes are distributed throughout the genome of ToNDV-K15 compared to ToNDV-R in RNA1 and RNA2, but most are synonymous mutations in both RNAs suggesting that ToNDV-K15 is a quasispecies of ToNDV-R?

Phylogenetic analysis

Phylogenetic analyses based on the nucleotide and the amino acid sequences for RNA 1 have revealed the closest virus to ToNDV-K15 is the ToNDV-R. ToMarV (isolate PRI-TMarV0601) is the next closest related to the ToNDV isolates (Fig 4). The phylogenetic analysis utilizing the amino acids of the ProPol region show consistent results with the previous literature (Fig 4 and 5), as ToNDV-K15 is closely related to ToNDV-R and both viruses are related to ToMarV. ToChSV is related to ToMarV and ToNDV. The tomato-infecting torradoviruses cluster together for RNA 1 both at nt and aa sequences. Similarly, RNA 2 phylogenetic analyses reveal that TI torradoviruses cluster together while NTIs torradoviruses cluster in a different clade .

In the case of both RNAs ToTV is more divergent from the new world torradoviruses (Figure 4 and 5). The ToTV branch is still in the TI torradoviruses, however, it is more distant from ToMarV from Mexico, ToNDV from southern California and, ToChV and ToChSV from Guatemala. The propol and CP regions, which are the ones used by the ICTV for species demarcation, for both RNA 1 and RNA 2 show a consistent clustering with two groups, the TI and NTI torradoviruses. However, SCLSV CPs cluster closer with the TI torradoviruses and away from the NTI torradoviruses. In summary, both RNAs of torradoviruses show strong clustering of the tomato-infecting and non-tomato infecting, independent of the gene analyzed (Fig 4 and 5)[should be consistent [Fig. or Figure?].

Host range of ToNDV-K15

To determine the host range of ToNDV-K15, I used agroinoculation method. Consistent with previous results, susceptible tomato varieties N6394, N6385 and H5608 agroinoculated with ToNDV-K15 developed necrosis at the basal portion of newly emergent leaves by approximately five dpi and strong scorching as the infection progressed (Fig. 3). Tomato plants agroinoculated with the vector control, showed no symptoms. Beans (top crop) and Chenopodium species were negative in the RT-PCR with the ToNDV specific primers at 15dpi. Notably, we detected asymptomatic infections in *Nicotiana tabacum* and *N. glutinosa*. *N benthamiana* showed epinasty and a slight discoloration in the newly emergent leaves. Some tomato varieties were not susceptible to ToNDV infection by agroinoculation, which shows the occurrence of natural resistance, i.e., a variety that is resistant to TYLCV. Eggplants (*Solanum melongena* vr. Black beauty) also tested positive for the agroclone, confirming the preference of ToNDV for members of the *Solanaceae* family, even though the plants showed no symptoms of infection. The cDNA clones for RNA 1 and RNA 2 of ToNDV show to be efficient infecting tomato and *N. benthamiana*. Out of 5 plants

inoculated with the virus, we obtained 100% infection with susceptible tomato plants. Non-susceptible varieties showed no infection by agroinoculation. These experiments were repeated two more times with the same results. The partial host range of this virus shows that Solanaceae family members are the preferred hosts, as these were the only plants infected by ToNDV-K15 (Table 1).

Variety evaluation

I next used the ToNDV agroinoculation system to assess the response of a group of tomato cultivars. All the varieties developed some ToNDD symptoms, incidence varied from 11% to 100% infections. Of the lines tested, 6 had < 26% infection, including three with 11% infection, that may show resistance to infection to ToNDV-K15 (Table 2). The positive control showed 100% infection in the trials, the mock control and resistant control showed no symptoms of infection.

Discussion

My results presented in this chapter provide evidence of the reappearance of ToNDV, the causal agent of ToNDD, in Southern California in 2015 in Kern Co. This disease caused substantial losses in processing tomato production in the Imperial Valley in the 1970s and 80s and this promoted processing tomato production out of this region. Thus, ToNDD has not been observed for 20-30 years. The detection of ToNDV in 2015 demonstrated that the virus has persisted, although the source is not clear. One possibility is that reemergence of an old disease can occur when the causal agent can persist in local weeds (sleeping virus). Evidence of this comes from the recent detection of ToNDV infecting nightshade samples collected in the Imperial Valley in 2016 (Wintermantel et al., 2018). Additionally, the host range study showed that ToNDV only infects

solanaceous hosts, including the weed *Datura stramonium* and there are several weeds of the Solanaceae family that can function as hosts in this region. A similar case would likely occur if processing tomato production was initiated again in Imperial Valley unless it was planted early (Jan-Feb) before whitefly populations increases. The ToNDD could reemerge along with another sleeping virus in weeds, TYLCV, that could result in severe disease and economic losses. Thus in situations where high populations of the polyphagous MEAM1 *B. tabaci*, the "sleeping" virus from the weeds can be acquired and transmitted to the crop and cause economic losses. An alternative explanation is that the virus was moved long-distance from Mexico via migrating whiteflies. This seems to be less likely because (i) there is no clear evidence of such long-distance spread of whiteflies (ii) ToNDV has not been reported in Mexico. The available information suggests that ToNDV has persisted in Southern California since it was first collected from the fields in Imperial Valley in the 1970s.

After having ruled out the involvement of common viruses associated with necrosis symptoms in tomato in California, a torradovirus etiology was considered based on the similarity with symptoms of chocolate spot disease caused by ToChSV (Batuman et al., 2010). The amplification of the expected-size fragments from most of the samples with the unknown necrosis associated disease suggested a torradovirus etiology and thus was confirmed based on the sequences of these fragments showing highest identities with ToNDV. Here, it is important to note that researchers at the USDA-ARS in Salinas CA had maintained the causal agent of ToNDD by whitefly transmission 40-50 years, and recently it was confirmed to be a torradovirus which was named ToNDV (Wintermantel et al., 2018). Furthermore, the complete nt sequences of the RNA1 and RNA2 of ToNDV-R isolate were determined. Together, these results suggested that this unknown disease of tomatoes in Kern Co, CA in 2015 was TONDD caused by ToNDV. Studies

performed with ToChSV in Guatemala revealed a common co-infection with tobamoviruses, necessitating the elimination of this tobamovirus by grafting a tobamovirus resistant scion (Batuman et al., 2010). In the present study, co-infection with tobamovirus was subsequently determined based on results of mechanical transmission experiments and RT-PCR tests. Because efforts failed to separate ToNDV-K15 and the tobamovirus using tobamovirus resistant tobacco plants, it was necessary to use whitefly transmission. This allowed for the separation of the co-infecting tobamovirus in *Datura stramonium* and subsequent sap transmission to *N. benthamiana* and these plants were used for characterization of ToNDV-K15 and additional experiments, including confirming whitefly transmission of progeny virus in plants infected from the infectious clones.

Using infectious clones and agroinoculation method, the development of ToNDD symptoms in tomato plants completed Koch's postulates for ToNDD and confirmed this was the cause of the "unknown" necrosis-associated disease observed in Kern Co in 2015. This is the third agroinoculation system developed for torradoviruses, the others being for ToTV and ToMarV (Ferriol et al.,, 2016; Wieczorek et al., 2014). The ToNDV-K15 agroinoculation system was then used to show that the host range of ToNDV-K15 was limited to the Solanaceae with the most severe symptoms induced in tomato. In contrast, ToNDV induced mild symptoms in *N. benthamiana* and *D. stramonium* and a symptomless infection in *N. tabacum*. This revealed interesting differences in the virus-host interaction. Although the sequence identity is high between ToNDV-K15 and ToNDV-R, they differ in a species of the partial host range we tested. ToNDV-K15 was only detected in species of the *Solanaceae* family in contrast to ToNDV-R, which was present, although scarcely, in other species like *C. quinoa* and *C. annum*. The capacity of ToNDV-K15 to infect eggplant is in agreement with previous results of torradoviruses being capable of

infecting eggplant (Amari et al., 2008), although ToNDV-K15 induced an asymptomatic infection. Other torradoviruses can infect pepper (*C. annum*), in our case the agroclone showed no infection on pepper of the cayenne variety. It is important to consider that in the partial host range evaluation of ToNDV-R, the inoculation was mechanical, therefore use of an agroclone might explain some of the differences in the host range. This wide range of symptom phenotypes in plants makes identification of hosts other than tomato difficult and requiring RT-PCR detection, such as with the primer pair developed in the present study. Finally, the system was used to screen tomato cultivars and identify some with promising resistance. This is also consistent with previous reports of resistant torradovirus (Gómez et al., 2012). In the case of torradoviruses, Agroinoculation overcomes problems in low rates of mechanical transmission in tomato.

Sequence and phylogenetic analyses performed with sequences of the infectious ToNDV-K15 RNA 1 and RNA 2 clones clearly revealed that ToNDV-K15 is an isolate of TONDV, i.e. identities >98% and placement in a strongly supported clade. The NTI and TI torradoviruses placement in distinct lineages was in agreement with previous studies as was the OW ToTV being more divergent and on a distinct phylogenetic branch. In terms of the NW? TI viruses evidence was found for two possible lineages, one with ToChV from Guatemala and the other with the ToNDV clade, ToMarV from Mexico and ToChSV from Guatemala.

Taking the relative lower sequence identities, the relationships revealed in the phylogenetic trees and the geographic separation, it seems reasonable that they represent distinct species (which ones are you indicating here?). However, it also has been proposed that the viruses in this clade be considered strains of ToMarV (van der Vlugt et al., 2015). ToNDV-K15 sequence and protein identity show it is a member of the torradovirus genus and an isolate of ToNDV-R (Wintermantel et al. 2018). ICTV species demarcation criteria for the Torradovirus genus establishes a threshold

of identity of < 80% for the ProPol region and under 75% of the CP region. Amino acid sequence information clusters ToNDV-K15 close to ToMarV in the CP region with a 92.5% of identity, however the ProPol region shows lower identity than the threshold established by the ICTV with a 77% identity between viruses, therefore further information is needed to assign ToNDV as a distinct species or a divergent isolate of ToMarV. A clear delineation about species and isolates is needed for clarifying ambiguous cases such as ToNDV and based on the current ICTV standards for establishing a new torradovirus, it is not clear whether these are strains or species.

With the development of Agroinoculation systems, it would be interesting to conduct experiments using pseudorecombinats to further investigate the relationship between ToNDV and ToMarV. Finally, with more TI sequences becoming available, it would seem appropriate to re-visit the species demarcation values for torradoviruses. In conclusion, I documented the re-emergence of ToNDD in a late-planted tomato field in Kern Co that showed virus-like symptoms and whitefly infestation. It is shown that this outbreak was caused by an isolate of ToNDV (ToNDV-K15). An agroinoculation system was developed for ToNDV-K15 and used to (i) fulfil Koch's postulates for ToNDD, (ii) investigate host range and (iii) screen tomato cultivars for resistance. These results and the tools generated in this study should allow rapid identification of future outbreaks. It is clear that ToNDV has persisted in Southern California, most likely in weed hosts, but outbreaks require a combination of factors that are not typically found in commercially (late planting and high whitefly pressure) production in Kern Co. However, as noted earlier, this could change if there are efforts to grow processing tomatoes in Imperial County. Worldwide there is reason for more concern and need for broad spectrum torradovirus-resistant varieties. For example, ToChSV and ToChV cause torradoviruses disease in Guatemala, and the detection of ToTV in the new world (Colombia and Panama), and Australia.

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Tables

Table 1. Partial host range of a new isolate of Tomato necrotic dwarf virus determined by agroinoculation with the infectious clone ToNDV-K15 RNA 1 and RNA 2. Five plants of each species or variety were agroinoculated with the ToNDV-K15 infectious clone. One plant of each variety/species was inoculated with the pJL89 vector only as a negative control. These experiments were repeated three independent times.

Host plant species	**Infected[a]**	**Symptoms**
Chenopodium quinoa	0/5	None
Chenopodium amaranticolor	0/5	None
Capsicum annum	0/5	None
Datura stramonium	5/5	Yellow specks on leaves
Nicotiana benthamiana	5/5	Epinasty and discoloration
Nicotiana glutinosa	5/5	Symptomless
Nicotiana tabacum	5/5	Symptomless
Phaseolus vulgaris cv Topcrop	0/5	None
Solanum melongena cv Black beauty	5/5	Symptomless
Solanum lycopersicum N6394	5/5	Necrosis in the base of leaves and stems
Solanum lycopersicum N6385	5/5	Necrosis in the base of leaves and stems
Solanum lycopersicum H5608	5/5	Necrosis in the base of leaves and stems
Solanum lycopersicum cv Glamour	1/5	Only one plant per inoculation presented symptoms of necrosis in the base of the leaves and stems

[a]Infectivity based on positive detection in RT-PCR tests with ToNDV specific primers from RNA extracted from tissue collected from the newly emerged leaves.

Table 2. Screening of tomato varieties for response to a new isolate of tomato necrotic dwarf virus (ToNDV) from a late-planted tomato plot on Kern Co in 2015 by agroinoculation of the infectious cloned RNA 1 and RNA 2. Two inoculations were performed, with the second one performed 14 days after the first inoculation. The symptoms were recorded one month after second inoculation.

Entry	Number of plants with symptoms/total inoculated (%)
1	10 (53)
2	11(58)
3	11 (58)
4	2(11)
5	4(22)
6	11(58)
7	6(32)
8	6(32)
9	11(58)
10	7(37)
11	15(79)
12	9(47)
13	8(42)
14	8(42)
15	8(42)
16	9(47)
17	15(79)
18	2(11)
19	16(84)
20	6(32)
21	4(21)
22	7(39)
23	11(58)
24	13(68)
25	did not germinate
26	15(79)
27	9(47)
28	12(63)
29	5(26)
30	8(42)
31	7(37)
32	8(42)
33	19(100)
34	2(11)
35	19(100)
36	19(100)
37	9(47)
38	19(100)
6394 (+ control)	10(10)
6428 (+ control)	10(10)
LA-3474-R (- control)	0(10)

Table 3. List of primers used in this study

Primers	Sequence (5'-3')	Application
ToR1F	GCWGAYTAYTCMAGYTTTGATGG	DG for detection of torradovirus RNA 1
ToR1R	GGWACWGCMACHAGRTTGTCATC	DG for detection of torradoviruses of RNA 1
ToR2F	TGGGATGARTGYAATGTKCT	DG for detection of torradoviruses RNA 2
ToR2R	CCWGTCCACCAYTTGCAATT	DG for detection of torradoviruses RNA2
ToMV-F	GATCTGTCAAAGTCTGAGAAACTT C	Primer specific for detection of ToMV
ToMV-R	CTCCATCGTTCACACTCGTTACT	Primer specific for detection of ToMV
ToNDVR1F	GTGGTTTTGTGATTTGGTTGATG	ToNDV RNA 1 specific RdRp
ToNDVR1R	ATCACCAGGCTTTACACCTATA	ToNDV RNA 1 specific RdRp
ToNDVR2F	TAGCTACATTGTATGCCAGAAAG	ToNDV RNA 2 specific CP1 (Vp35)
ToNDVR2R	CTGCAGTGTGCCATCACTCTC	ToNDV RNA 2 specific CP1 (Vp35)
pJL89R1-F	GTTCATTTCATTTGGAGAGGTTAAAAGAATTATATACCGA	Amplification RNA 1
pJL89R1-R	TGGAGATGCCATGCCGACCCTTTTTTTTTTTTTTTTTTTTAAA ATAAAAGAAAAGAAAATCTTTT	Amplification RNA 1
pJL89R2-F	GTTCATTTCATTTGGAGAGGTTAAAAGAATTTTAATATAT	Amplification RNA 2
pJL89R2-R	TGGAGATGCCATGCCGACCCTTTTTTTTTTTTTTTTTTTTAAA ATAAAAAAAAAAAAAAATCTTTTTG	Amplification RNA 2
pJL89-F	CCTCTCCAAATGAAATGAACTTCCTTATATAGAGGA	Amplification pJL89 vector
pJL89-R	GGGTCGGCATGGCATCTC	Amplification pJL89 vector
R1-1082	GTGATCCAGCTACCAGGTTG	Sequencing of infectious clone
R1-1836	CAAGTGTCAGGTTCATGCTGAG	Sequencing of infectious clone
R1-2382	CATTGATTTGCATCCAACAGATG	Sequencing of infectious clone
R1-3'	GCTTGGTTAGCTGGAATTTGTC	Sequencing of infectious clone
R1-3063	GTGTGCTTCACCAAAGGTTC	Sequencing of infectious clone
R1-3646	GTCATTAACGGTGAGGATTGAAGG	Sequencing of infectious clone
R1-3898	CATCCTGTGGTTACCCTTTCAAC	Sequencing of infectious clone
R1-4263	GTTGTTGGCAAGAAGAGACCACTCTG	Sequencing of infectious clone
R1-4406	GTGTGCCACTGTCATTATCCAC	Sequencing of infectious clone
R1-4930	CTCACGAAGGCCAGTTTCCAC	Sequencing of infectious clone
R1-5'	GATCTGTGGACAACGACGTAG	Sequencing of infectious clone
R1-526	GTGAGGATGTAGATATCGTAG	Sequencing of infectious clone
R1-5700	CATTTCCATTGATAAGTCACCGTG	Sequencing of infectious clone
R1-6398	GTGTGAATATGATTCCATCATGCA	Sequencing of infectious clone
R2-1150	GTCTTGGGCCATTGAAGCACTC	Sequencing of infectious clone

R2-1657	CATTGACTTCCCTGATGATTGGAC	Sequencing of infectious clone
R2-2792	CGGAGGCTGTAATATGTTTGTAG	Sequencing of infectious clone
R2-3’	GTGGCGATAGAAAGGGTTTG	Sequencing of infectious clone
R2-3’.2	CAAGAAGTAATGAATGGCAC	Sequencing of infectious clone
R2-3500	CAATGACCATATACTGGCTTGACAG	Sequencing of infectious clone
R2-3970	CATACGAGTGTGCCTCCTAGGAAAG	Sequencing of infectious clone
R2-4067	GAACTAACAATGGCTGCAGGTG	Sequencing of infectious clone
R2-5’	GTAGGTCGCACAACGACGTGG	Sequencing of infectious clone
R2-620	GGCAGAGCAGAAATCTGAGAAC	Sequencing of infectious clone
R2-656	CAACTTCAACTGTCTAATCAG	Sequencing of infectious clone

ToNDV tomato necrotic dwarf virus, DG degenerate primer

Primer sequences developed for ToNDV were designed from the Genebank accession numbers NC_027926.1 and NC_027927.1 for RNA 1 and RNA 2 respectively. In the primer sequences, K =g/T; M=A/C; Y=C/T; W=A/T; S=C/g and V=A/C/g.

Table 4. Percentage amino acid sequence identity of NTI and TI torradoviruses compared with ToNDV-K15. ICTV species demarcation criteria establish <75% identity in CPs aa sequences and ProPol region <80% aa sequence identity.

Total RNA 1	ToNDV-R	ToMarV	ToChSV	ToChV	ToTV	LNLCV	MYMoV	SCLSV	CaTV1
ProPol	98.1	78.1	76.0	68.4	57.2	39.3	39.2	35.0	40.1

Total RNA 2	ToNDV-R	ToMarV	ToChSV	ToChV	ToTV	LNLCV	MYMoV	SCLSV	CaTV1
3CPs	94.5	79.0	75.4	71.7	66.7	42.8	46.9	46.7	48.3
ORF1	98.9	80.0	73.5	64.0	60.8	24.0	30.1	21.1	25.9

Figures

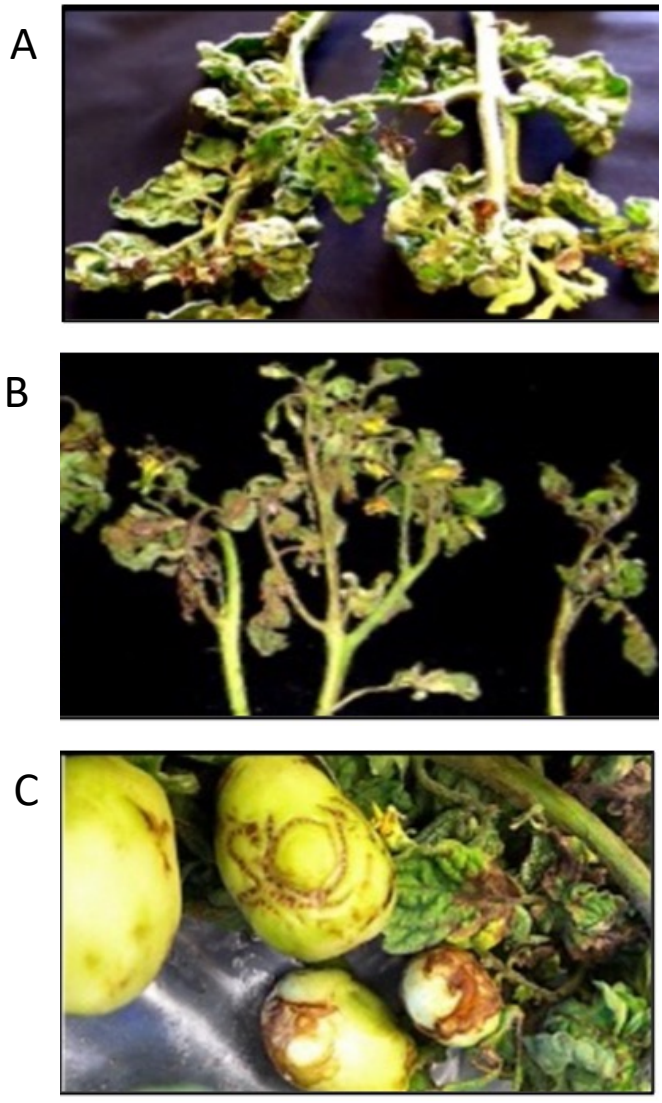

Figure 1. Virus-like disease symptoms observed in a late-planted plot of processing tomatoes in Kern County in 2015. Tomato plants were stunted and showed distorted growth and severe crumpling and necrosis of leaves and necrosis of petioles and stems (A, B). The fruits showed ring spots with etched borders (C). It should also be noted that this fields had high populations of whiteflies, believed to be *Bemisia tabaci*.

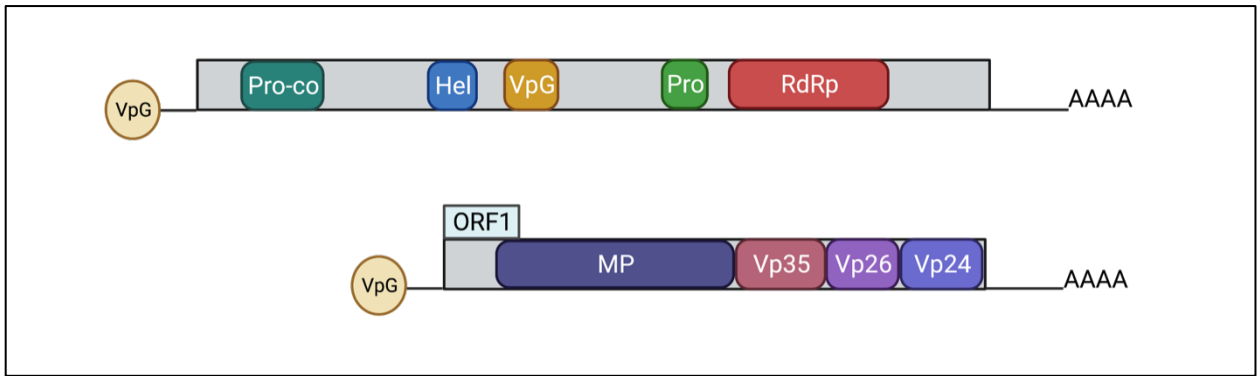

Figure 2. Schematization of the genome organization of a new isolate of tomato necrotic dwarf virus (ToNDV-K15) recovered from a late-plant tomato field in Kern Co, CA in 2015. RNA1 is 7246 bp and predicted to have a VpG bound to the 5' untranslated region (UTR) and a polyA tail in the 3' end. There is a single large ORF in RNA 1 which encodes for a 241.3 kDa polyprotein that is cleaved into the RdRp, protease, helicase, protease cofactor and VpG. RNA2 is 4912 bp a has two open reading frames, ORF1 and a large downstream ORF1 encoding a polyprotein. ORf1 encodes a 23.3 kDa protein, whereas the polyprotein will be cleaved into the movement protein and three CPs. RNA 2 has a predicted VpG at the 5' end and a polyA tail at the 3' end.

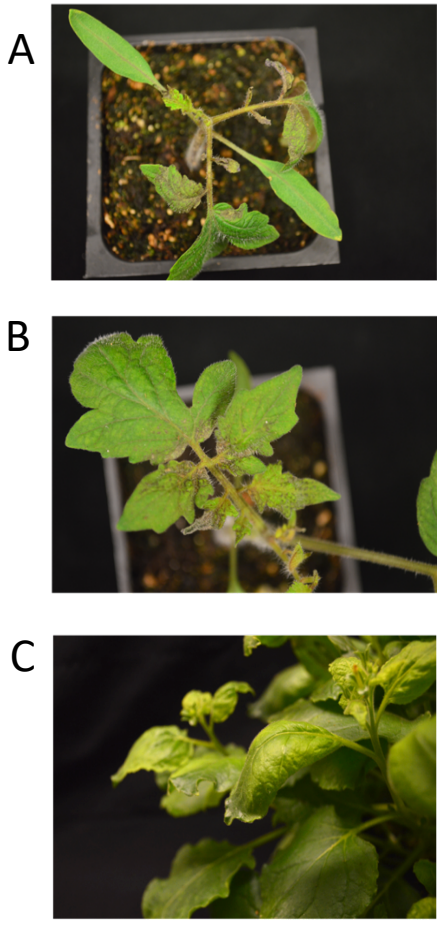

Figure 3. Symptoms induced by the full-length infectious clone of a new isolate of tomato necrotic dwarf virus recovered from late-planted tomatoes in Kern Co CA in 2015, at 14 dpi. (A) Necrosis symptoms in the basal part of leaves on tomato (*Solanum lycopersicum*) plants of cv 6394 (B) More severe necrosis in tomato plants (cv 6485) newly emerged leaves of *Solannum lycopersicum* 6485 (C) *Nicotiana benthamiana* plants with epinasty and mottling in leaves

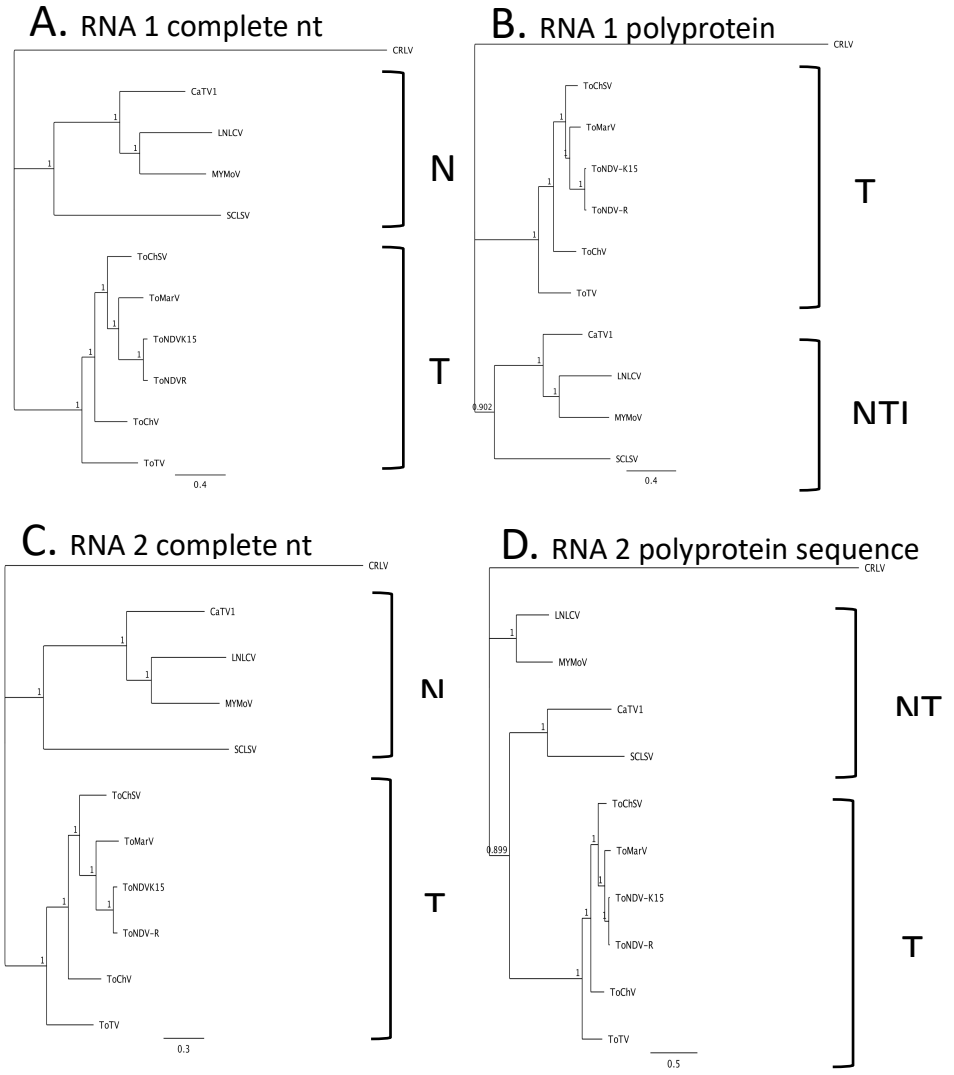

Figure 4. Phylogenetic analysis of RNA 1 and RNA 2 sequences of a new isolate of tomato necrotic dwarf virus (ToNDV) and other torradoviruses (A) Nucleotide based phylogenetic analysis of the complete sequence of RNA 1, (B) amino acid based phylogenetic analysis of the polyprotein of RNA 1 (C) Phylogenetic analysis of RNA 2-ORF 2 based on nucleotide sequences (D) Phylogenetic analysis of RNA 2, ORF 2 based on amino acid sequences. Analyses were made in Geneious, using a MAFFT alignment, a GTR substitution model in MrBayes with 100,000 iterations and a burn of 10,000 MCMC. Non tomato infecting species (NTI), Tomato infecting species (TI). *Cherry raspleaf virus* (CRLV) was used as root for all of the trees.

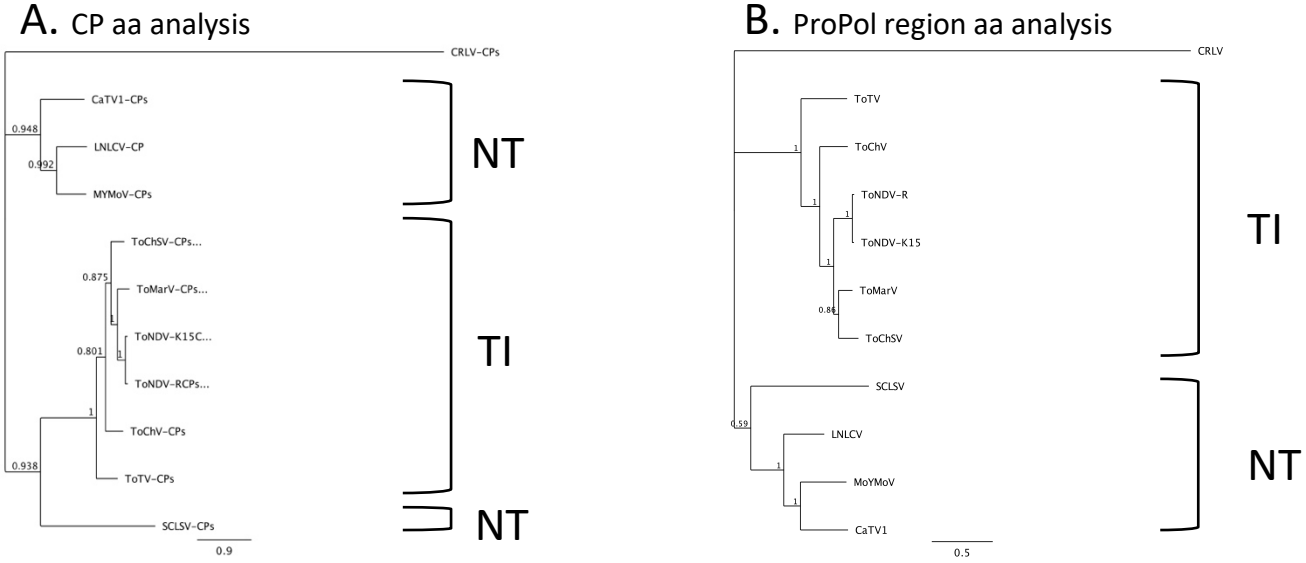

Figure 5. Phylogenetic analysis of RNA 2 sequences of a new isolate of tomato necrotic dwarf virus (ToNDV-K15) recovered from a late planted field in Kern Co in 2015. Analysis of the CP (A) and ProPol (B) regions of the *Torradovirus* genus. Non tomato infecting (NTI) and tomato infecting (TI) viruses in the genus. The bayesian trees were generated with a mafft alignment and a GTR model with 10000 bootstraps and the percentage is shown in the branches. CRLV was used as an outgroup.

Chapter III

Genetic and functional analyses of the genes and proteins of a new isolate of the torradovirus tomato necrotic dwarf virus (ToNDV) from Southern California, USA

Genetic and functional analyses of the genes and proteins of a new isolate of the torradovirus tomato necrotic dwarf virus (ToNDV) from Southern California, USA

Abstract

Torradoviruses (family *Secoviridae*) are an emerging group of viruses with a bipartite genome. The RNA1 (~7Kb) encodes proteins involved in virus replication and RNA2 (~5 kb) encodes proteins associated with movement [ORF1 and movement protein (MP)] and the three capsid proteins (CP), VP35, VP26 and VP24. Since very little is known about the mechanism (s) by which torradovirus moves in plants and the role of ORF1 and MP in movement, I investigated genetic and functional properties of these proteins from a new isolate of tomato necrotic dwarf virus from Kern Co, CA (ToNDV-K15). Using infectious RNA1 and RNA2 clones and mutagenesis, essential role of ORF1 in cell-to-cell movement was established, and motifs important for this process were identified in ORF1, MP and CP. Protein structure modelling provided insights into how these mutations affect protein structure and function. Subcellular localization experiments with GFP fusion showed ORF1 and MP accumulated in the plasma membrane and in structures resembling plasmodesmata (PD). Phylogenetic analyses revealed that the MP of ToNDV-K15 is related to the MP of Polerovirus and that the two viruses might be using a two-protein strategy for cell-to-cell and long-distance movement. RNA binding assays showed ORF1 protein binds ssRNA in a non-specific manner. Together, these results provide further insight into the functions of RNA2 encoded proteins of torradoviruses in virus movement.

Introduction

Plant viruses infect their hosts and alter the cellular machinery to replicate and spread within plants (Singh & Singh, 2018). This often involves altering normal cellular functions that lead to the development of disease symptoms. Given their small genomes, viruses encode multifunctional proteins that are essential for successful viral infection. Knowledge of the functions of viral proteins and their interactions with host factors can lead to new effective strategies to control spread of viral diseases (Romay & Bragard, 2017).

The major steps in viral infection of plants are (i) viral replication and gene expression, (ii) cell-to-cell movement, and (iii) long distance (systemic) spread. Thus, the capacity to move within the plant is essential and involves overcoming numerous barriers. For cell-to-cell movement, viruses use plasmodesmata (PD), the membrane-lined channels that interconnect plant cells (Lucas et al., 2009). However, PDs typically are nanochannels (1-3 nm) that are too narrow to allow movement of virions or viral nucleic acids. These channels are also regulated and dynamic structures that can change size. The size exclusion limit (SEL) reveals the aperture of the PD nanochannels, which impacts macromolecular transport.

Therefore, plant viruses have evolved specialized proteins to modify the SEL and facilitate cell-to-cell movement of viral nucleic acids, often moving though PD in the process (Noueiry et al., 1994). Those are referred of as movement proteins (MP); and are defined as non-structural proteins that mediate movement typically through PD. For some viruses, capsid proteins (CP) might also play a role in long-distance movement (Rojas et al., 2016a), either in the form of virions or providing an additional role in movement, e.g., CP of Tomato yellow leaf curl virus (TYLCV) (Rojas et al., 2001).

Thus, cell-to-cell movement of many plant viruses occurs between cells via PD of interconnected cells to allow the virus to move out from initially infected cells and eventually, to the phloem for long-distance movement/transport to tissues where active cell division is underway, e.g., shoot and root apices. Thus, viruses utilize the vascular system of the plant for systemic infection, this occurs via source to sink manner and allows viruses to access cells that are progenitors of phloem cells (Vuorinen et al., 2011). Finally, it should be noted that long-distance movement must be differentiated from long-distance movement of RNAi, siRNA as some long distance MPs have turned out to be suppressors of silencing that can facilitate long-distance movement, e.g. 2b protein of cucumber mosaic virus (CMV) (Nemes et al., 2014). Two major mechanisms of cell-to-cell movement of plant viruses have been recognized: (i) via virus-specific tubules through which virions move or (ii) MP-mediated SEL increase that typically does not involve structural modification of PD. The tubules are generated by modifications of PD or *de novo* and are composed of MP and CPs, through which a non-virion MP-viral nucleic acid complex may move.

The *Secoviridae* family is a large and diverse family of plant infecting plus-sense ssRNA viruses. Many of these viruses have several members which use the tubule mode of movement to establish infection, e.g., members of the genera comovirus, nepovirus and cheravirus (Amari et al., 2011; Kasteel et al., 1997; Rojas et al., 2016b; Van Lent et al., 1991). Whereas the PD SEL modification is used by geminiviruses, cucumoviruses, dianthoviruses, potyviruses and tobamoviruses (Deng et al., 2015; Mushegian & Elena, 2015; Rojas et al., 2016b). *Cowpea mosaic virus* (CPMV) (Pouwels et al., 2003; Van Lent et al. , 1991) was one of the first virus shown to utilize the tubule mechanism and demonstrated a role for the MP and CP interaction in this process (Carvalho et al., 2004). The genus Torradovirus was recently established and is in the family

Secoviridae and there is little information about how these viruses move in plants. Thus, the torradovirus, ToNDV described in chapter 2 was used as a tool to expand the knowledge on these viruses. In the present study, I investigated the role of RNA2 encoded proteins, ORF1 and MP, involved in movement of ToNDV. Here, I describe genetic and functional analyses of these proteins with mutational, subcellular, and nucleic acid binding analyses. To understand the biological properties and possible interactions during infection of torradoviruses, sequence comparisons were used to infer putative functions of these proteins. Subcellular localization experiments informed about possible interactions with plant proteins in the vicinity and possible function. I identified motifs in the proteins, e.g., myristoylation domains, that may be involved in anchoring these proteins to cellular locations, e.g., membranes, and specific residues in the proteins that allows the ORF1 and MP of torradoviruses to function (Wang et al., 2021). The information obtained in this study with a new isolate of ToNDV has revealed novel roles and possible functions of these proteins in torradovirus movement.

Materials and Methods

Generation of mutants

The infectious RNA2 clone of ToNDV-K15 in recombinant plasmid (ToNDV-R2-pJL89) was used as the template for introducing mutations. This was accomplished by introducing mutations in the primer sequences and using them for PCR amplification of the entire recombinant plasmid RNA2 construct. The PCR amplification was conducted with 1X CloneAmp HiFi PCR premix (Clonetech) with 1 ul (60 ng/ul) of RNA 2-pJL89. The PCR reaction involved an initial denaturation step of 98 C for 30 s; followed by 35 cycles of 98 C for 30 s, annealing at 60 C for

30s and elongation at 72 C for one min. The PCR products were analyzed by agarose gel electrophoresis in 0.8% TAE buffer. The expected-size DNA fragments were purified from the gel and quantified with a Nanodrop (Thermo Fisher Scientific). 200 ng of each of the PCR amplified mutated RNA 2 were used for the Gibson Assembly, incubated at 50 C for 1 h with 200 ng of the pJL89 PCR-amplified fragment. Five ul of the Gibson Assembly reaction was transformed into *E. coli* competent cells, which were spread onto solid LB media with kanamycin and incubated overnight at 28 C. The resulting colonies were picked and individually grown at 28 C in LB liquid media with antibiotics overnight. The cells were then concentrated by centrifugation, and total plasmid DNA was extracted the according to manufacturer's instructions (Zymo Co). Recombinant plasmids from selected transformants were sequenced to confirm the presence of the appropriate mutation.

Modeling in silico of proteins of interest

A neural network-based model, AlphaFold was used to predict *in silico* tertiary structure of the proteins and to estimate accuracy of the prediction in a google colab notebook (https://colab.research.google.com/github/deepmind/alphafold/blob/main/notebooks/AlphaFold.ipynb). The predictions were visualized in ChimeraX-1.2.5. Lipophilicity analysis of ORF1 was performed in ChimeraX-1.2.5 with the MLPP plugin (Laguerre et al., 1997).

Agroinoculation of tomato and *Nicotiana benthamiana* plants

Recombinant binary plasmids with the RNA2 mutations (Table 1) were transformed into *Agrobacterium tumefaciens* (C58) cells by electroporation, spread and grown in LB plates supplemented with kanamycin at 28 C for 48 h. Representative colonies were selected and grown

in liquid LB media with kanamycin overnight and stored at -80^0 C. To test for infectivity, five *N. benthamiana* and tomato (variety 6394) plants were co-agroinoculated with the wild-type RNA1 and the mutant RNA2 constructs (OD_{600}=1 in a 1:1 ratio) by needle puncture of the shoot apex. Positive and negative control plants were co-agroinoculated with wild-type RNA1 and RNA2, or with the empty pJL89 vector, respectively. The plants were maintained in a controlled environment chamber at 22^0 C with a photoperiod of 16 h light and 8 h of dark. At 21 days post-inoculation (dpi) plants were evaluated for disease symptoms and samples taken from newly emerged leaves were used for extraction of total RNA to be used in RT-PCR test for ToNDV-K15 infection. Three independent experiments were conducted for each mutant.

Sequence analyses and phylogenetic analyses

Torradovirus genus sequences were obtained from GenBank and aligned in Geneious with the MAFFT algorithm. For the split MP phylogenetic analysis, 531 movement protein sequences were obtained from GenBank and aligned with the MAFFT algorithm in Geneiuos. The phylogenetic relationships were revealed with the Neighbor Joining method with 1000 boostraps and Jukes-Cantor genetic distance model with the GeneiousTree Builder for the N- and C-termini of the MP of ToNDV-K15 and visualized in Geneious.

Leaf disc assays for assessing replication and cell movement of RNA2 mutants

To assess replication and cell-to-cell movement properties, each RNA2 mutant was co-agroinfiltrated with wild-type RNA 1 into patches in *N. benthamiana* leaves that were outlined with a marker. At 0, 1, 3, 5, and 7 dpi, three leaf discs (10 mm diameter) were taken from within and 5 mm away from of the infiltrated patch. Total RNA was extracted with the Qiagen RNeasy

kit according to manufacturer's instructions. The samples were treated with DNase and RT-qPCR was performed in a BioRad CFX-96 thermocycler with the iTaq universal SYBR Green One-Step Kit (Biorad) and RNA1-specific primer pair qPCR4-R1F 5' GGTCCTGCTGAAACTGCTC 3' and qPCR4-R1R5'GAGGGTTTCAAAGGACAATCTG3'. The RT-qPCR reaction consisted of an initial RT step of 10 min at 50 C followed by a denaturing step at 95 C for 1 min followed by 40 cycles of denaturation at 95 C for 30s, annealing, and extension at 60.5 C for 30 s. The detection of the amplification of the target of ToNDV-K15 RNA 1 fragment was determined with the ΔCT method with actin as a reference gene [primer pair: ACT-F(5'TCCTGATGGGCAAGTGATTAC3') and ACT-R (5'TTGTATGTGGTCTCGTGGATTC 3') (Liu et al., 2012)] and the same RT-qPCR conditions.

Constructs expressing GFP fusion proteins

ToNDV genes encoding proteins of interest and GFP were amplified by PCR with primers that included CACC in the 5' end of the forward primer to facilitate cloning into the pENTR vector (Themo Fisher Scientific). The PCR-amplified fragments were ligated for 1 h with the pENTR vector at room temperature and 5 ul of the ligation was transformed into *E. coli* cells (Top 10, Thermo Fisher). Following the incubation, 200 ul of the transformation was spread onto LB media plates and grown overnight at 37 C. Thirty colonies (transformants) were grown overnight in liquid LB media with kanamycin. Plasmid DNA was extracted with the Zymo miniprep kit (Zymo) and analyzed by agarose gel (0.8%) electrophoresis in TAE buffer. Potential clones were selected based on plasmid size and sequenced. After confirmation by sequencing, the pENTRY clones were used in an LR (recombination) reaction according to manufacturer's instructions (Thermo Fisher) to introduce the gene of interest to a pSITE vector such that the GFP gene (fluorescent tag) is fused

to the 3' end of the gene of interest, such that the fusion proteins have a GFP tag at the C-terminus. Five ul of the LR reaction was transformed into Top 10 (Thermo Fisher) *E. coli* cells and cells were spread on LB agar medium with spectinomycin and incubated overnight at 37 C. The resulting colonies were grown in liquid media overnight at 37 C and plasmids were extracted and potential clones were sequenced to confirm the identity and correct reading frame.

Confocal microscopy

A. tumefaciens strains carrying the recombinant plasmids, ORF1-GFP, MP-GFP were infiltrated alone or in various combinations into leaf patches of *N. benthamiana* plants. Cell suspensions of OD_{600}=0.3 having been induced with acetosyringone for 2 h were used for infiltration. Plants were kept in the dark for 24 h and moved to a controlled environment chamber at 22 C with a 16 h light and 8 dark photoperiod. Time course preliminary experiments were performed with the ORF1-GFP and MP-GFP fluorescently-tagged proteins, in which expression was monitored at 24, 36, 48 and 72 h. The leaf discs were collected from inside the infiltrated area and observed with the confocal microscope. In addition, ORF1-GFP and MP-GFP were co-infiltrated in a 1:1 ratio with cellular-organelle markers tagged with mCherry for ER, Golgi, plastids, nucleus and plasma membrane. Confocal microscopy was performed with a Leica SP8 confocal laser scanning microscope (CLSM) with a 63X oil immersion objective. The eGFP was excited at 488 nm and the emission was gathered at 500-550 nm, whereas mCherry was excited at 550 nm and the emission was gathered at 600 nm.

Protein expression and immunoprecipitation in plants

A. tumefaciens C58 carrying the ORF1-GFP, MP-GFP, GFP-ORF1 and GFP-MP were infiltrated into *N. benthamiana* leaves as described. After 48 hpi three leaf discs (10 mm diameter) were ground in Laemmli buffer and analyzed by polyacrylamide gel electrophoresis (SDS-PAGE) in 10% gel. The proteins were transferred into nylon membranes, and the membrane was blocked for 4 h and left overnight with the primary GFP antibody (Invitrogen). The next day, the membrane was washed and incubated for 1.5 h with the secondary antibody (BioRad). The membrane was visualized by chemiluminescence in a Chemidoc (BioRad).

Protein expression and purification in *E. coli*

ORF1 and MP of ToNDV were PCR amplified and cloned into the pRSET-A (Thermo Fisher Scientific). The constructs were sequenced to confirm identity and reading frame. The ORF1 and MP pRSET-A constructs were transformed into *E. coli* strain BL21 and the protein were expressed according to manufacturer's instructions (Thermo Fisher). Cells were centrifuged and the pellets were stored at -80 C until protein was extracted. Proteins were extracted according to manufacturer's instructions (Qiagen expressionist) and recovered with a Ni-NTA agarose resin according to manufacturer's instructions (Qiagen). Recovered proteins were renatured by stepwise dialysis against buffer E (Qiagen) pH 4.5 (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea) and used in binding assays.

Gel mobility-Shift assays

Protein-RNA binding assays were performed by combining different quantities of selected proteins and nucleic acids in a total volume of 20 ul, which was incubated for 30 min at 22 C in

binding buffer (10 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA, 10% [v/v] glycerol, 1 mM PMSF and 1 mM DTT). Binding reactions were then analyzed by agarose gel electrophoresis on 0.8% agarose gel in TAE buffer (Rojas et al., 1998).

Results

Mutational analysis

To identify locations in the ToNDV-K15 ORF1 and MP for mutagenesis, I searched for highly conserved regions and amino acids, including those previously reported as essential for infection in *Tomato torrado virus* (ToTV) and *Tomato marchitez virus* (ToMarV). Cleavage sites and functional motifs such as RNA binding motifs and myristoilation domains were emphasizes in these searches. In the case of MP similarities with sequences from viruses in other families were included in the analysis.

Six mutations were introduced into ORF1 (Table 1). I first altered the start codon of ORF1 [ATG to CTG], and this ORF1 knock-out mutant (ORF1-KO) did not induce symptoms at 21 dpi in either *N. benthamiana* or tomato. In contrast, *N. benthamiana* or tomato plants co-agroinoculated with wild-type RNA 1 and RNA 2 developed ToNDV symptoms after 5 dpi. No symptoms were observed in plants agroinoculated with the empty vector control. These results indicated that the ORF1 protein is required for infection of ToNDV.

Several possible myristoilation domains were identified in the N-terminus of ORF1 of ToNDV-K15. Myristoilation domains are usually associated with signal transduction, membrane binding and protein stability (Turnbull & Hemsley, 2017), and target vital proteins to the cell periphery (Rojas et al., 2001; Wang et al., 2021). Therefore, I hypothesized that these motifs play

an essential role in ORF1 targeting and function. I selected the two myristoilation motifs in the ORF1 protein for mutation and named them, MYR1 and MYR2 (Fig 1-2). The same mutation was introduced into each motif [G-X-X-X-**S/T**-X-X-X to G-X-X-X-**A**-X-X-X]. I also generated a double MYR mutant (MYR 3) in which both of these myristoilation sites were altered. The *N. benthamiana* and tomato plants co-inoculated with wild-type RNA 1 and all the ORF1-MYR mutants developed typical symptoms of ToNDV infection by 5 dpi, similar to the wild-type ToNDV. Thus, by 14 dpi the ORF1 MYR1, MYR2 and MYR3 mutants induced symptoms indistinguishable from those of wild-type ToNDV in both hosts. These results indicated that these MYR domains are not essential for infectivity or virulence of ToNDV and presumably ORF1 function.

I continued searching for conserved domains in ORF1 by sequence comparisons and alignments including regions of conservation between tomato and non-tomato infecting viruses. A cluster of three amino acid residues were highly conserved in the ORF1 amino acid sequences of all torradoviruses, L42, D43 and F44 (Figure 2). Therefore, I generated an alanine replacement mutant in which this triad is converted to alanine residues and this mutant is referred to as the ORF1 LDF (Fig 1). By 14 dpi, *N. benthamiana* and tomato plants inoculated with the LDF mutant had developed no symptoms, whereas typical symptoms developed in plants inoculated with the wild-type ToNDV RNA 1 and RNA 2. Furthermore, RT-PCR tests, with an RNA 1 primer pair, of the newly emergent leaves of these plants revealed no amplification of the target sequences for the LDF mutant, whereas the fragment was amplified from the symptomatic newly emerged leaves of plants inoculated with wild-type RNA 1 and RNA 2.

Because torradoviruses utilize a polyprotein genome strategy, I considered the possibility that the ORF1 protein is cleaved into smaller functional proteins. I examined the ORF1 aa sequence

for putative cleavage sites, and one Q/I was located to aa 70/71 (Fig 1). Therefore, I generated a mutant in which these residues were substituted with A/A, and the mutant is referred to as ORF70 (aa70). When this ORF1 cleavage mutant was co-inoculated with wild-type RNA 1 into *N. benthamiana* and tomato plants, typical wild-type symptoms had been developed by 14 dpi, similar to plants inoculated with wild-type RNA 1 and RNA 2. Sequencing of this mutated location in a fragment amplified by RT-PCR confirmed the presence of the mutation. This shows that these amino acids are not necessary for infection and that the ORF1 protein is unlikely to be cleaved.

A similar approach was used for the MP in which the amino acid sequences of TI and NTI torradoviruses were aligned and analyzed. It is important to note overlap between the 3' end of ORF1 and the 5' end of MP and downstream impact on CP expression did not allow for alteration of the start codon of the MP. Therefore, I focused on identifying functional domains by aligning torradovirus MPs and searching for conserved areas. A total of 7 mutations were introduced into the MP (Table 1). Conserved amino acids were identified at positions 263N, 266F and 270Q (Fig 3). I substituted each of these amino acids as well as all three with alanine (A) residues. *N. benthamiana* and tomato plants co-agroinoculated with RNA 1 wild-type and each of the the RNA 2-MP mutants developed wild-type symptoms and RT-PCR and sequencing of infected newly emerged leaves confirmed infection and of the presence of the introduced mutations. These results show that the conserved amino acids at positions 263, 266 and 270 of the ToNDV (and presumably those of other torradovirus) are not essential for infection.

Other conserved regions of the MP of ToNDV-K15 also were analyzed, including a phenylalanine (F) residue to position 210 in the MP of ToTV that was a pathogenicity determinant, as a mutation in that site resulted in a symptomless infection (Wieczorek & Obrepalska-Steplowska, 2016). The 210F residue is conserved in the MP of ToNDV-K15, and I exchanged

the 210F residue in the ToNDV-K15 MP with an alanine residue (Fig 3) referred to as mutant MP210. When co-agroinoculated with wild-type RNA 1, the MP210 mutant did not induce symptoms by 14 dpi, nor was viral infection detected in newly emergent leaves with RT-PCR tests. This result show that F210 in the MP of ToNDV is necessary for ToNDV infection.

The MP is the first protein in the ORF2 polyprotein of the RNA 2 (Fig 1). However, there is no evidence that the MP is, in fact, expressed as single ~50 kDa protein. Based on results of N-terminal sequencing of the three CPs of ToMarV and *Tomato chocolate spot virus* (ToChSV) it appears that ToNDV-K15 has similar cleavage sites, including the site to release MP from VP35 (Ferriol et al., 2016). Therefore, I used the CP cleavage site information to investigate the MP. Furthermore, in performing my alignments and comparison with the MP aa sequence, noted that the protein may be composed of two domains. There is a N-terminal domain related to 3a-type movement proteins of bromovirus such as brome mosaic virus and cucumber mosaic virus and a C-terminal domain related to the luteoviruses (Fig 2). Therefore, I performed a conserved domain BLAST analysis (CD-BLAST) analysis, which confirmed this and indicated that ToNDV MP aa residue 225 was the end of the N-terminal domain (Fig 2). To further investigate this potential hybrid MP, I performed phylogenetic analyses with the aa sequences of the N- and C-terminal parts of the ToNDV-MP and MP sequences of 530 viruses (Fig 4 and 5). As shown in Fig 4, the torradovirus MP sequences are placed in a larger clade (0.40 support) with MPs of bromoviruses (3a-type), secoviruses and luteoviruses. Within this clade there a two strongly supported sub clades, one with the bromovirus MPs (0.89) and one with the secovirids and luteovirus MP (0.90) (Fig 4). Interestingly, in the subclade with secoviruses and luteoviruses there are three clades, two with secoviruses and one with luteovirus and MP sequences of torradoviruses. This result was not in agreement with the CD-BLAST results, but they suggest a role for recombination in the

evolution of these MP, including those of Torradoviruses. The results of the tree generated with the C-terminal ToNDV MP sequences placed these sequences in a strongly supported clade with MP sequences of members of the genus Polerovirus (family *Sobemoviridae*) and one other member of the family Secoviridae, the nepovirus Caraway yellows virus. Together these results are consistent with the MP gene/protein being a hybrid that arose via recombination events in plants having mixed infections. I hypothesized that the MP may be cleaved after aa 225, and I identified two potential cleavage sites aa 228 (Q/S) and aa271 (Q/S) and generated alanine replacement mutants, A228Q and A271Q; these were named MPMID and MPMID2, respectively (Fig 1). Each mutant was co-agroinoculated with wild-type RNA 1 into *N. benthamiana* and tomato plants. By 14 dpi, wild-type symptoms were present in newly emerged leaves and RT-PCR tests confirmed ToNDV infection and presence of the mutation. Thus, these results showed these putative cleavage sites were not essential for viral infection and that MP most likely functions as a complete protein, rather than being cleaved into two functional proteins.

In addition to the MP, the RNA 2 polyprotein includes the three CPs, Vp35, Vp26 and Vp24 (Fig 1). I aligned the amino acid sequences of the CP portion of the polyprotein including those of all TI and NTI torradoviruses and noticed the cleavage sites were highly conserved among all species, including the CPs of ToNDV-K15. I also identified a MYR domain in the N-terminus of the largest capsid protein Vp35, which is cleaved from the MP (Fig 1). I mutated this MYR domain by alanine replacement as previously described to create the MYR35 mutant. When the Vp35 MYR mutant was co-agroinoculated with wild-type RNA 1, no symptoms were observed by 14 dpi in *N. benthamiana* or tomato plants, not was infection detected by RT-PCR.

My search also revealed potential essential regions in the other CPs. Vp24, the smallest CP, has a region of positively-charged amino acids (RRHTRMR). Positively charged amino acids

in the CP of N-terminus bromoviruses has been associated with RNA binding and are referred to as the ARM domain (Schmitz & Rao, 1998). Moreover, aa sequence comparisons revealed, the positively charged amino acids in Vp24 of ToNDV resembled the RNA binding site (ARM) in bromoviruses (SRTSRRRRRPRR) (Schmitz & Rao, 1998). Therefore, I investigated the role of this positively charged region of the Vp24 by generating an ARM mutant by alanine substitution of all seven of the residues in this motif (RRHTRMR). This ARM mutant was co-inoculated with wild-type RNA1 and, 14 dpi no symptoms were observed in the *N. benthamiana* or tomato plants.

Assessment of whether non-infectious mutants are impaired in replication of movement cell-to-cell

After identifying mutants that abolished infection, I next determined whether the mutation impacted replication or cell-to-cell movement of ToNDV-K15. This was done by RT-qPCR analyses of leaf discs taken from within and 5 mm outside of the infiltration patches at 0, 1, 3, 5, and 7 dpi. The mutants tested were: (i) ORF1-KO, (ii) ORF1-LDF, (iii) MP-210, (iv) VP35-MYR4 and (v) VP24-ARM. Co-agroinoculated wild-type RNA 1 and RNA 2 and agroinfiltration of the empty vector were positive and negative controls, respectively. Actin was used as a reference gene for all tests.

The RT-qPCR analysis showed that all the mutants replicated inside the infiltrated patches to levels similar or somewhat lower than those of the wild-type ToNDV, replication was initially detected at 1 dpi and increased until 5 dpi and then decreased at 7 dpi (Fig. 6). The RT-qPCR analyses results also showed that none of the mutants moved outside the infiltrated area based on failure to detect viral replication of RNA 1 in discs taken 5 mm outside of the infiltrated patch at

any of the time points, including 7 dpi. For the leaf discs outside the infiltrated patch, the wild-type was detected at 7 dpi (Fig 6).

In contrast, the wild-type virus replication was detected in discs collected outside of the infiltrated patch at 5 dpi and 7 dpi, and at substantially higher levels than detected inside the infiltrated area (Fig 6). For the empty vector negative control, no viral replication was detected in leaf disks from within and outside the infiltrated patch.

In silico modelling of wild-type functional and non-functional mutant proteins.

To gain insight into how some of the mutations may have impaired protein function, the impact on structure on non-functional mutant proteins was assessed by generating models of the wild-type ORF1, MP, Vp35 and Vp24 proteins. These were compared with models of the mutated proteins of the ORF1-LDF, MP-210, Vp35-MYR4 and Vp24-ARM mutants. This allowed me to predict how these mutations might interfere with viral infection.

The model of the ORF1-LDF showed that the triple alanine substitution with the amino acids L42A, D43A and F44A, caused a decrease in the lipophilicity of this region of the protein (Fig. 7). Furthermore, this change in protein property was highly supported according to the alphafold method with pLDDT values <90 and a predicted error close to 0 (Fig 7). This change can alter the subcellular localization or protein-protein interactions.

I next examined the impact of the MYR domain of the wild-type and mutated Vp35 proteins. The model in Fig 4, revealed no obvious structural changes at this region of the protein. However, because Vp35 is a CP and a component of the virion, I next modeled the structure of a subunit of the virion, which includes a single nucleotide of each CP (Fig. 10) and compared this subunit model with that generated with the VP35-MYR and VP24-ARM in addition to wild-type

Vp26 protein (Fig 8). The results showed that the mutations may cause a chance in the Vp35-MYR position and the exposure of the ARM motif (Fig. 10) which could result in unstable virions that are impaired in cell-to-cell movement.

I next modelled and compared the wild-type Vp24 and Vp24-ARM mutant proteins. The support of the model of the region with the mutation is high (Fig 9). This revealed evidence of a change in the exposure of the ARM residues in the ARM mutant model. The residues in the ARM region of the wild-type protein are inside a pocket (Fig 9). However, the change in charge caused by the mutation results in the disappearance of this pocket disappears, making the residues in this position more exposed (Fig 9).

Subcellular localization

To gain further insight into ORF1 and MP function, I performed subcellular localization analyses with GFP fusion tag. The GFP-tagged ORF1 or MP were observed with confocal laser scanning microscopy to determine their subcellular localization and co-localization with cellular organelles following agroinfiltration of *N. benthamiana* leaf patches as described in materials and methods. I first established the optimum time to observe the localization of these protein by conducting a time course experiment in which expression was evaluated at 24, 48 and 72 hpi. The optimal visualization time was 48 hpi, based on sufficient signal strength but not overexpressed and aggregated which obscured localization or cellular structures.

Constructs were initially generated to express MP-GFP and ORF1-GFP. The ORF1-GFP fusion protein localized to the cell periphery and to PD-like structures in the cell wall (Fig 11). Furthermore, small and vesicle-like structures were observed moving rapidly inside the cell often appearing to be targeted to the cell periphery for a short period of time (Fig 11). I next used

fluorescently-labeled markers to tag diverse plant organelles such as the plasma membrane, plastids, endoplasmic reticulum (ER), Golgi and the nucleus. I observed co-localization of ORF1-GFP with the PM marker. ORF1-GFP also accumulated in the nuclear periphery and might be accumulating close to ER strands in the cell (Fig 11).

The confocal visualization of the MP-GFP was also preceded by a time course of images at 24, 48 and 72 hr. I determined that 48 hpi was the optimal time because the signal was strong but allowed for distinction of details in the cell. MP-GFP localized to the cell-periphery and in PD-like structures in the cell wall (Fig 13). In contrast to the ORF1-GFP, MP-GFP did not transiently accumulated in the structures, nor did it appear to move as vesicles. Thus, although both ORF1-GFP and MP-GFP localized in the PM and PD, it was in different ways.

I used the organelle markers tagged with mCherry fluorescent protein to identify organelles to which MP might be localizing. The MP-GFP co-localized to the PM, similarly to ORF1 (Fig 13). The MP-GFP of ToNDV-K15 did not localize to other organelles such as ER, mitochondria, plastids, Golgi or nucleus. The BC1 geminiviral protein is also a plasma membrane localized protein, when compared with localization of ToNDV-K15, the pattern was similar and fluorescence appeared to extend into adjacent cells, possibly via PD (Fig 14). Additionally, MP-GFP localize closely with TMV-MP-mCherry protein when observed by confocal microscopy (Fig 14).

Expression of ORF1-GFP and MP-GFP fusion proteins was confirmed by western blot by 72 dpi with an anti-GFP antibody (Fig 15A) but levels of protein expression were low. I attempted to concentrate the protein by immunoprecipitation with chromotek anti-GFP beads. This allowed for increased recovery of the proteins and confirmed the size of the GFP-tagged ORF1 and MP (Fig 15B). My results revealed the expected size of ~50 kDa and ~75 kDa fusion proteins

consistent with the size of ORF1-GFP and MP-GFP expression in infiltrated leaf patches, respectively (Figs 12-13).

RNA binding

For expression of ORF1 and MP in *E. coli*, I successfully expressed and purified ORF1 but due to challenges in expression and purification of the MP, binding experiments were performed only with ORF1. To investigate if ORF1 possesses RNA binding properties, I performed a ssRNA gel shift experiment with *E. coli*-expressed ORF1 protein (Fig 16). The protein was expressed, extracted, purified, and dialyzed prior to being used (Rojas et al., 1998b). As a negative control, I used SDS denaturalized protein. The results show that at concentrations of 3 until 6 ug of protein, the ORF1 protein bound with the ssRNA marker caused a shift in migration and there was a tendency to preferentially bind larger fragments in the ~7 kb size range (Fig. 16). Together these results suggest that ORF1 can bind the ssRNA and in the size range of ToNDV RNA 1 and RNA 2. Similar gel shift assay experiment was performed with the ORF1 protein and dsDNA markers. Here, I observed no evidence of binding as there was no shift of bands migration in the presence of ORF1 (up to 7ug) (data not shown).

Discussion

In the present study, I investigated the role of ORF1 and MP of ToNDV in viral movement using genetic and functional approaches. This involved mutational analyses using the infectious clones of ToNDV-K15 RNA 1 and RNA 2 described in Chapter 2, subcellular localization, and RNA binding experiments. The impact on protein structure of three mutations that abolished cell-

to-cell movement was further investigated with a novel approach that models tertiary protein structure.

In the mutational analyses, I determined (i) whether the mutation was essential for infectivity and (ii) for mutants necessary for infection, whether the impact was on replication or cell-to-cell movement using a leaf disc agroinfiltration assay and RT-qPCR to detect ToNDV at different time points. The ORF1 KO mutant replicated in *N. benthamiana* leaves but failed to be detected in leaf discs 5 mm from the infiltrated patch, indicating a role for ORF1 in cell-to-cell movement of ToNDV. Previous research has established a role for this protein on movement- in an isolate of ToMarV (ToANV), in this case, a ORF1 KO mutant moved cell-to-cell but failed to move long-distance or induce symptoms (Ferriol et al., 2017). These results support the involvement of the ORF1 protein in movement of these two torradoviruses but may reveal functional differences in cell-to-cell movement and function for ORF1 proteins. My results also showed that these ToNDV-K15 RNA 2 encoded proteins are not necessary for replication, consistent with replication functions provided by RNA 1 (Ferriol et al., 2016).

It has previously been reported that ORF1 function in movement of ToMarV and my results with ToNDV-K15 agree with this. I further hypothesized that N-terminal MYR motifs in ORF1 were important for functions involving membrane targeting, as has been reported for other movement associated proteins (Rojas et al., 2001; Wang et al., 2021). However, this hypothesis was not supported by the mutational analyses, as all the ORF1 MYR mutants induced wild-type symptoms. Thus, if ORF1 binds to membranes it must use other mechanisms. In this regard it would be interesting to assess the subcellular localization of the MYR mutants.

I did identify a very conserved amino acid motif in ORF1 that is essential for ToNDV-K15 infection in *N. benthamiana* and tomato, and cell-to-cell movement in *N. benthamiana*. The LDF

mutant is essential and the conservation among all torradoviruses is consistent with an essential role in infection. Importantly, I confirmed the mutant was replication competent and deficient in cell-to-cell movement, further supporting the notion that ORF1 is involved in movement of ToNDV-K15. The effect of the LDF mutation on the tertiary structure was modelled and compared with the wild-type protein. These analyses showed very similar tertiary structures, but reduced lipophilicity for the LDF mutant in the mutated area. High lipophilicity is associated with binding to membranes and other proteins (Laliberté & Zheng, 2014), and could be a means by which ORF1 binds to membranes. Thus, it will be interesting to determine if membrane localization is altered with subcellular localization studies. Other than membranes, viral proteins can interact with other viral and plant proteins to indirectly mediate cell-to-cell movement such as those involved in PD gating, silencing suppression and immune signaling (Hong & Ju, 2017; Rojas et al., 2016a). However, my results suggest a more essential role in cell-to-cell movement. The LDF domain in the ORF1 is conserved among all torradoviruses (Fig 2) and the LDF mutant abolished cell-to-cell movement, which indicates possible interference with ORF 1. These results show the essential role of the LDF domain amino acids during ToNDV-K15 infection.

Another RNA 2-encoded protein that is essential for cell-to-cell movement of torradovirus is the MP (Ferriol et al., 2018). Here it is important to note that it was not possible to make and MP KO mutant because it is part of the polyprotein that includes the CPs, which also maybe essential for cell-to-cell movement. One of the very interesting findings that emerged from my sequencing and phylogenetic analyses of the MP was that this protein is related to those of the members of the genus Polerovirus (family *Luteoviridae*), and this was found with analyses of the N- and C- terminal regions of the ToNDV-K15 MP. Interestingly, a conserved ORF3a was discovered to be conserved in luteoviruses and poleroviruses in studies of *Turnip yellows virus*

(TuYV) and it was revealed to be involved in long-distance movement localized to PD (Smirnova et al., 2015). Poleroviruses have another movement-related protein called P4, which is required for cell-to-cell movement and it is a weak silencing suppressor (Fusaro et al., 2017). This dual MP system of poleroviruses could provide insight into function of ORF1/MP of torradoviruses. ToMarV ORF1 protein mutant was able to move cell-to-cell, but not long-distance (systemically). (Ferriol et al., 2017), This might indicate that ORF1 has a similar role to the P3a protein, and that a dual MP system might be acting in the torradovirus cell-to-cell movement. This hypothesis gain support from (i) both proteins are essential for cell-to-cell movement and (ii) both proteins localized to the cell periphery and more specifically to PD.

The essential role of the MP in cell-to-cell movement of ToNDV-K15 was shown when I abolished cell-to-cell movement by mutations at the highly conserved aa 210F residue. As A and F residues differ in mass and side chain, this region may be important for proper structure or function of this important residue. The conservation of this residue in the ToTV was previously noted, and mutation F210 indicated it is a symptom determinant of ToTV, i.e., the mutant induced symptomless infections in tomato plants (Wieczorek & Obrepalska-Steplowska, 2016). Thus, my results were consistent a role of the conserved F residue in cell-to-cell movement, although I failed to identify a symptomless phenotype with the ToNDV-K15 mutant.

The protein immediately following the MP in ToNDV-K15 RNA 2 polyprotein, is the largest CP, VP35. In my efforts to find regions essential for infection, I identified a MYR domain in the N-terminus of Vp35 (Fig 1A). As done with the MYR motifs for ORF1, a Vp35-MYR mutant was generated. In contrast to results for the MYR mutants of ORF1, those in the N-terminus of ORF1, the mutant of Vp35 abolished symptom development and infection. Moreover, this mutation did not impact viral replication, but did abolish cell-to-cell movement. This result show

that the myristoilation domain of Vp35 is essential for infection. This could be due to effects on encapsidation, and the essential role for virions in cell-to-cell movement of some secoviruses is well established for CPMV which moves cell-to-cell as virions through tubules made with the MP (Pouwels et al., 2002; Wellink & Van Kammen, 1989). TMV does not need CP to move cell to cell, but requires it for efficient systemic movement (Taliansky et al., 2003). Alternatively, it could be involved in interaction with ORF1 or MP or host factors. The modelling performed with the wild-type and mutated Vp35 showed that the mutation in the Vp35 might cause an altered interaction in the virion and impair formation of CP subunits (Fig 10), indicating improper protein-protein interactions. For ToNDV-K15, it is not known if tubules are involved as is the case for CPMV and *Alfalfa mosaic virus* (AMV *Bromoviridae*) (Sánchez-Navarro & Bol, 2007; Van Lent et al., 1991). Thus, the mechanism in torradovirus cell-to-cell movement still needs to be determined.

I also made a mutation in the small CP subunit, Vp24 based on identifying a positively charged region of amino acids, starting at aa 139, that includes four arginine (R) residues and is similar to the ARM involved in RNA binding in several viruses, e.g., CMV (family *Bromoviridae*) (Schmitz & Rao, 1998). Converting all the amino acids of Vp24-ARM with alanines, did not impact replication, but abolished cell-to-cell movement. The 3D modeling of the wild-type Vp24 and the Vp24-ARM mutant (Fig. 9) revealed that the mutant protein no longer formed a pocket that contained the charged residues. This mutant also changed the structure of the CP subunit formed by the CPs. It is likely that this change in these amino acids affected virion assembly, structure, and interaction with RNA. It would be useful to develop an encapsidation assay to investigate impact of mutant on virion formation.

Another method to infer potential functions of viral movement-associated proteins is subcellular localization of tagged proteins. I used this approach for ORF1 and MP individually and in combination with cellular markers. Notably, although both ORF1-GFP and MP-GFP targeted PD, ORF1-GFP accumulation was transient and this was associated with the active formation and movement of numerous small vesicles, which moved continually close to the cell plasma membrane and in some cases to the PD in the cell wall (Fig 11). The MP also localized to the membrane, and in structures located in the cell wall that resemble PD (Fig 11). However, in contrast to ORF1, accumulation of MP-GFP was not transient in terms of association with the PM and PD. As noted, 3D confocal images appeared to show the MP-GFP transversing PM-lined PD (Fig 14). Further evidence that the ToNDV-K15 MP localization to PD came from the co-localization of the MP-GFP and TMVMP-mCherry, a well-known PD localized protein (Mushegian & Elena, 2015).

My results with the ToNDV CP mutants indicated an important role for virions in cell-to-cell and most likely long-distance movement. What is less clear is whether virion movement occurs via tubules or MP-modified PR. Considering common feature of ToNDV MPs with CMV, viruses that utilize tubules for cell-to-cell movement, it is possible ToNDV utilizes tubules across the PD for cell-to-cell movement (Canto & Palukaitis, 2007; Pouwels et al., 2003).

The co-localization with other cellular organelle markers showed that ORF1-GFP and MP-GFP do not clearly localize to ER, mitochondria, Golgi or plastids. Here it should be noted that attempting to investigate possible co-localization of ORF1 and MP was not possible due to low signal of the RFP tag. Western blot and immunoprecipitation analyses allowed for the confirmation of the expression of the expected-size ORF1-GFP and MP-GFP proteins, which shows that the GFP observed in the analyses was associated with the fusion protein and not eGFP or cleaved

fusion proteins, although some such degradation was observed. Furthermore, those challenges also made it difficult to test the interaction of these proteins by Co-IP.

To explore further functions of ORF1, I evaluated the RNA binding capacity of this protein. Proteins involved in cell-to-cell movement of RNA viruses, such as MP of TMV, bind ssRNA, but not dsRNA or DNA and typically in a sequence non-specific matter (Otulak & Garbaczewska, 2011). This allows the viral genomic RNA to move cell to cell (Citovsky et al., 1992). The geminiviral BC1 movement protein from *Bean dwarf mosaic virus* (BDMV) binds DNA in a size and form specific manner (Rojas et al., 1998a). In the case of a small twinned icosahedral virus such as BDMV, the size preference of the BV1 or BC1 MPs might determine the maximum genome size compatible with virion formation. My results show that ToNDV ORF1 protein binds a ssRNA and in the size range (~7 kb) of the viral RNAs. This could indicate a role for ORF1 in transporting viral RNA to PD, perhaps for virion independent movement. However, this was not supported by my results showing that the CPs were essential for cell-to-cell movement. ORF1 may play a role in genome size selection and transport, and the result showing that ORF1 does not bind dsDNA, is consistent with the ORF1 selectivity according to nucleic acid type.

In conclusion, my experiments allowed me to gain more insight into characteristics and functional properties of the ORF1 and MP proteins encoded by the RNA 2 of ToNDV-K15. This investigation revealed essential regions in the ORF1 and MP, as well as capsid proteins involved in cell-to-cell movement of the virus. For some mutants, that abolished cell-to-cell movement, modelling of the protein tertiary structure was used to predict impact of mutations on protein function. More studies need to be conducted to further the mechanism(s) by which these viruses move cell to cell and long distance.

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Tables

Table 1. Names and properties of alanine replacement mutants in the RNA 2 encoded ORF1, MP and CP genes of a new isolate of tomato necrotic dwarf virus (ToNDV-K15).

Protein mutated	Mutant ID	Symptoms (necrosis in the base of the leaves)	Detected by PCR in newly emergent leaves	Sequencing of PCR fragments (no reversion)
ORF1				
	K.O.	No (0/5)	No (0/5)	No amplification in PCR
	LDF	No (0/5)	No (0/5)	No amplification in PCR
	MYR1	Yes (5/5)	Yes (5/5)	Yes (5/5)
	MYR2	Yes (5/5)	Yes (5/5)	Yes (5/5)
	MYR3	Yes (5/5)	Yes (5/5)	Yes (5/5)
	ORF70	Yes (5/5)	Yes (5/5)	Yes (5/5)
Movement protein (MP)				
	MP210	No (0/5)	No (0/5)	No amplification in PCR
	MP263	Yes (5/5)	Yes (5/5)	Yes (5/5)
	MP266	Yes (5/5)	Yes (5/5)	Yes (5/5)
	MP270	Yes (5/5)	Yes (5/5)	Yes (5/5)
	MP3D	Yes (5/5)	Yes (5/5)	Yes (5/5)
	MPMID	Yes (5/5)	Yes (5/5)	Yes (5/5)
	MPMID2	Yes (5/5)	Yes (5/5)	Yes (5/5)
Vp35 (CP1)				
	MYR4	No (0/5)	No (0/5)	No amplification in PCR
VP24(CP3)				
	ARM	No (0/5)	No (0/5)	No amplification in PCR

Figures

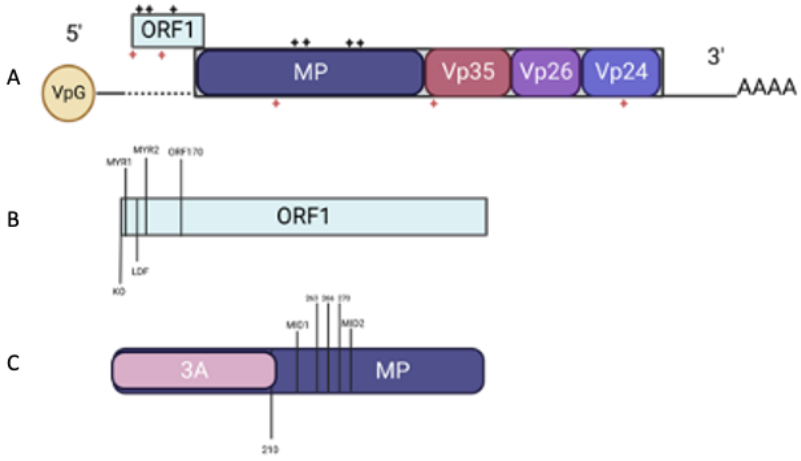


Figure 1. A. Map of complete RNA 2 of tomato necrotic dwarf virus (ToNDV-K15) showing genes and locations of alainne replacement mutations are shown with stars, on top genes having no effect on infection or symptoms and those bellow shoing those that abolished infection and cell-to-cell movement. B. Map of ORF1 showing the mutations in more detail. C. Map of MP showing the mutations in greater detail.

Figure 2. Amino acid sequence alignment showing the conserved MYR1(A), MYR2 (B) and LDF (c) motifs identified in ORF1 proteins of tomato-infecting and non-tomato infecting torradoviruses. The red boxes define the size of each of the motifs. The shading of the amino acids shows the level of conservation, the darker the color, the higher the conservation.

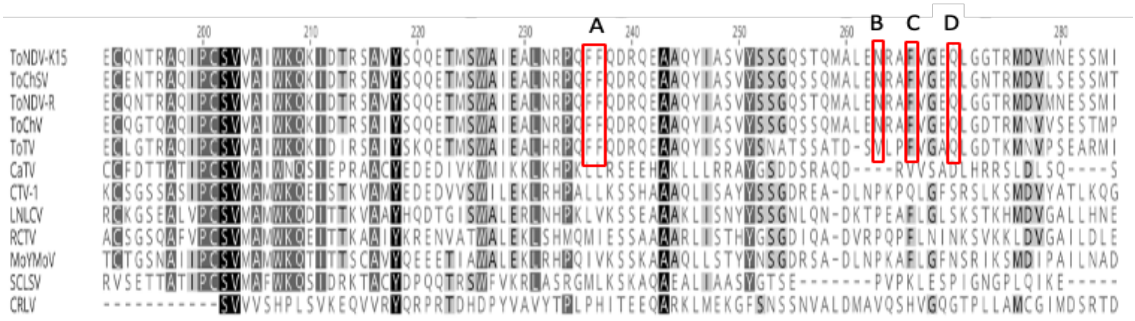


Figure 3. Amino acid alignment of tomato-infecting and non-tomato infecting torradovirus MP sequences. The box in red highlights the region of the MP210F, MP263N, MP266F and MP270Q residues that were mutated. Only MP210F caused no symptoms or infection after 14 dpi in tomato or *N. benthamiana.*

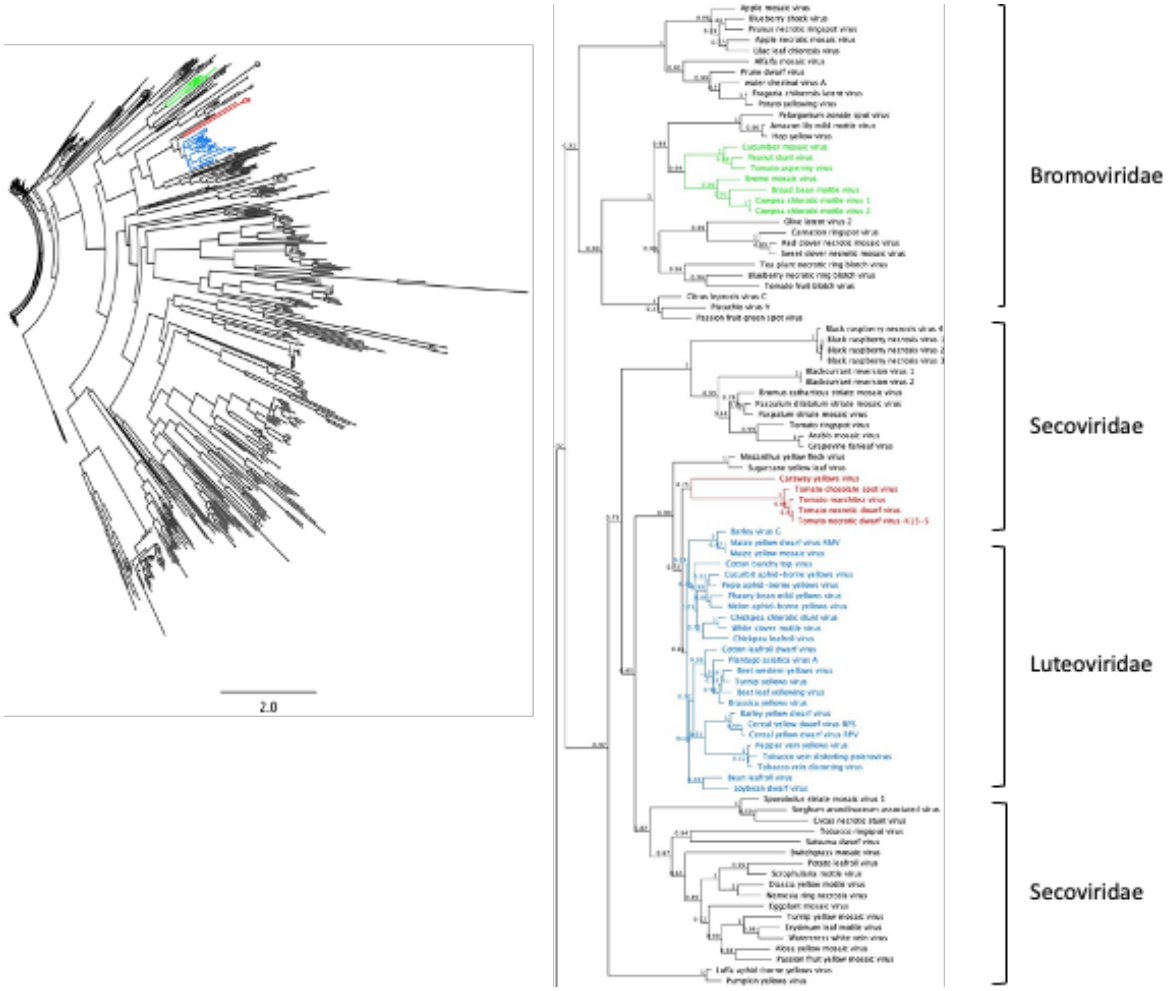


Figure 4. Phylogenetic trees generated with the N-terminus amino acid sequences of ToNDV movement protein (MP) and those of 531 viral MPs. The torradovirus cluster is shown in red. Highlighted in green are the MP sequences of members of the family Bromoviridae, in which the 3a protein is present.

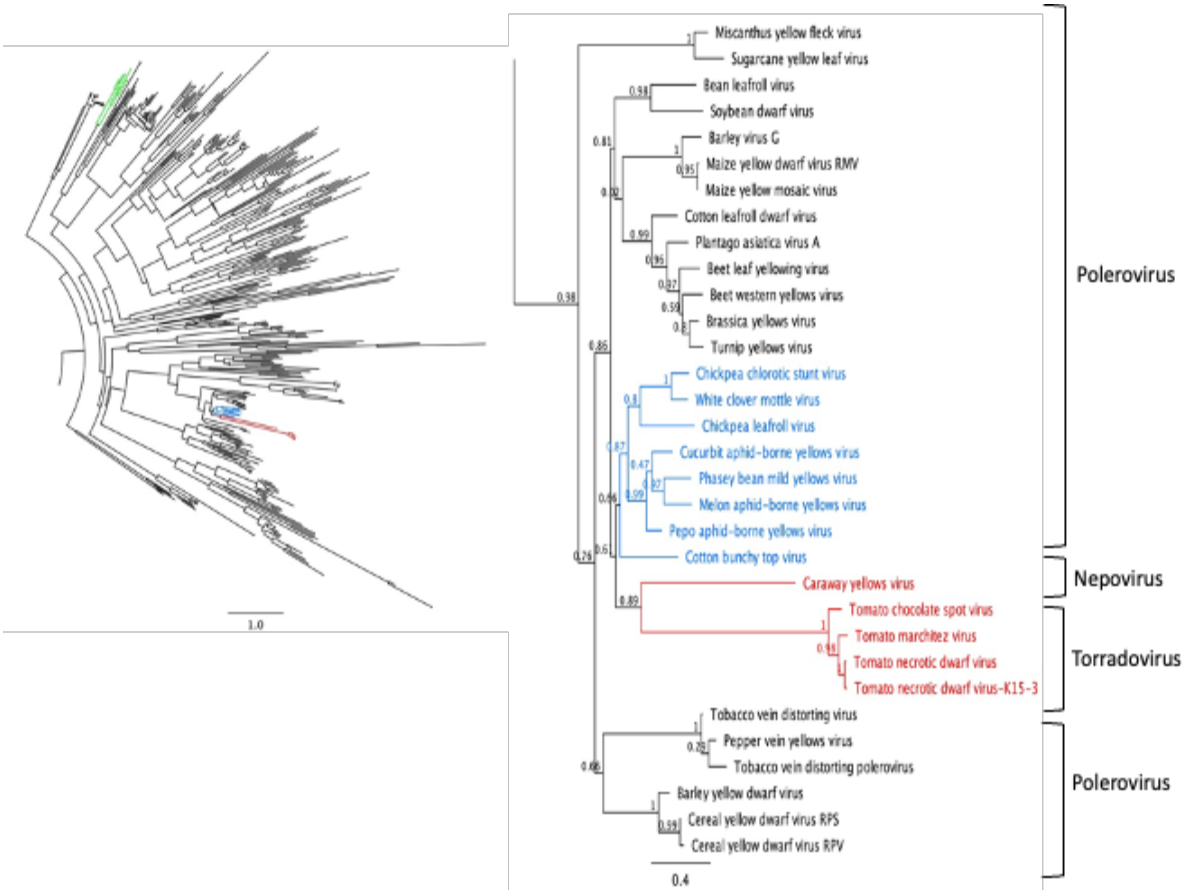


Figure 5. Phylogenetic tree generated with the C-terminus amino acid sequences of tomato necrotic dwarf virus isolate K15 (ToNDV-K15) MP and those MPs of 531 viruses. The torradoviral sequences are shown in red. Other notable sequences are the MP from the Nepovirus Caraway yellows virus, also shown in red and 3a-type MPs of *Bromoviridae*, which are shown in green. The sequences most closely related to the torradoviruses are those of the MPs of members of the genus Polerovirus in the *Solemoviridae* family, e.g., as Cotton bunchy top virus.

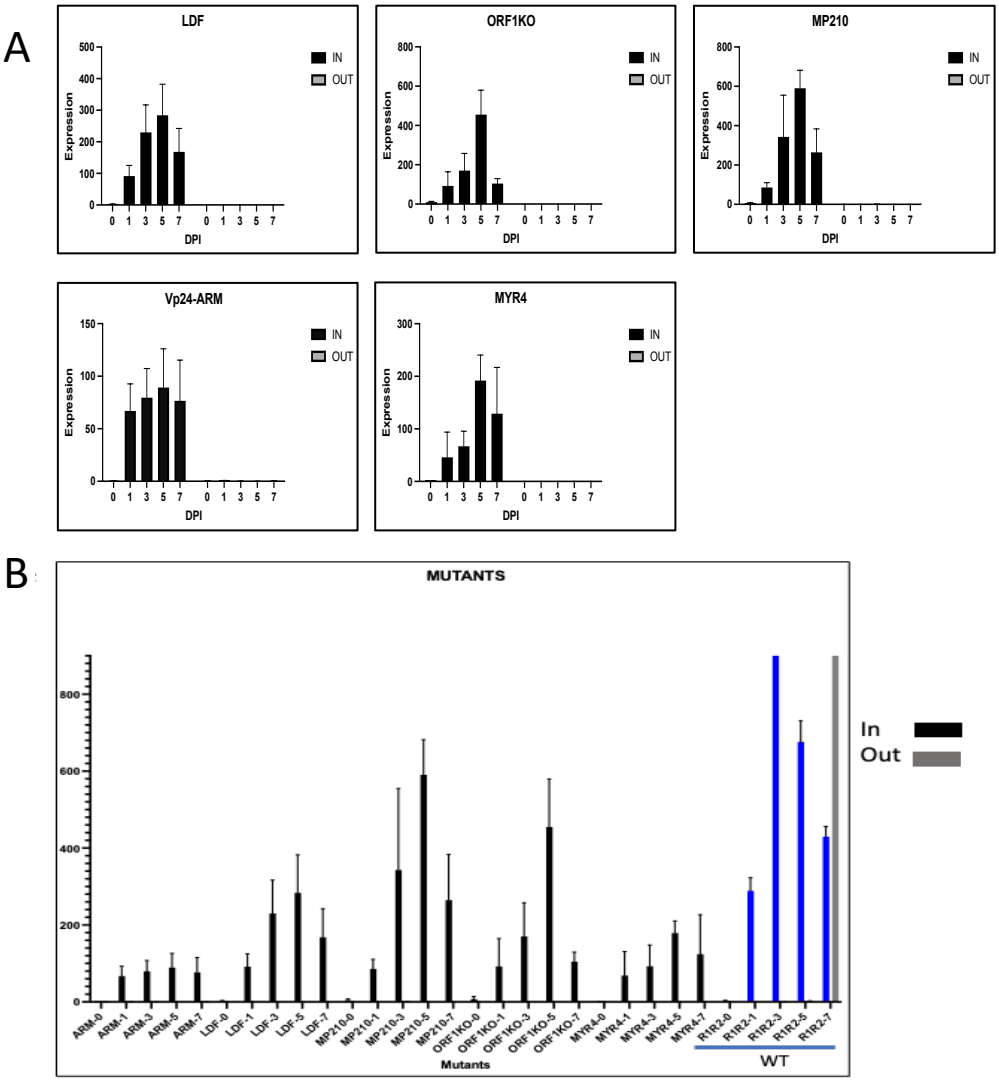
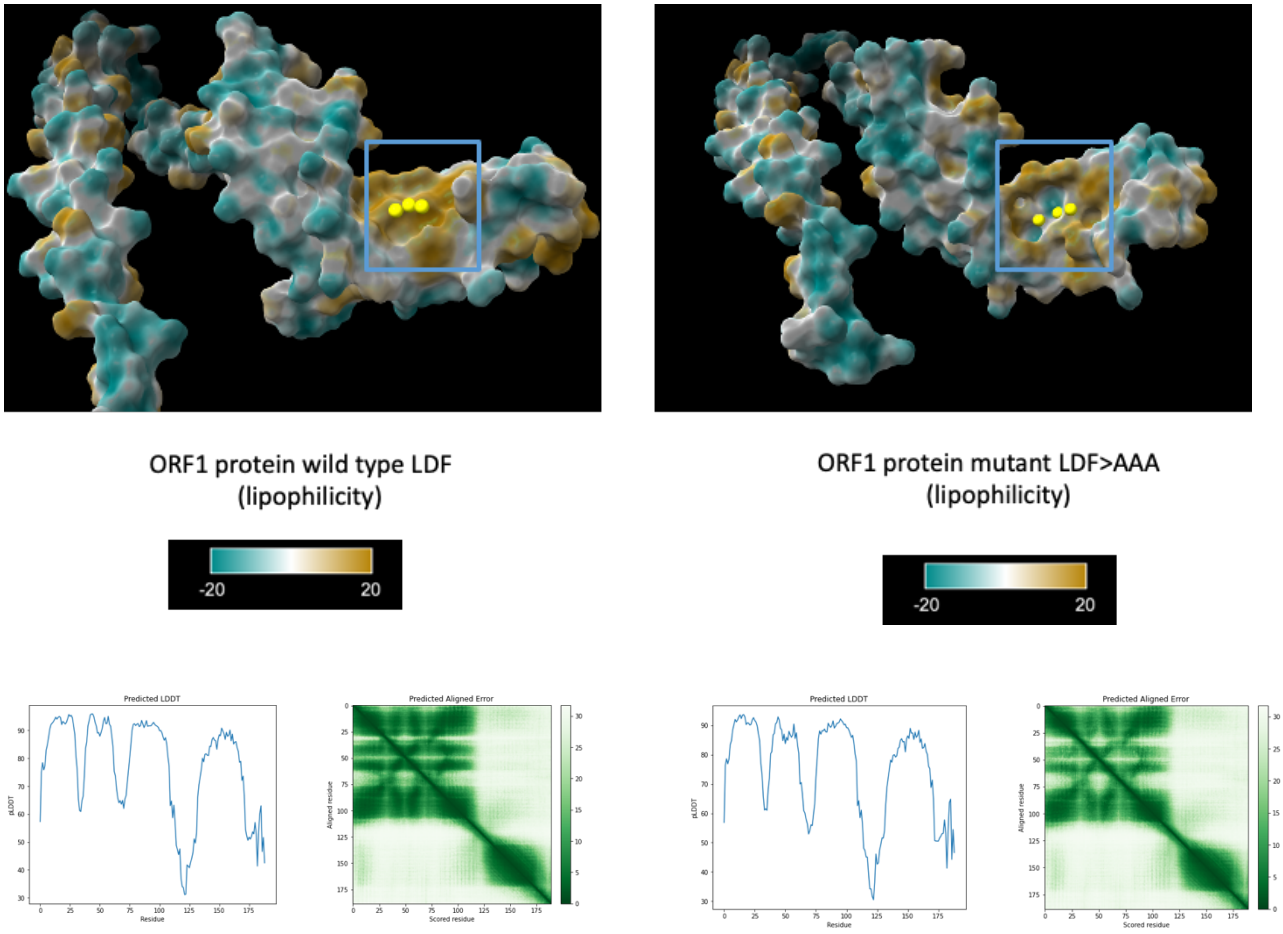


Figure 6. Results of the RT-qPCR tests for detection of the RNA1 of tomato necrotic dwarf virus isolate K-15 on leaf discs of *N. benthamiana* taken inside and outside of patches agroinfiltrated with mutants and wild-type at 0 hr, 1, 3, 5, and 7 days post infiltration. Numbers represent totals for three independent experiments.



pLDDT>90

Figure 7. Protein modelling of the wild-type and the LDF mutant of ORF1. The wild-type residues and mutated residues are shown with yellow dots inside a blue box. Predicted confidence in the model is expressed as the result of a predicted local-distance difference test (pLDDT) and predicted error by residue is visualized in green.

Vp35 wild type

Vp35-MYR4

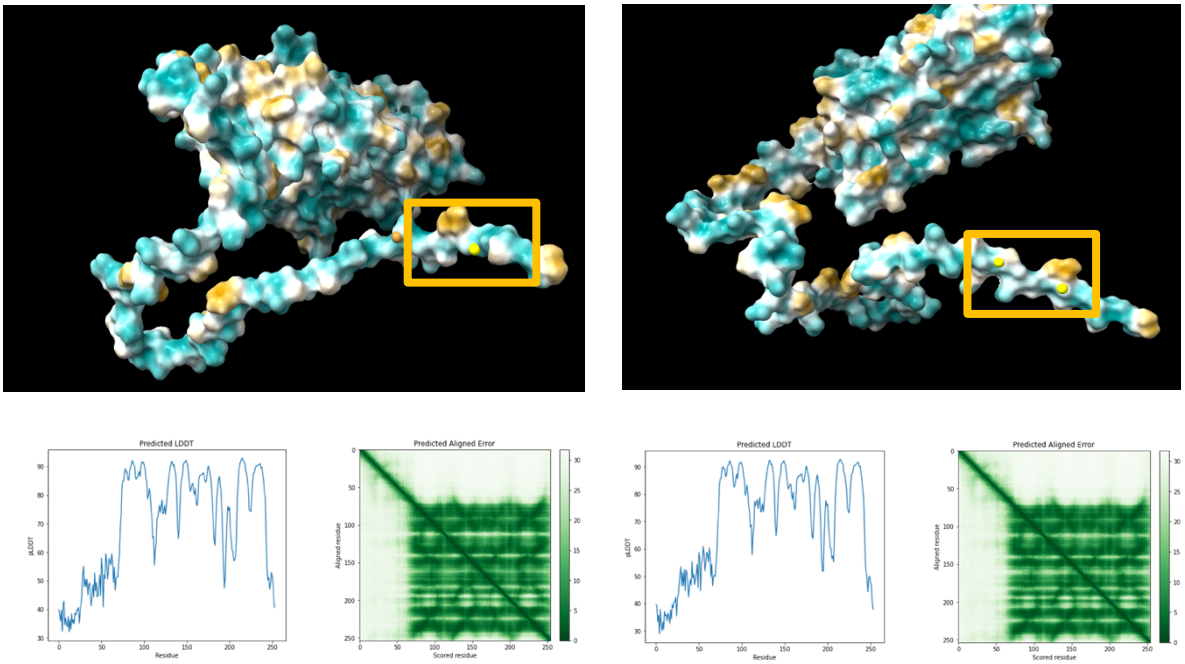


Figure 8. Protein modeling of the capsid protein, Vp35, in comparison with the mutant Vp35-MYR, mutated and wild-type MYR4 amino acids are shown in yellow as dots and inside the yellow boxes. The confidence in the modeling is expressed as the result of the predicted local-distance difference test (pLDDT>40) and predicted error by residue is visualized in green.

VP24 Vp24-ARM

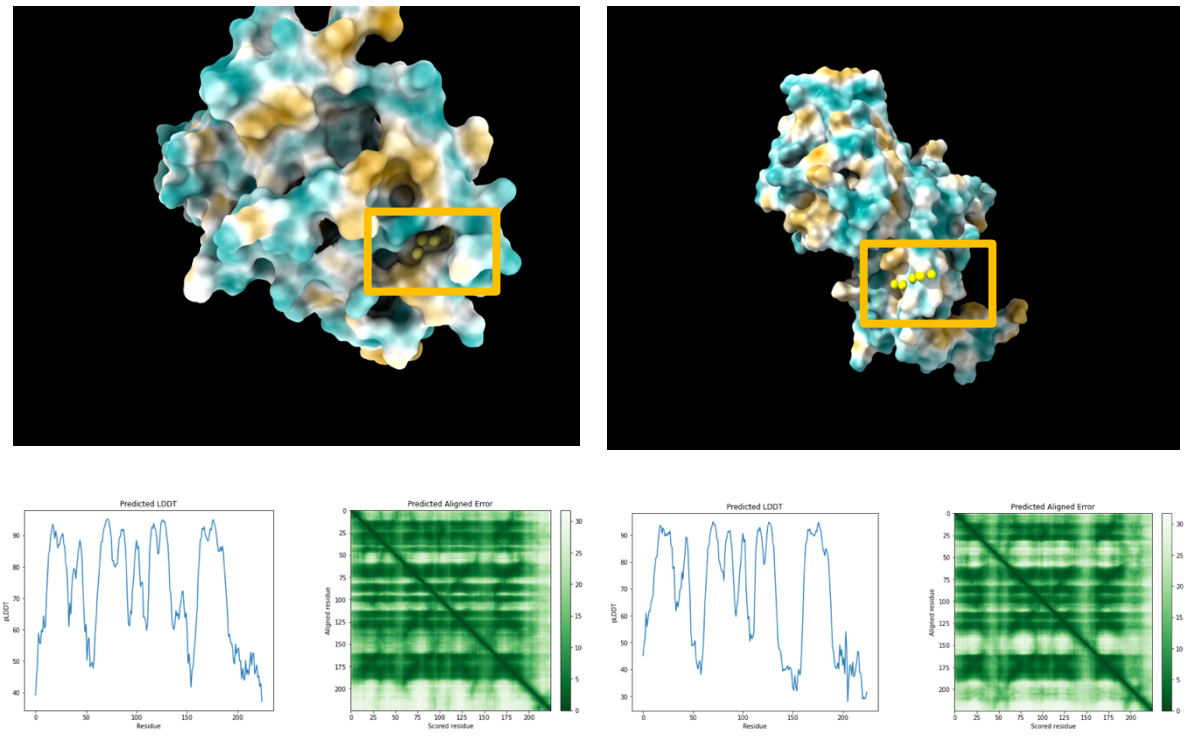


Figure 9. Protein modeling of the wild-type and mutant of capsid protein Vp24, Vp24-ARM. Mutated amino acids and their wild-type form are shown in yellow as dots and inside yellow boxes. Predicted confidence in the modeling is expressed as the result of a predicted local-distance difference test (pLDDT>70) and predicted error by residue is visualized in green.

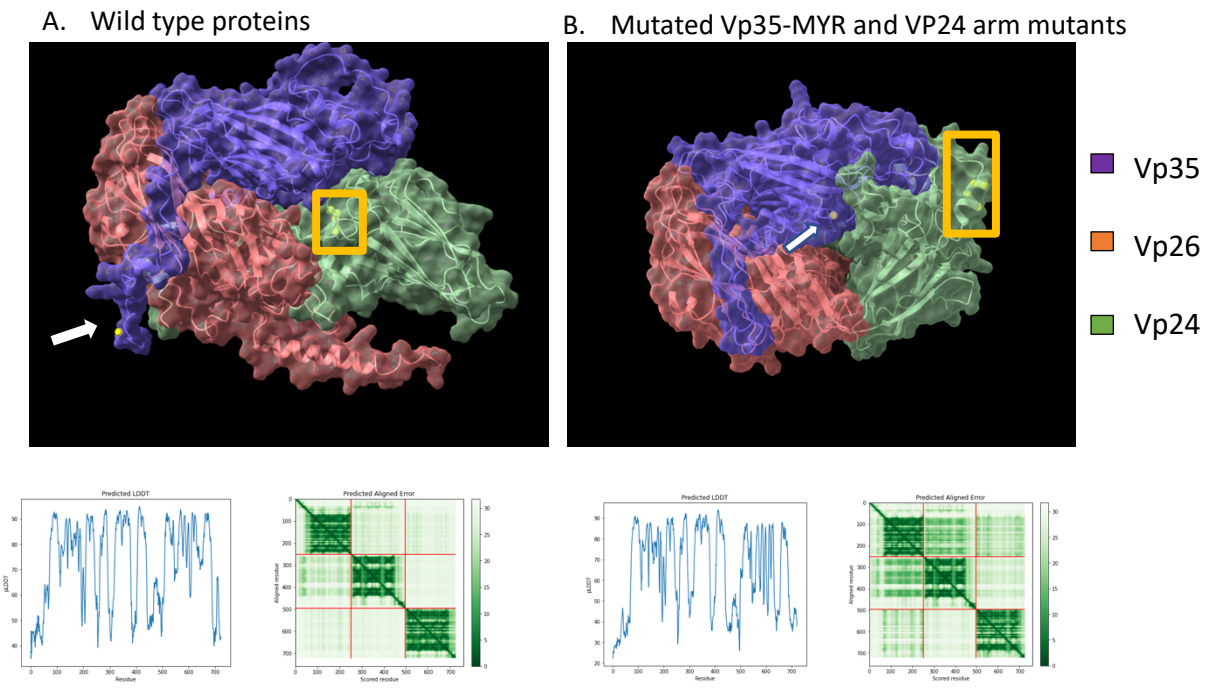


Figure 10. Protein modeling of the structure of the wild-type and mutant capsid proteins of tomato necrotic dwarf virus isolate K15 (ToNDV-K15) A) assembly of the three wild-type capsid proteins (CPs), Vp35, Vp26 and Vp24 and B) assembly of the mutated capsid protein Vp35-MYR and Vp24-ARM, with wild-type Vp26 CP. Mutation in Vp35-MYR is labeled as a yellow do and Vp24-ARM mutant residues are inside the yellow box. Predicted confidence in the model is expressed as the result of the predicted local-distance difference test pLDDT (pLDDT>70-90) and predicted error by residue is visualized in green.

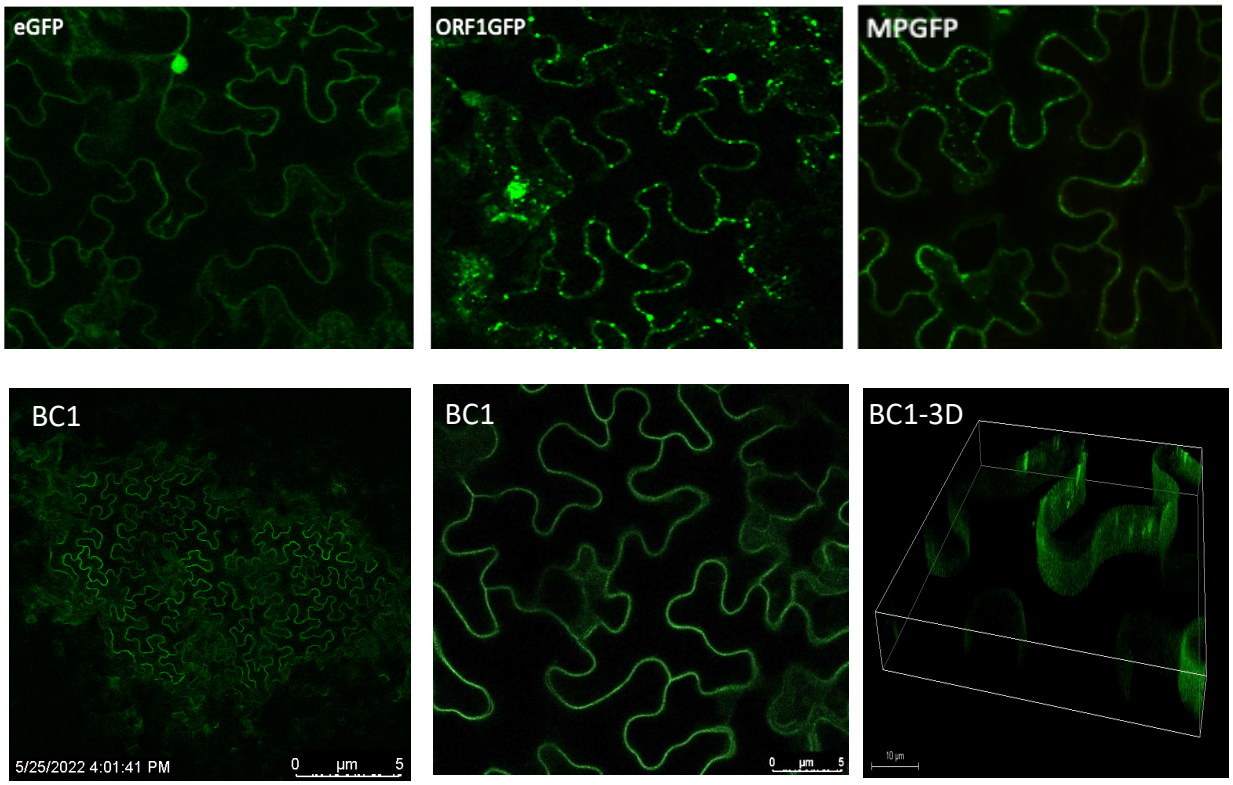


Figure 11. Subcellular localization of transiently expressed constructs of ORF1GFP and MPGFP proteins of tomato necrotic dwarf virus (ToNDV-K15) in agroinfiltrated *N. benthamiana* epidermal cells 48 hpi. eGFP is a control of free GFP and BC1 MP of begomoviruses was used as a control for plasma membrane and more specifically PD localization.

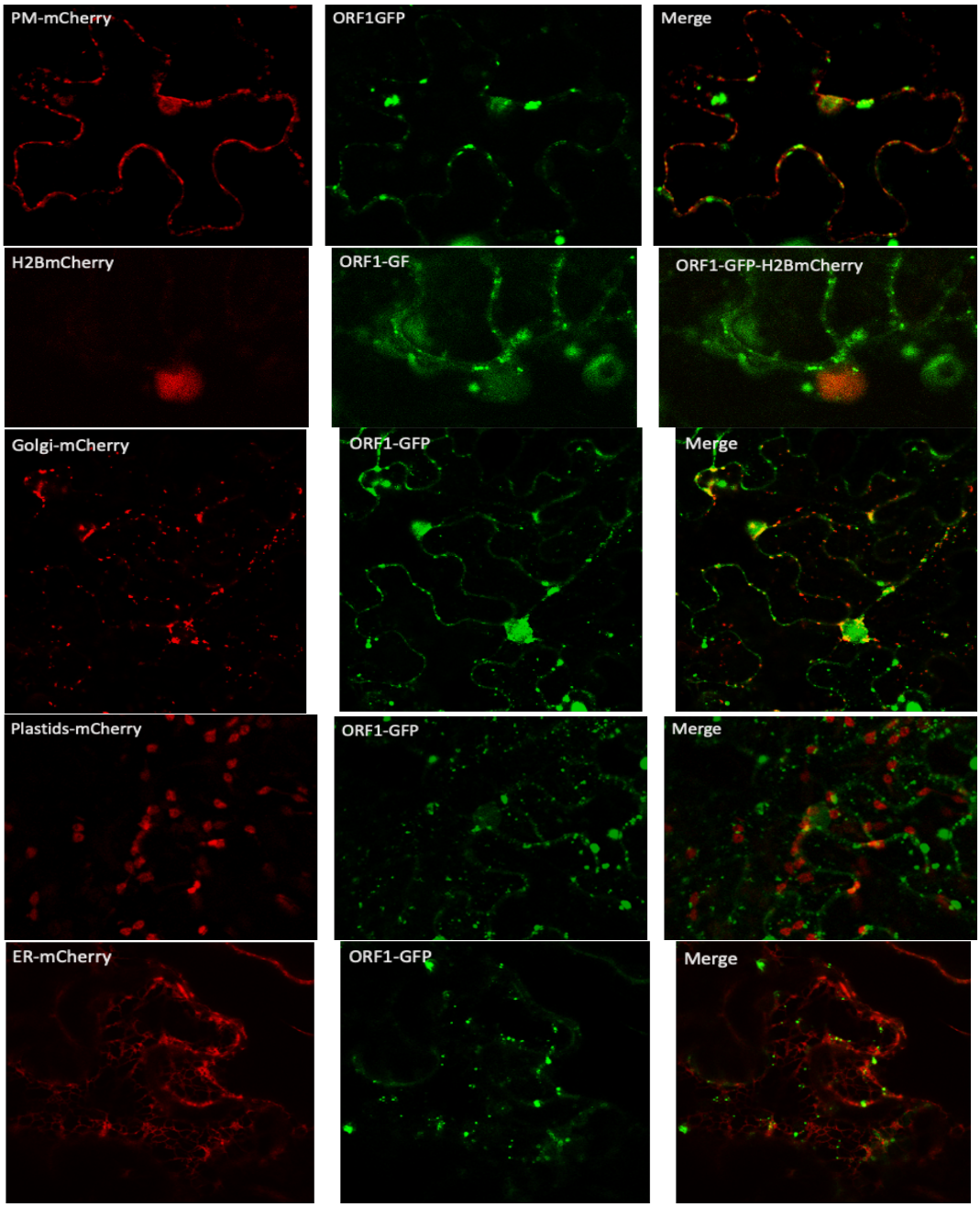


Figure 12. Confocal laser scanning microscopy of the subcellular co-localization of transiently expressed constructs of ORF1-GFP protein of tomato necrotic dwarf virus (ToNDV-K15) in *N. benthamiana* epidermal cells at 48 hpi with mCherry-labeled plant organelles for plasma membrane (PM), nucleus, golgi, plastis and endoplamic reticulum (ER).

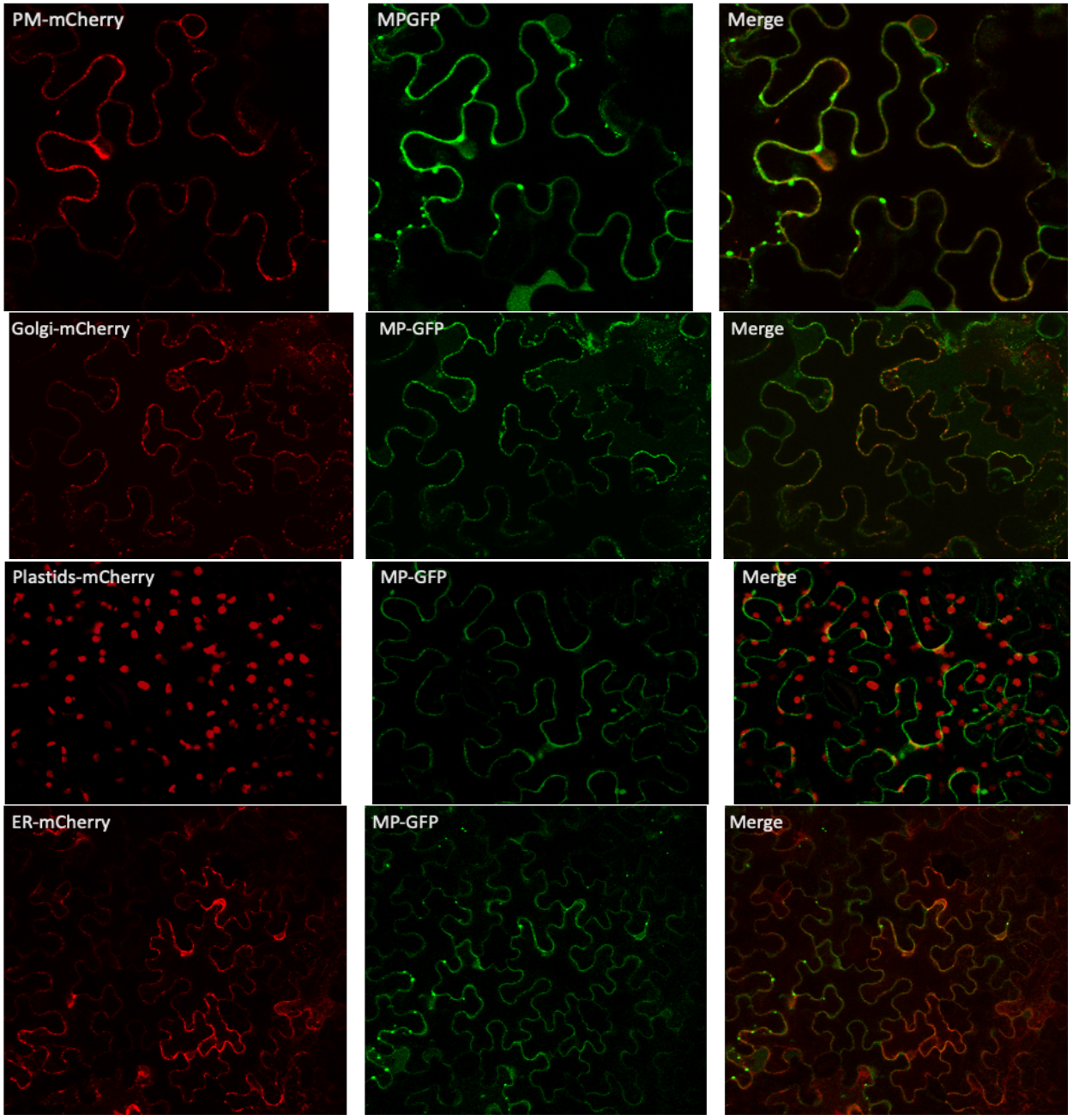


Figure 13. Confocal laser scanning microscopy of the subcellular co-localization of transiently expressed constructs of MP-GFP protein of tomato necrotic dwarf virus (ToNDV-K15) in *N. benthamiana* epidermal cells at 48 hpi with mCherry-labeled plant organelles for plasma membrane (PM), golgi, plastis and endoplamic reticulum (ER).

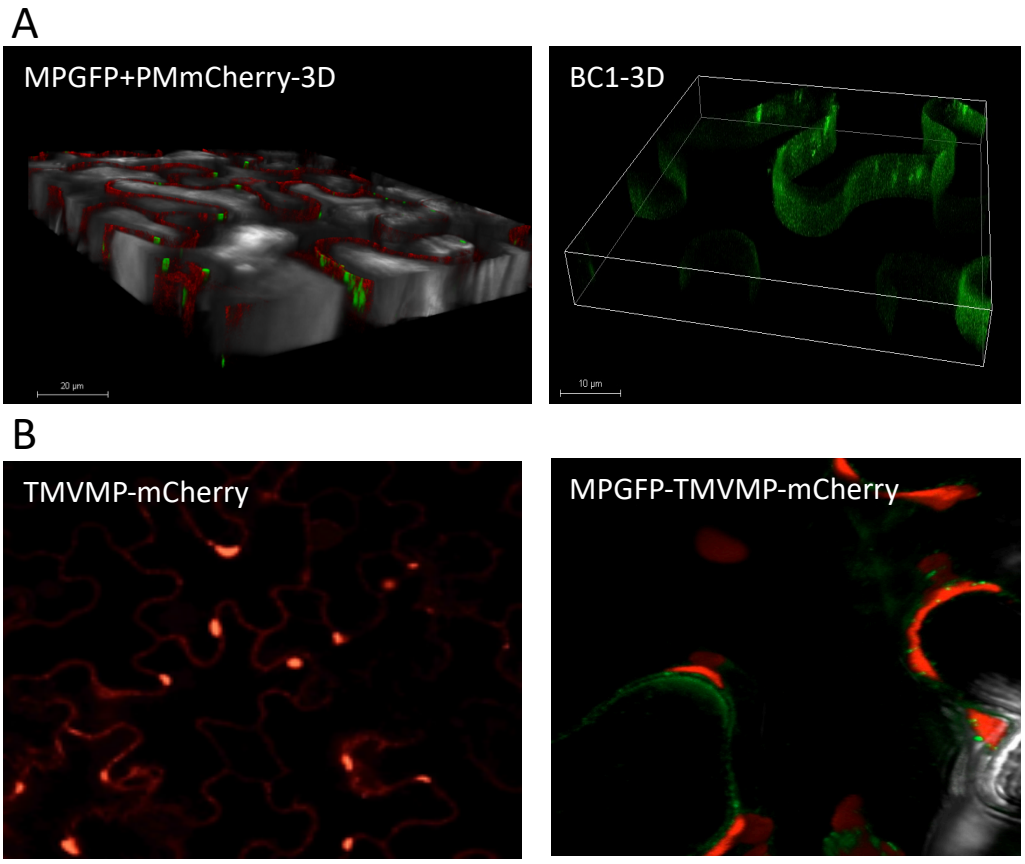


Figure 14. 3D reconstructions of Z-stacks of images from confocal laser scanning microscopy of tomato necrotic dwarf virus (ToNDV-K15) in *N. benthamiana* epidermal cells by 48 hpi showing (A) MP-GFP co-localization with the plasma membrane marker (PMmCherry) in *N. benthamiana* epidermal cells at 48 hpi and the BC1 geminiviral protein showing similar localization to the MPGFP though the plasma membrane and (B) TMVMP-mCherry localization of the individual protein and the co-localization of the MPGFP with the TMV-MPmCherry protein.

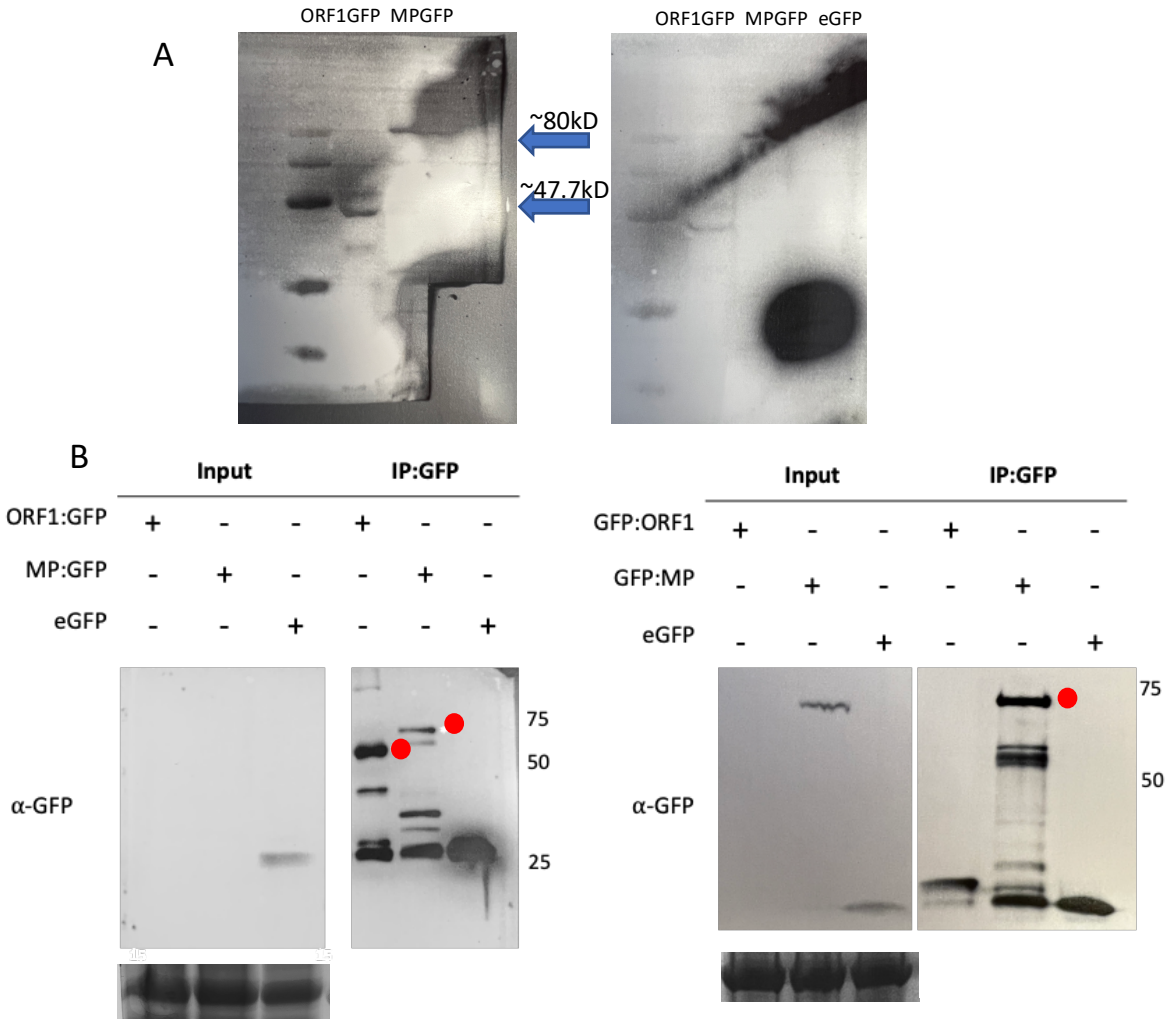


Figure 15. Detection of transient expression of GFP-tagged tomato necrotic dwarf virus-K15 RNA-2 encoded proteins, ORF1-GFP and MP-GFP in *N. benthamiana* leaf patches. Western blot with anti-GFP antibody of the input ORF1GFP and MPGFP is shown in (A). On the left, the eGFP control protein was excised from the membrane to allow for better visualization of proteins with lower concentration. On the right is the original membrane with the eGFP control. (B) The ORF1GFP, MPGFP, GFPORF1 and GFPMP fusion proteins were extracted from *N. benthamiana* leaf patches and immunoprecipitated with chromotek anti-GFP agarose beads and visualized by western blot with anti-GFP primary antibody and HRP chemiluminescence. The red circles mark the expected size protein.

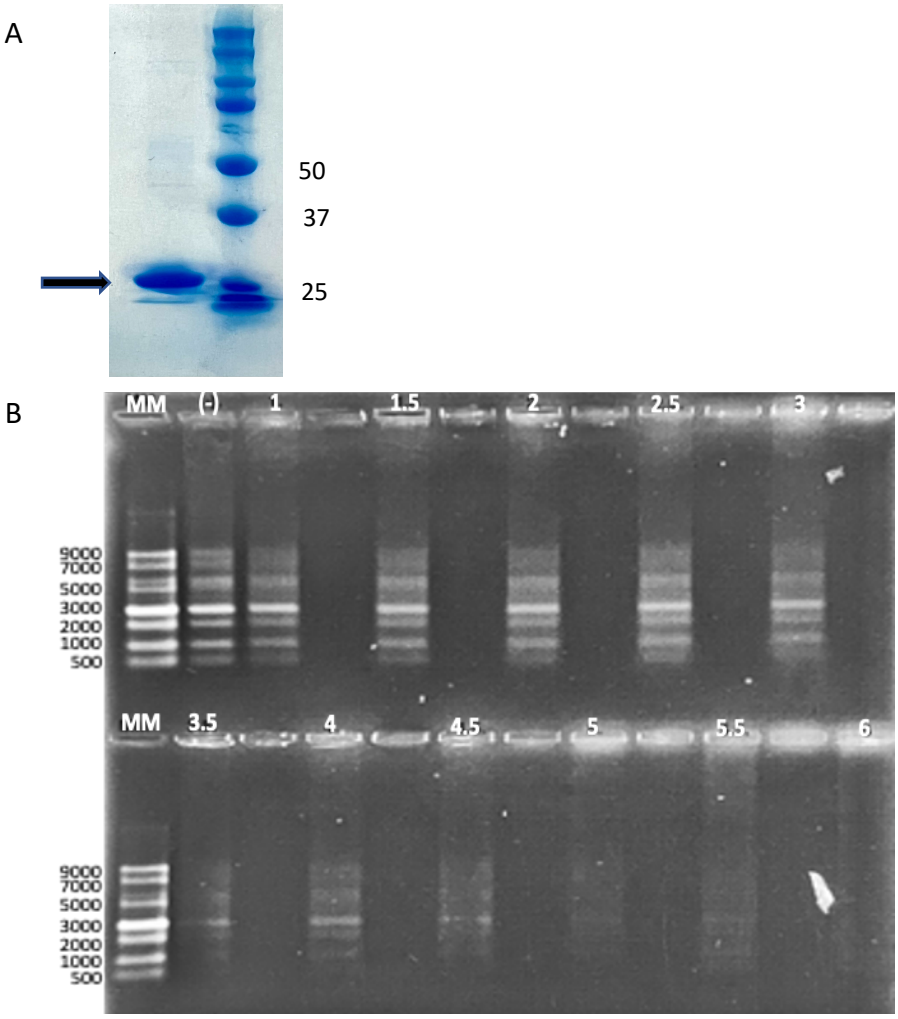


Figure 16. Results of gel-shift experiments performed with the *E.coli* expressed ORF1 protein of tomato necrotic dwarf virus isolate K15 (ToNDV-K15) and single stranded RNA standards. (A) RNA-2 expressed and purified ORF1-His protein of ToNDV-K15 from *E. coli*. The purified protein was dialyzed and used for nucleic acid binding experiments. (B) Gel-shift assay to detect capacity of different concentrations of the *E. coli* expressed ORF1-HIS protein to bind 2.5 ul of ssRNA marker (0.5 to 9 kb, NEB). The ORF1 protein concentrations are showed on top of each lane. ORF1 protein and the ssRNA were incubated for 30 min in binding buffer (Rojas et al., 1998b), after which they were visualized in a 0.8% agarose gel.

Chapter IV

Analysis of the complete genome of an isolate of *Squash vein yellowing virus* from California (SqVYV-CA) reveals a recombination event between genera that facilitated development of a specific RT-PCR test

Abstract

Squash vein yellowing virus (SqVYV) is a species in the genus *Ipomovirus* (family *Potyviridae*) that infects cucurbits and causes the highly damaging watermelon vein decline disease. The virus was first reported in Florida in 2007 and is transmitted by whiteflies and mechanically. In 2014 a SqVYV-like virus was detected in cucurbits within the Imperial Valley of California and a partial sequence was analyzed in Batuman et al. (2015).

Here, I present the analysis of genome sequences of an isolate of SqVYV from California, (SqVYV-CA). The plus-sense ssRNA genome of SqVYV-CA is 9909 nucleotides (nt) and encodes a predicted polyprotein of 3200 aa (364.6 kDa). The genome organization is typical of SqVYV-like Ipomoviruses, i.e., including no HC-Pro gene and P1a and P1b genes encoding serine proteases at the 5' end of the genome. However, pairwise sequence comparisons revealed evidence of recombination, as most of the genome (~90%) was nearly identical to that of a SqVYV isolate from Israel (SqVYV-IL), but the 5' end at the N-terminus of the P1a gene was highly divergent from those of other SqVYV isolates and other ipomoviruses. It was further shown that the recombinant sequence came from a yet to be identified member of the genus *Potyvirus,* such that the in-frame fusion leads to expression of a hybrid P1a protein, with the N-terminal half composed of potyvirus P1 sequence and the C-terminal half of the P1a from an isolate of SqVYV most closely related to SqVYV-IL. The sequence of the recombination region was used to generate a primer pair for specific RT-PCR detection of SqVYV-CA. This test was used in 2021 to detect SqVYV-CA in field-collected melon leaves showing yellowing from late-planted melon fields in Fresno, CA, which was the first report of the virus in the Fresno area.

Introduction

The family *Potyviridae* is the largest and most diverse group of plant-infecting RNA viruses. Many of these viruses cause economically important plant diseases of crops and ornamentals worldwide. Outbreaks of these viruses can result in 100% loss in crops such as cucurbits, papaya, and peppers. Symptoms caused by these viruses typically include mosaic, mottling and distortion of leaves and deformation and reduced size of fruits (Ali, 2020). Members of this family are non-enveloped and possess a genome composed of plus-sense single-stranded RNA. The virions are long flexuous rods that measure ~680 to 900 nm in length and 11-20 nm in width, and encapsidates the genomic RNA, which is ~8-11 kb (Inoue-Nagata et al., 2022). Most members are monopartite, except for those of the genus *Bymovirus*, which are bipartite (Inoue-Nagata et al., 2022).

The family *Potyviridae* currently is composed of 11 genera established based on host range, vector, genomic features and phylogeny (Inoue-Nagata et al., 2022). The genus *Potyvirus* is the largest (~150 species) and all members are vectored by aphids in a non-persistent manner. The other genera have far fewer species (6-11), and some have vectors other than aphids. Thus , the 11 species of the genus *Macluravirus* are vectored by aphids (non-persistent) (Wylie et al., 2017), whereas the six species of the genus *Tritimovirus* have eriophyid mite vectors and transmission is semi-persistent (Tatineni and Hein, 2018). Members of the genus *Ipomovirus* are vectored by whiteflies of the *Bemisia tabaci* cryptic species complex and in a semi-persistent manner (Gilbertson et al, 2015; Inoue-Nagata et al., 2022).

There are nine ICTV-recognized species in the genus *Ipomovirus*, most of which occur in the Old World (OW), mostly in Africa (Gilbertson et al., 2015). The exception in SqVYV, which was first identified infecting cucurbits in the New World (NW), i.e., Florida US in 2007 (Adkins

et al., 2007). Here, it is also important to note that ipomovirus species are relatively divergent in sequence and genome organization. Three genome organizations have been recognized for Ipomovirus species based on divergence in the 5' proximal P1-type and HC-Pro genes and proteins (Dombrovsky et al., 2014): (i) *Sweet potato mild mottle virus* (SPMMV)-type with a large P1 gene/protein and HC-Pro; (ii) SqVYV-type, with P1a and P1b genes/proteins, but no HC-Pro gene; and (iii) *Cassava brown streak virus* (CBSV)-type, with only a P1b gene/protein and no HC-Pro.

The first identification of SqVYV was from Florida USA in 2003, when the virus was associated with yellowing symptoms observed in fields of squash (*Cucurbita pepo*). Potyvirus-like virions were observed by electron microscopy in preparations of these leaves and potyvirus-like pinwheel-type inclusion bodies were observed in cells of infected leaves (Adkins et al., 2007). Molecular characterization (sequencing) revealed that the potyvirus-like virus was actually a new type of potyvirus-like virus, which was named SqVYV (Adkins et al., 2007). The virus was initially shown to be mechanically transmissible and, subsequently, by whiteflies and not aphids. The host-range of SqVYV is narrow and limited to members of the Cucurbitaceae family, and there is no evidence of seed transmission (Adkins et al. 2007). Importantly, SqVYV induces the highly destructive vine decline disease of watermelon, whereas it causes vein yellowing and mosaic in melon and squash.

Beginning in 2014, there have been several new reports of SqVYV in different geographical regions of the world. In the Spring of 2014, watermelon plants in a field in Israel showed symptoms of wilting, yellowing and necrosis of leaves and stems, as well as severe damage (internal necrosis) of fruits. It was established that this was watermelon vein decline disease caused by a distinct strain of SqVYV, SqVYV-IL (Reingold et al. 2016). In September 2020, a SqVYV

isolate was detected infecting butternut squash, in the Lower Rio Grande Valley of Texas, but the strain was not identified (Hernandez et al., 2021).

The subject of this chapter of my thesis is the outbreak of a SqVYV-like virus that was detected in pumpkin plants with severe stunting and leaves with crumpling epinasty and yellowing at the DREC in Holtville, California in the Imperial Valley in the fall of 2014 (Batuman et al. 2015). The first clue of Ipomovirus infection of these plants came from RT-PCR tests with degenerate potyvirus primers in which a positive result was obtained with a CI primer pair but negative with a HC-Pro primer pair, and the observation of long flexuous rods similar to that of potyviruses virions were observed in leaf dip preparation viewed by EM (Batuman et al., 2015). Sequencing of the RT-PCR amplified CI fragment and, subsequently, a capsid protein (CP) gene amplified with a SqVYV primer pair revealed 99% identity of SqVYV-IL. This was the first report of SqVYV infecting cucurbits in a state other than Florida and indicated that the virus was spreading. Here, I present the completion and analysis of the complete sequence of the genomic RNA of an isolate of SqVYV-CA and development of a specific RT-PCR test for the virus, which was used to show SqVYV-CA has spread into Fresno Co in 2021.

Materials and Methods

Source tissue and virus propagation

Fresh or stored archival samples of symptomatic pumpkin leaf samples infected with SqVYV-CA collected in 2014 in the Imperial Valley were the source of the virus. These samples were used to mechanically transmit SqVYV-CA to giant pumpkin seedlings (*Cucurbita maxima*). Sap was prepared by grinding tissue in ice-cold 0.1 M potassium phosphate buffer (pH 7) in a 1:10

wt/vol ratio, and mechanically (rub) inoculated onto celite-dusted cotyledonary leaves of pumpkin seedlings. At 21 dpi, symptoms were assessed, and samples collected for testing and storage.

In October 2021, melon leaf samples with yellow symptoms were received from fields in four locations in Fresno Co. Symptomatic leaf tissue from these samples was used for RT-PCR tests, sap transmission and stored at -80 C.

RNA extraction, cDNA and PCR

Total RNA was extracted from leaf tissue or FTA cards with the RNAeasy extraction kit (Qiagen) according to manufacturer's instructions. For RT-PCR tests, the cDNA was synthesized with random primers and MMLV reversetranscription enzyme (Takara Clonetech). First, 2 mM reverse random primer mix (2 ul) and 9 ul of RNA extract were mixed and incubated 3 min at 70 C and then placed in ice for 5 min. MMLV enzyme buffer (4 ul), 2 ul of MMLV enzyme and water were added to the reaction final volume of 20 ul and incubated 90 min at 42 C. PCR was then performed with selected primer pairs and involved an initial denaturation step of 98 C for 30 s that was followed by 35 cycles of 98 C for 30 s, annealing at 55 C for 30 s and elongation at 72 C for one min. The PCR was analyzed by agarose gel electrophoresis in 0.8% TAE buffer. RT-PCR-amplified DNA fragments were excised and recovered with the Zymo kit according to manufacturer's instructions and sent for sequencing to QuintaraBio. Because members of the family *Potyviridae* have a 3' poly-A tail, a poly-T primer was used to perform the RT of the genomic RNA.

To confirm the precise 5' end of the genomic RNA of SqVYV-CA, rapid amplification of cDNA ends (RACE) was performed. The cDNA for RACE-PCR was synthesized with primer GPS-R2 that anneals at nt 970 (Table 1). Then a poly-C tail was added to the 5' end using terminal

transferase with dCTPs according to manufacturer's instructions (New England BioLabs). The PCR was performed with the Abridge Anchor primer AAP (Table 1) and specific reverse primer GPS-R3 that binds to nt 520. The resulting 5'-RACE PCR fragments were cloned into the pGEM-T Easy Vector (Promega), and sequences of representative clones were determined at Quintara with the M13F (-21) and M13R sequencing primers (Table 1). To obtain a consensus sequence, an alignment sequence from 5 cloned RACE-PCR fragments was generated and aligned with 5' sequences of SqVYV-IL, SqVYV-IR and SqVYV-FL (Fig 2).

Phylogenetic analysis

Alignments of nucleotide and amino acid sequences was done with the MAFFT algorithm (Katoh and Standley, 2013) and MrBayes was used for the phylogenetic analyses in Geneious (Biomate) with a poisson matrix and 1000 bootstrap replications. Complete sequences of the following ipomovirus species were used: SqVYV-CA [this study], SqVYV-FL [YP_001788991.1], SqVYV-IL [ALN38790.1], SqVYV-IR [AOY33888.1], SPMMV [CAA97466.1], *Cucumber vein yellowing virus* (CVYV) [QGW63298.1], CBSV [ADR73022.1], *Ugandan cassava brown streak virus* (UCBSV) [ACM48176.1], *Coccinia mottle virus* (CocMoV) [AOC84052.1], *Tomato mild mottle virus* (TMMoV) [CCD57807.1]. In addition, the complete seuqnece of *Papaya ringspot virus* (PRSV) [NP_056758.1] was included as well as sequences of *Triticum mosaic virus* (TriMV) [ACT53745.1], a member of the genus *Tritimovirus* was used as an outgroup.

Results

Characterization and phylogenetic analysis of the SqVYV-CA isolate

Pumpkin seedlings mechanically inoculated with sap prepared from pumpkin leaves with crumpling and yellowing from field-collected and an archival sample collected in 2014 in Imperial Valley of California, developed symptoms of vein-yellowing, crumpling and yellow mosaic/mottle typical of SqVYV infection. These plants were confirmed to be infected by SqVYV with RT-PCR tests with the SqVYV capsid protein (CP) primer pair and not with other known mechanically transmissible viruses, e.g., cucumber mosaic virus or potyviruses (data not shown). It was from these plants that tissue was collected and RNA was extracted and used for cDNA generation and amplification of the complete genome PCR, with the Gibson Assembly method. In this way, putative full-length clones of SqVYV-CA were generated in the binary vector pJL89. One clone, pJL89-SqVYV-CA-4 was selected for sequencing by primer walking. The RACE-PCR was used to confirm the sequence of the 5'-end of the genomic RNA of SqVYV-CA. The expected ~700 bp fragment was generated, cloned and sequenced. Sequences of five cloned fragments were identical. Comparisons with 5' ends of other SqVYV isolates revealed high level of divergence m e.g., 77.5% and 71.1% identity with those of SqVYV-IL and -FL respectively (Fig 2). Thus, based on my 5' end determination, the complete genome sequence of SqVYV-CA-4 of SqVYV is 9909 nt and includes a single large ORF of 3200 aa and a 5' and 3' non-translated regions of 103 and 202 nt, respectively (Fig 1).

Thus the 5' untranslated region (UTR) of SqVYV-CA was confirmed by RACE and was 103 nt, compared with 112 nt for SqVYV-IL and 118 nt for SqVYV-FL. The precise 5' end sequence is 5' AAAATAAACATTACATGAACATT 3'. Notably, the sequence of the SqVYV-CA 5' UTR is only 74.8% and 71.3% identical to those of SqVYV-IL and SqVYV-FL,

respectively, indicating that it was derived from a different virus. The 3'UTR is larger in general and is , e.g., is 200 nt for SqVYV-CA compared with 191 nt for SqVYV-IL and 199 nt for SqVYV-FL. Sequence comparisons showed that the SqVYV-CA 3'UTR sequence is 97.4% and 87.5% identical to those of SqVYV-IL and SqVYV-FL.

The single large ORF was predicted to be translated into a polyprotein of 346.6 kDa, which is similar in size to those reported for other isolates of SqVYV and ipomoviruses. The polyprotein was further predicted to be cleaved into P1a, P1b, P3, CI, VPg, protease, replicase (RdRp) and CP (Fig 1). Thus, the SqVYV-CA genome organization is typical of an ipomovirus. Pairwise sequence comparisons performed with the polyprotein aa sequence of SqVYV-CA showed highest identity (90.2%) with that of SqVYV-IL, followed by SqVYV-FL (85.2%) and, with a much lower value (57%) with the highly divergent SqVYV-IR (Table 2). The ipomovirus species with the highest identities were CVYV (53.3%) and CocMoV (53%), slightly lower than for SqVYV-IR. The phylogenetic tree generated with the complete polyprotein aa sequences (Fig 3) and those of the conserved CP (Fig 4) placed the four SqVYV sequences in a strong supported clade (100%), in which SqVYV-CA was placed on a distinct branch (Fig 3). However, the phylogenetic tree generated with the highly conserved CPaa sequence placed SqVYV-CA together with SqVYV-IL in a strong supported clade (100%) (Fig 4). Thus, these phylogenetic analyses showed that SqVYV-CA was more closely related to SqVYV-IL than to SqVYV-FL or SqVYV-IR and that the closest virus to SqVYV is CVYV (Fig 3).

Determination of the recombination event in SqVYV-CA P1a/P1b gene/protein

Although the genome organization of SqVYV-CA is similar to those of other isolates of SqVYV, pairwise comparisons performed with predicted aa sequences of individual proteins revealed evidence of a recombination event in the P1a gene of SqVYV-CA resulting in a hybrid gene composed roughly of two halves: the 5' 1013 nt from a yet to be characterized member of the genus *Potyvirus* and the 3' of 788 nt from a SqVYV-like virus, most identical with SqVYV-IL (91.2%) (Table 2). The resulting in-frame hybrid P1a protein is predicted to be 64 kDa, which is slightly larger than the P1a proteins of other SqVYV isolates, e.g., 61.4 kDa SqVYV-IL and 61.3 kDa SqVYV-FL. The SqVYV-CA P1a protein retains the typical proteolytic domain (H-7X-D-X-S) of a serine protease, which is found in other ipomoviruses (Mbanzibwa et al., 2009). To determine the nature of the two halves of the hybrid SqVYV-CA P1a gene/protein, I next performed (i) a BLASTp search with the sequence of each half, (ii) sequence comparison with each half and (iii) phylogenetic analyses of the complete P1a aa sequence and those of each half. The BLASTp analyses showed that the sequence of the N-terminal half had the highest identity (57.8%, Evalue: $2e^{-121}$) with P1 aa sequence of *Papaya ringspot virus* (PRSV) and *Zucchini tigre mosaic virus* (57.5%, Evalue:$2e^{-113}$), which are species of the genus *Potyvirus.* The 3' C-terminal half had the highest identities (93.3%, Evalue: $2e^{-134}$) with SqVYV-IL. These results were consistent with those for the split aa sequence comparisons (Table 4). Finally, phylogenetic analyses performed with the complete P1a aa sequences placed SqVYV-CA in a distinct clade nearest to the potyviruses (Fig 6). Furthermore, the phylogenetic tree generatedwith the N-terminal half placed SqVYV sequences in a highly supported distinct from other ipomoviruses. Within this SqVYV clade, there was a subclade with SqVYV-IL, -FL and -IR and a second subclade with SqVYV-CA and PRSV (Fig 7). Thus, these results show that SqVYV-CA P1a gene is a hybrid

composed of a 5' half from a potyvirus P1 gene and a 3' half from SqVYV-like half (Figs 7-8). The recombination event in SqVYV-CA full genome was precisely mapped to nt 1034 of the SqVYV-IL and further showed the homology between the ipomovirus P1a and potyvirus P1 sequences (Fig 5).

Development of SqVYV-CA specific primer pair for detection of the California isolate

I then utilized the high divergent potyvirus P1 of the SqVYV-CA hybrid P1a gene to design a specific primer pair for detection of SqVYV-CA by RT-PCR. Sequence alignments revealed suitable sequences in the recombination region between nts 674 and 976, and a primer pair was designed to direct the amplification of a ~200 nt fragment (Table 1). I then performed RT-PCR tests to optimize parameters, i.e., direct a single 200 bp fragment. In the final RT-PCR test, the SqVYV-CA primer pair directed the amplification of the expected 200 bp fragments, whereas no fragment was amplified from an RNA extract of uninfected leaves (Fig 9). In RT-PCR tests of squash leaves infected with SqVYV-CA via mechanical inoculation, the expected size of ~200 nt fragment was also amplified, whereas no fragment was amplified from an RNA extract prepared from uninfected leaves (data not shown).

The SqVYV-CA-specific primers were not predicted to direct the amplification of the target fragment from the genome of SqVYV-IL, SqVYV-FL and SqVYV-IR based on *in silico* analyses. However, to further confirm this, I obtained a samples of squash leaves infected with SqVYV-FL on an FTA card, kindly provided by Dr. Scott Adkins. I then performed RT-PCR tests with the general SqVYV CP primer pair (Table 1) and the SqVYV-CA-specific primer pair with total RNA extracts of (i) plants infected with SqVYV-CA, (ii) plants infected with SqVYV-FL and (iii) an uninfected control. In the tests performed with the general SqVYV CP primer pair, the

expected-size ~850 bp fragment was amplified from extracts of plants infected with SqVYV-FL or SqVYV-CA and not from the extract of the uninfected control (Fig 9). In equivalent RT-PCR tests with the SqVYV-CA-specific primer pair, the expected-size ~200 bp fragment was amplified only from the extract of the sample infected with SqVYV-CA and not from extracts of plants infected with SqVYV-FL or uninfected controls (Fig 9). These results indicate that this RT-PCR tests can be used for specific detection of SqVYV-CA.

In 2021, I had the opportunity to test the SqVYV-CA-specific RT-PCR test with field-collected samples. At the end of the 2021 growing season, we received melon leaf samples with yellowing symptoms collected from four fields in Fresno Co CA, and these were tested by RT-PCR with the (i) general SqVYV CP primer pair and (ii) the SqVYV-CA-specific primer pair. Two of the samples tested, 21-607 from Huron and 21-609 from Firebaugh, were positive in RT-PCR tests with CP primers, i.e., the ~850 bp fragment was amplified, and with the SqVYV-CA-specific primer pair, i.e., the ~200 bp fragment was amplified (Fig 10). The other two samples were negative for SqVYV infection with both tests. To further confirm the identity of the ~200 bp fragment amplified with the SqVYV-CA-specific primer pair, sequencing was performed and comparisons revealed 99% nucleotide identity with the sequence of SqVYV-CA for both samples, thereby confirming infection with SqVYV-CA. Thus, these primers can be used in the RT-PCR to monitor for the spread of SqVYV-CA.

Discussion

Since it was first detected in 2003, SqVYV has become a problem for cucurbit crops in Florida, especially in watermelon where it induces vine decline and appears in mixed infections with other viruses (Baker et al., 2008; Egel and Adkins, 2007; Gilbertson et al., 2015). However,

more recent outbreaks of SqVYV in different regions of the OW and NW locations have made this an emerging virus of cucurbit crops worldwide (Acevedo et al., 2013; Egel and Adkins, 2007; Reingold et al., 2016; Turechek et al., 2010). Furthermore, characterization of strains involved in these outbreaks has also revealed higher levels of genetic diversity in this virus than previously recognized, and insights into the origin of the virus. Of particular relevance to the present study were two apparently unrelated outbreaks in 2014, one involving watermelon in Israel (Reingold et al., 2016) and the other involving yellowing in melons and pumpkins in the Imperial Valley of CA, USA (Batuman et al., 2015), that is the subject of the present study.

The first evidence that suggested ipomovirus infection in the pumpkins with yellowing symptoms observed in the Imperial Valley in 2014 was the result of a RT-PCR test with degenerate potyvirus primers, in which the target sequence was amplified with the CI primer pair, but not with the HC-Pro primer pair. Sequencing of the CI fragment revealed highest identity with CI sequences of SqVYV (83%), confirming infection with an ipomovirus, rather than a potyvirus (Batuman et al., 2015). Furthermore, much of the 3' end of SqVYV-CA was determined, but efforts to amplify the 5' end with primers based on SqVYV sequences were unsuccessful.

The strains involved in these outbreaks were substantially divergent from SqVYV-FL, indicating these outbreaks were not due to recent introductions of SqVYV-FL.

The Gibson assembly method allowed us to obtain a full-length SqVYV-CA clone and sequence analysis revealed a number of interesting findings with this virus. The finding that downstream of P1a and P1b genes, the SqVYV-CA genome sequence is nearly identical to that of SqVYV-IL (98%) is in agreement with results of Reingold et al. (2016) and suggests a common evolutionary origin, independent of SqVYV-FL. Thus, taken together with the high identities in the 3 UTRs indicates SqVYV-CA and SqVYV-IL had the same major parent.

The major difference between SqVYV-IL and SqVYV-CA and the one responsible for lower polyprotein identity and a different tree topology than with the conserved CP gene is the recombinant event discovered in the P1a gene (Fig 5). This recombination event involved the acquisition of 1013 nt of the P1 gene of a yet to be characterized member of the genus *Potyvirus* (based on 56.6% aa identity and < for ipomovirus P1a gene). Although it is not clear if the resulting hybrid P1a/P1b protein provides a selective advantage, it had no effect in the capacity of the virus to induce watermelon vine decline disease (Batuman et al., 2015). Furthermore, this recombination event is perhaps not surprising as it has been previously reported the sequence similarities between P1a protein of SqVYV and CVYV and P1 proteins of potyviruses (Valli et al., 2007). This may allow for template switching during replication and is consistent with the P1a/P1b hybrid protein being functional. Taken together with the divergent 5' UTR of SqVYV-CA, a model can be proposed in which this recombination event occurred in plants having mixed infection of SqVYV-IL and an uncharacterized potyvirus and SqVYV-IL. During the initial transcription of the 5' end strand of the potyvirus there is a template switch event at the middle of the P1a gene of SqVYV-IL resulting in the transcription of the rest of this genome including the 3' end UTR. This may have occurred in cucurbits, which often have mixed infections, including ipomovirus and potyviruses (Turechek et al., 2010). In fact, we detected mixed infection of SqVYV-CA and a potyvirus, *Watermelon mosaic virus* (WMV) and *Zucchini yellow mosaic virus* (ZYMV) in melon sample from California. It is also notable that the C-terminal aa sequence of the hybrid P1/P1a proteins was most identical to that of SqVYV-ILm but slightly lower (91.2% identity) than other 3' proteins. Because the potyvirus is uncharacterized, this recombinant sequence in SqVYV-CA provided limited insight into the origin of SqVYV-CA.

Within the genomes of some viruses are specific recombination hotspots, which mediate rapid viral evolution. In the case of ipomoviruses, this seems to be in the 5' proximal genes. The genomes of CYVV and SqVYV encodes two P1 proteins, P1a and P1b, which probably have a common origin, and arose via gene duplication (Valli et al., 2007). In the case of CVYV, function divergence of these proteins has been demonstrated, in which P1a (like the potyvirus P1) has protease activity that requires plant factors to function, whereas the P1b does not require a plant factor for protease activity and is a suppressor of gene silencing (Valli et al., 2006). Thus, the function of the ipomovirus P1a is less clear and it has been suggested to play a role in host adaptation (Valli et al., 2007). My results showing recombination within the SqVYV-CA P1a may reveal how this region is a recombination hotspot that allows for more rapid viral evolution via acquisition of sequences from species within the genus and even from members of other genera. In the case of SqVYV-CA, the minor parent was an unknown potyvirus and it is not clear if this recombination event provided a selective advantage, it is clear that SqVYV-CA is competitive based on establishment and spread in California. Recombination has also been shown in 5' and 3' proximal regions of the genome OF SPMMV and it has been reported that thirteen aas in the N-terminus of the P1 protein were under positive selection, whereas other genes (P3, 6K1 and CP) were under purifying selection (Tugume et al., 2010). The importance of the P1 region in a member of the genus Potyvirus comes from WMV in which recombination breakpoints were identified in the P1 gene as well as in the NIa-Pro and Nib-CP genes (Verma et al., 2020). Thus, my results indicate that ipomovirus diversity and evolution can be driven by recombination in the P1-type genes. The role of the recombination events in host adaptation remain to be determined.

I took advantage of the divergent potyvirus P1 sequence in the hybrid SqVYV-CA P1a gene to develop a primer pair of SqVYV-CA with an RT-PCR test. The specificity of this RT-

PCR test was shown by the failure to detect SqVYV-FL and lack of non-specific fragments in tests with RNA extracts of uninfected plants. Demonstration of the practical utility of the test came from the detection of SqVYV-CA infection in two field-collected melon samples with yellowing symptoms collected in Fresno in 2021. This is the first report of SqVYV-CA this far north in California and is a particular concern for watermelon production. It is likely that SqVYV-CA was introduced with viruliferous whiteflies migrating from desert production areas, e.g., Imperial Valley and Yuma, AZ. Therefore, an important question is whether the virus can persist and become established in the Central Valley. Thus, this SqVYV-CA test will be useful for monitoring for the establishment of the virus in this important melon production region. It should be noted the SqVYV-CA has not become a major component of the 'yellow complex' in cucurbits in desert production for the Southwestern US, though it has been regularly detected in samples since 2014 suggesting establishment and potential threat in the future.

In conclusion, the complete sequence of SqVYV-CA revealed a common origin with SqVYV-IL, but subsequent divergence via a unique recombination event in the P1a gene, in which the 5' end is derived from the P1 gene of a yet to be identified member of the genus *Potyvirus*. This divergent potyvirus-like sequence was used to develop a SqVYV-CA-specific RT-PCR test, which was used in 2021 to detect SqVYV-CA for the first time in the Fresno area. This introduction was most likely via whiteflies from desert melon production, and a major concern is that watermelon vine decline will appear in the Central Valley and become economically important. disease in California. Thus, it is important to keep monitoring and to further investigate SqVYV-CA.

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Tables

Table 1. Sequence of the oligonucleotide primers used in this study

Primers	Sequence (5'-3')	Application
SqVYVCP-F	CCCTCGGAGAACTTGATATGGAAGCAC	Diagnostic of SqVYV species
SqVYVCP-R	CGCGTCCTTCCTCTCCAGGCGCTG	Diagnostic of SqVYV species
SqVYV-CAF	GCTGATTATCTGGAGAAGCCAAC	Diagnostic of SqVYV California isolate
SqVYV-CAR	CTCAGCAACGGCTGCCTCC	Diagnostic of SqVYV California isolate
GPS-R2	CTGATACATGAGATCGCGCATTG	Specific primer for SqVYV for RACE cDNA synthesis
AAP	GCC CAC GCG TCG ACT AGT ACG GGI IGG GII GGG IIG	
GPS-R3	CTTGTCTTGCACCTTTTCTATGGCAG	Specific primer for sequencing of RACE
M13F(-21)	GTAAAACGACGGCCAGT	Invitrogen
M13R	CAGGAAACAGCTATGAC	Invitrogen

*In the primer sequences, I = inosine

Table 2. Percentage of identity determined by pairwise comparison of amino acids sequences of the complete polyprotein (ORF1) and individual mature proteins of SqVYV-CA with those of other ipomovirus and the potyvirus species, *Papaya ringspot virus* (PRSV). P1a-N is the N-terminal and P1a-C is the C-terminal region of the P1a protein of SqVYV-CA.

	ORF1	**P1a**	**P1a-N**	**P1a-C**	**P1b**	**P3**	**6K1**	**CI**	**6K2**	**VPg**	**Nia**	**Nib**	**CP**	**PIPO**
SqVYV-IL	90.2	49.6	18.5	91.2	98.8	98.4	100	99.2	100	97.4	100	99.6	98.9	89.8
SqVYV-FL	85.2	40.8	18.9	71.9	91.3	89.9	98.1	96.8	88.7	92.3	97.9	96.4	97.2	84.6
SqVYV-IR	57.0	26.6	13.4	44.1	60.6	60.4	53.8	68.4	58.5	68.0	62.7	61.5	63.3	56.4
CVYV	53.3	18.0	11.8	10.5	33.4	51.9	63.5	74.7	52.8	52.6	51.7	71.2	68.0	52.3
CocMoV	53.0	19.1	9.7	13.1	34.4	53.2	69.2	74.5	56.6	55.7	48.9	68.6	66.9	52.0
CBSV	42.3	3.0	2.3	13.9	31.4	45.6	59.6	63.8	37.7	53.1	47.0	64.9	60.7	41.0
UCBSV	42.1	2.0	1.7	15.0	32.8	45.0	55.8	66.3	34.0	49.0	44.9	63.5	61.7	40.7
SPMMV	30.6	13.5	9.6	13.8	24.4	12.6	36.5	49.4	26.4	34.5	30.6	55.8	35.2	30.1
TMMoV	25.3	2.3	0.6	14.7	22.1	9.0	20.0	40.8	34.0	28.7	29.9	51.3	32.0	24.6
PRSV	24.7	41.9	56.6	29.5	12.1	7.7	19.2	28.5	7.5	18.5	21.5	38.1	20.1	24.8

Figures

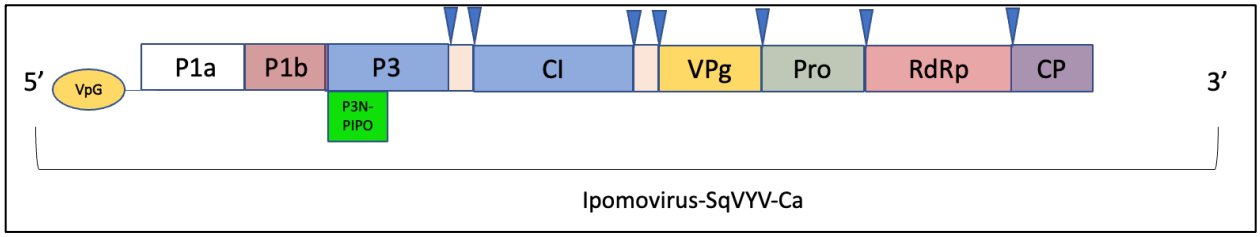

Figure 1. Diagram showing the organization of the genes in the polyprotein of an isolate of SqVYV from California (SqVYV-CA). Cleavage sites of the polyprotein are labeled with blue arrows.

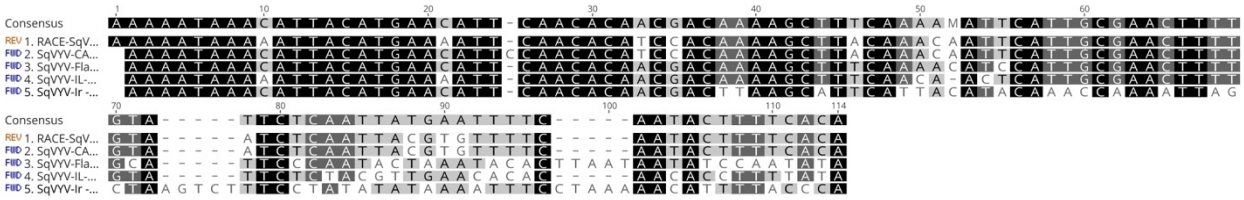

Figure 2. An alignment of 5'-end RACE sequences of cloned fragments of the strain of SqVYV from California (SqVYV-CA) and comparison with 5'end sequences of SqVYV-IL, SqVYV-FL and SqVYV-IR. The shading represents identity between sequences, with darker shading indicating higher identity.

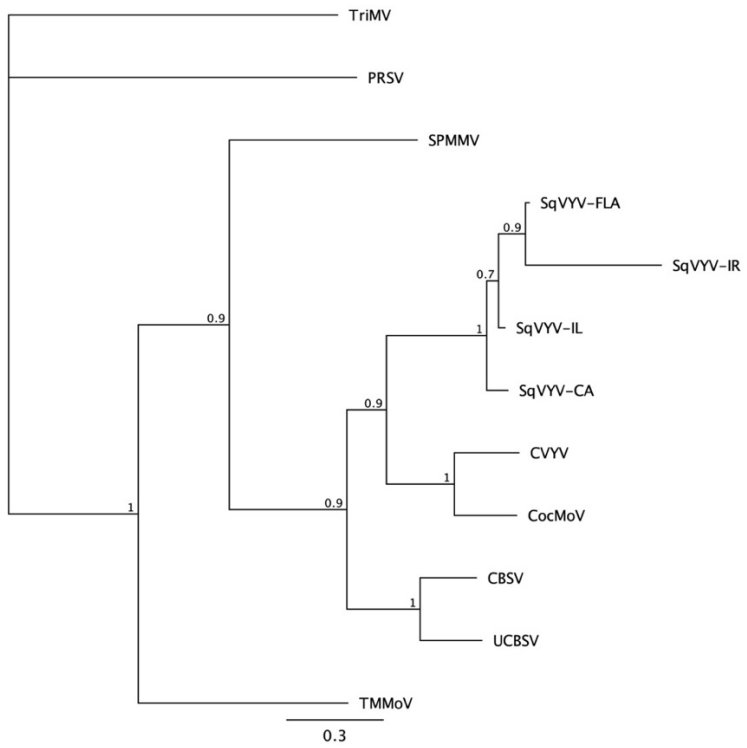

Figure 3. Bayesian phylogenetic tree generated with the complete amino acid sequences of the polyprotein of members of the genus *Ipomovirus* including SqVYV variants from Florida (FL), Israel (IL), Iran (IR) and California (CA). The species *Triticum mosaic virus* (TriMV) was used as an outgroup. The sequences were aligned in MAFFT and the phylogeny was calculated with a poisson model and 100000 bootstrap iterations in Geneious and percentages are shown on the branches.

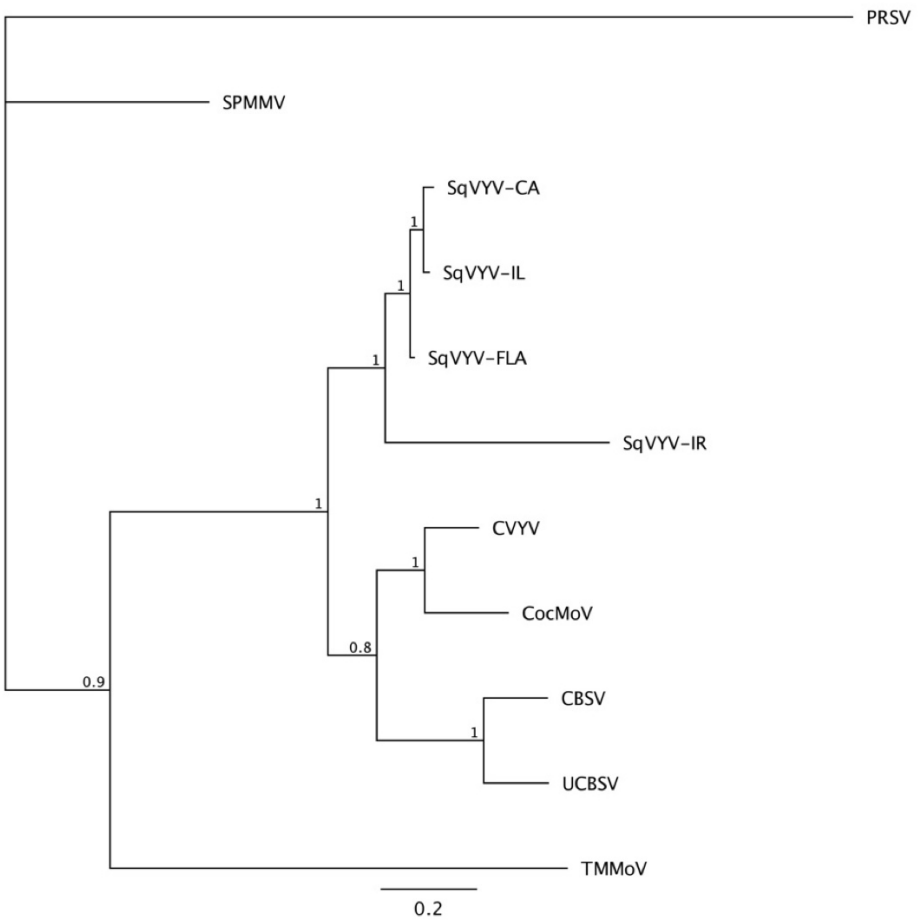

Figure 4. Bayesian phylogenetic tree generated for capsid protein (CP) amino acid sequences of members of the genus *Ipomovirus* including SqVYV variants from Florida (FL), Israel (IL), Iran (IR) and California (CA). *Papaya ringspot virus* (PRSV) was used as an outgroup. The sequences were aligned in MAFFT and the phylogeny was calculated with a poisson model and 100000 iterations in Geneious, percentages are shown on the branches.

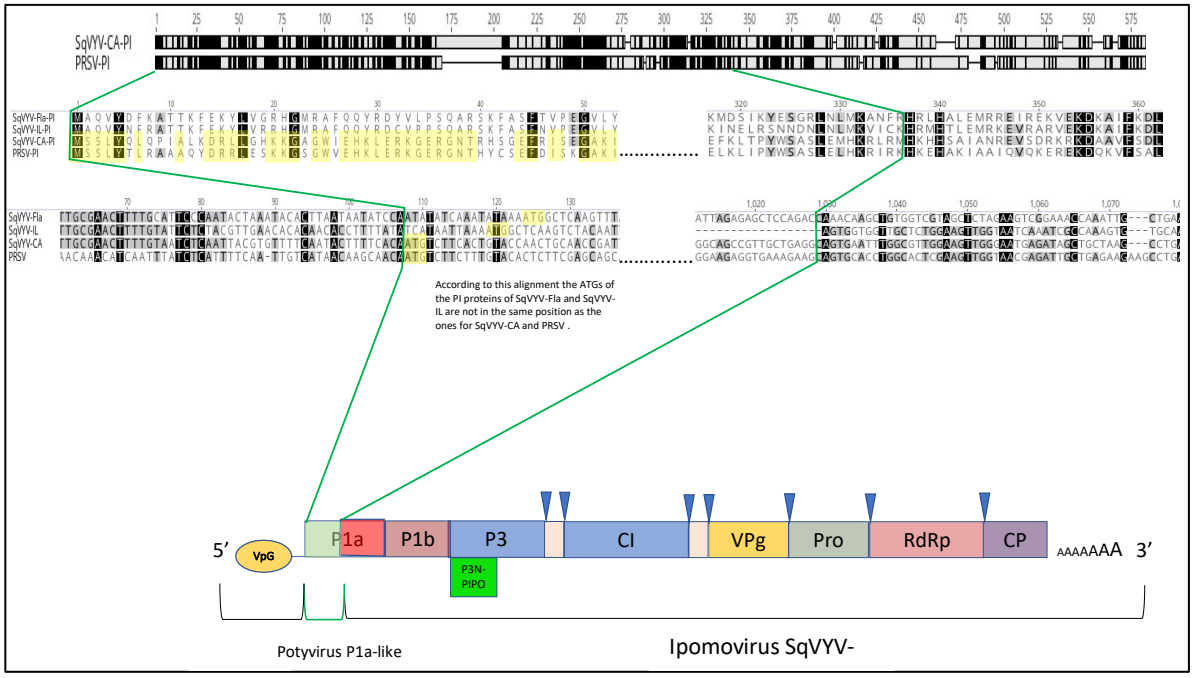

Figure 5. Identification and mapping of a recombination event in the P1a gene nucleotides of the isolate of Squash vein yellowing virus from California (SqVYV-CA). Amino acid alignments of this region of two divergent isolates of SqVYV, (SqVYV from Israel [SqVYV-IL] and Florida U [SqVYV-FL]) and the potyvirus papaya ringspot virus (PRSV). Shading intensity shows identity between the four sequences with the darker degree of shading reflects higher identities. The yellow shading shows sequences with 100% identity between SqVYV-CA and PRSV.

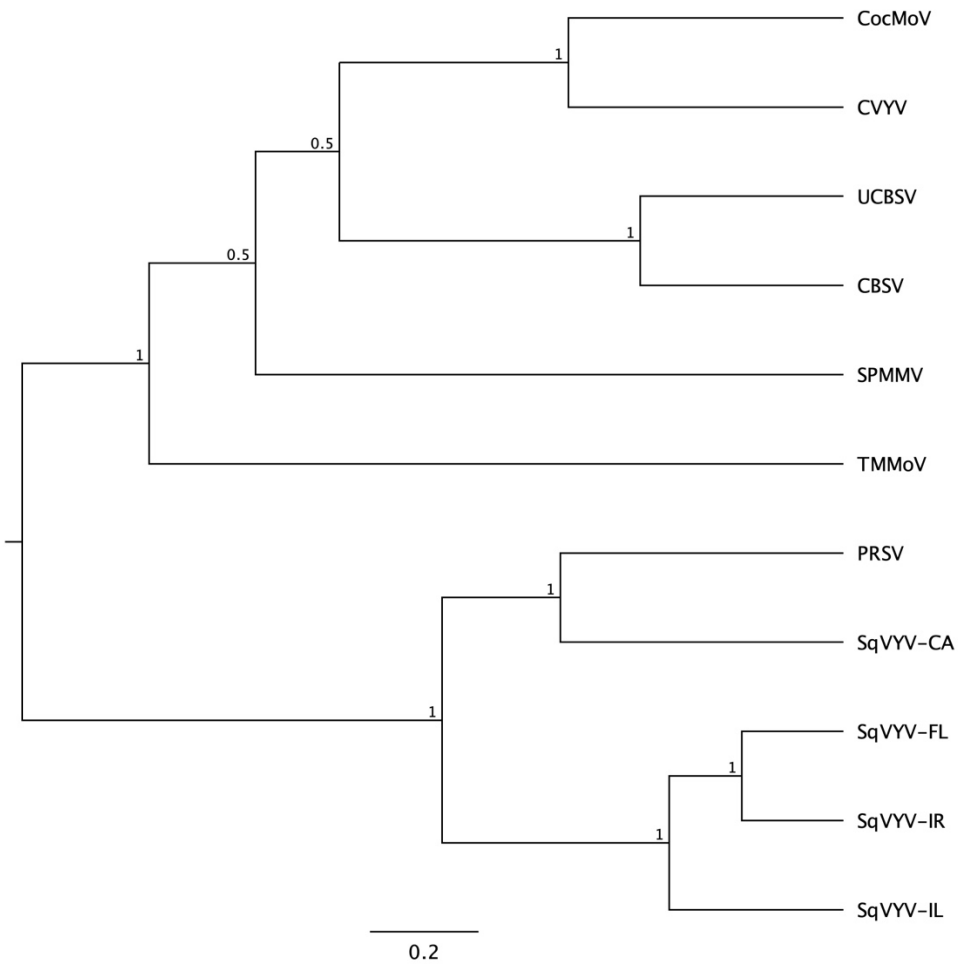

Figure 7. Bayesian phylogenetic tree of the amino acids of the complete P1a protein of Ipomovirus members and SqVYV isolates from Florida (FL), Israel (IL), Iran (IR) and California (CA). The matrix used was poisson with 1000 bootstraps. *Papaya ringspot virus* (PRSV) was used as an outgroup. The bootstrap percentages are shown in the branches.

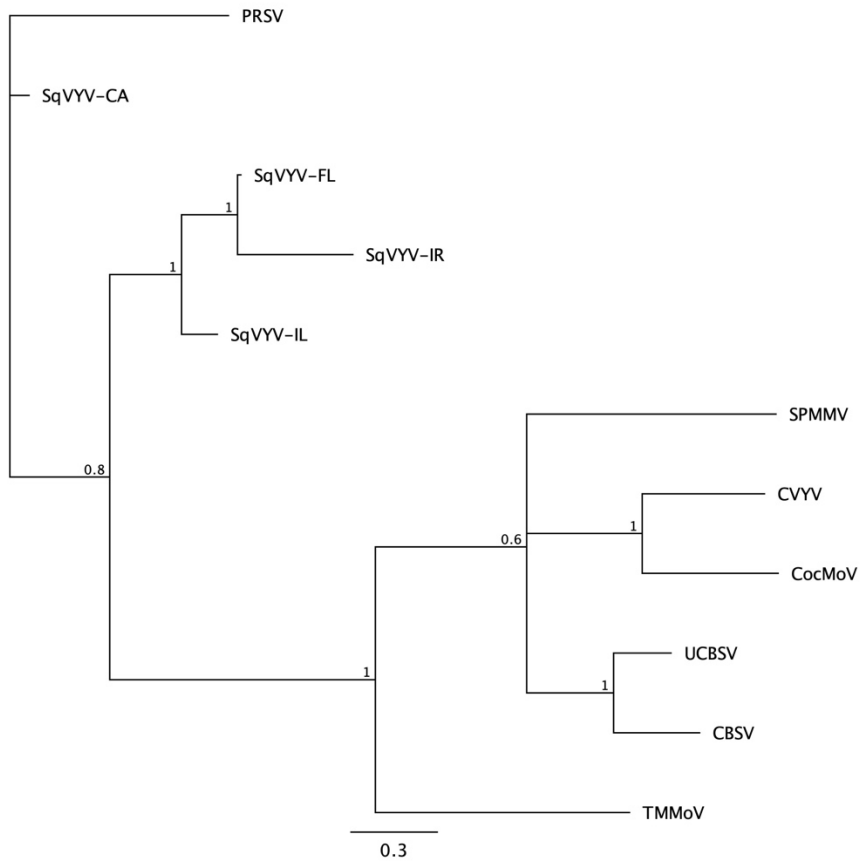

Figure 7. Bayesian phylogenetic tree generated with the N-terminus amino acid sequences of the P1a protein of ipomovirus species and SqVYV strains, SqVYV-CA, SqVYV-IL, SqVYV-IR and SqVYV-FL. The potyvirus PRSV was used as an outgroup The matrix used was poisson and the model GTR with 10000 bootstraps replications with percentages shown on the branches.

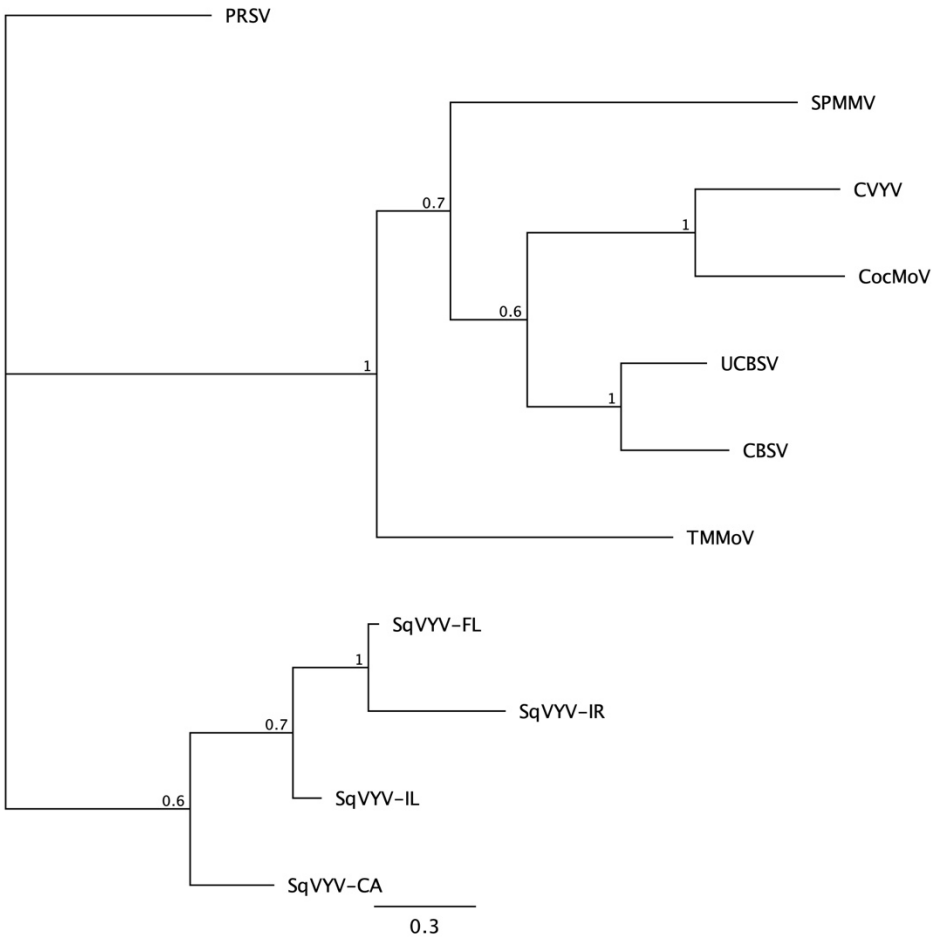

Figure 8. Bayesian phylogenetic tree of the C-terminus amino acids of the P1a C-terminus protein of ipomovirus members and SqVYV strains, SqVYV-CA, SqVYV-IL, SqVYV-IR and SqVYV-FL. The potyvirus PRSV was used as an outgroup. The matrix used was poisson and the model GTR with 10000 bootstraps and percentages are shown on the branches.

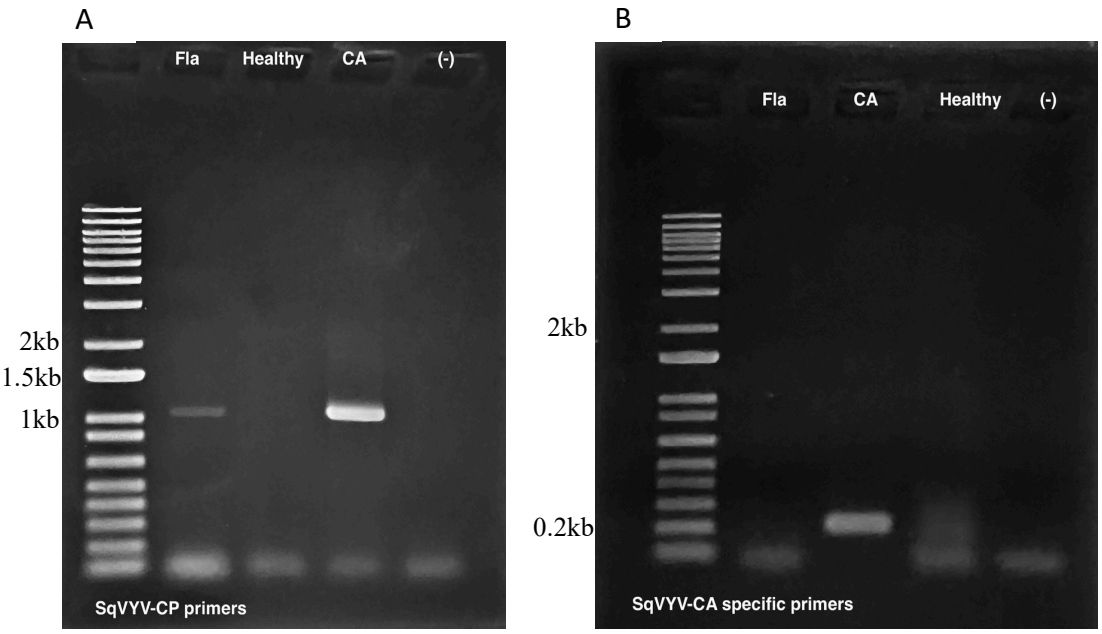

Figure 9. Evaluation of specificity of a primer pair (SqVYV-CAF and SqVYV-CAR) designed for the specific detection of SqVYV-CA. RT-PCR tests were performed with total RNA extracts of squash leaves infected with strains of SqVYV from Florida (SqVYV-FL, Fla) and SqVYV-CA as well as uninfected (healthy) squash leaves with (A) a general SqVYV capsid protein (CP) primer pair (amplified a ~850 bp fragment) and (B) the SqVYV-CA specific primer pair (amplified a ~200 bp fragment).

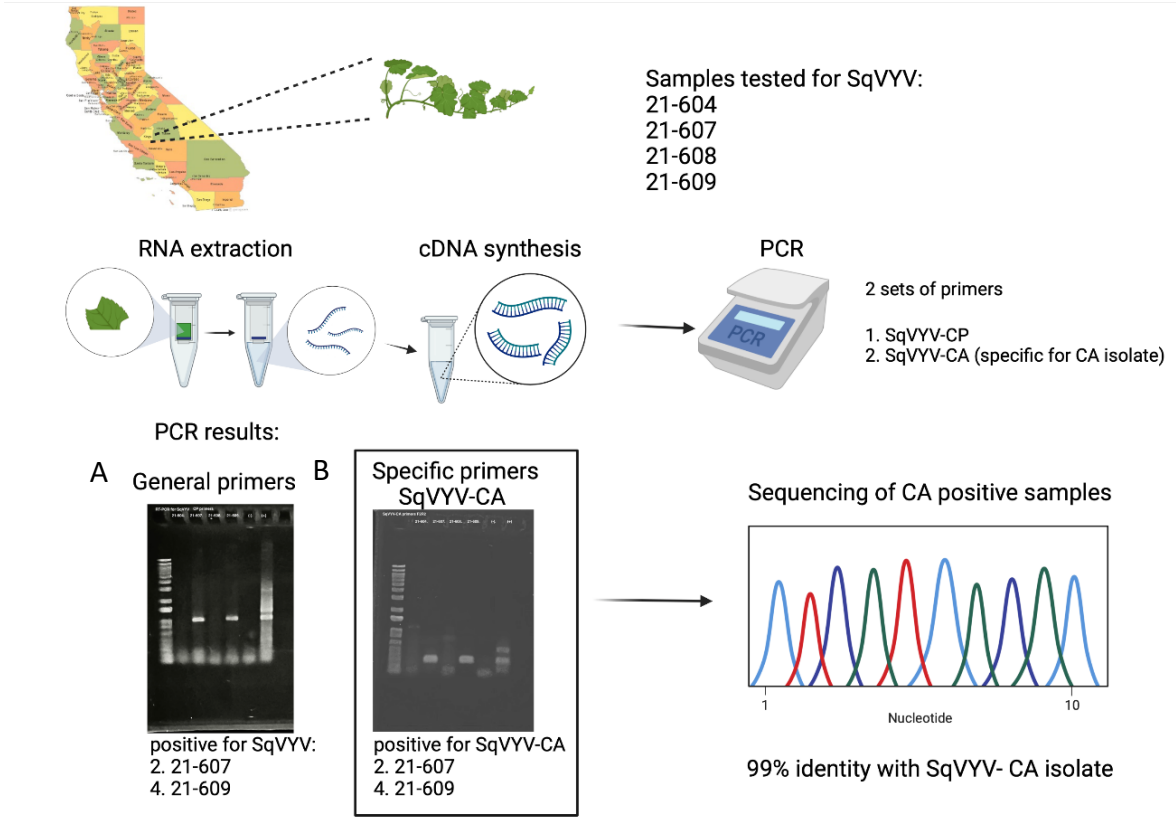

Figure 10. Steps in the RT-PCR detection of the California strain of squash vein yellowing virus (SqVYV-CA) in melon leaved with yellowing symptoms collected from 4 fields in Fresno Co. in October 2021. RT-PCR tests were conducted with, A) a general SqVYV primer pair that directs the amplification of a ~850 kb fragment and B) SqVYV-CA-specific primer pair that directs the amplification of an ~200 bp fragment.

Chapter V

Future directions

Chapter 2. **A late-season outbreak of a necrosis disease of tomato associated with high whitefly populations was caused by the reemergence of a Tomato necrotic dwarf virus (ToNDV) in Kern County, California, and application of an infectious clone for evaluation of resistance in tomato.**

In Chapter 2, I showed that an isolate of the torradovirus ToNDV, ToNDV-K15 was the causal agent of a disease outbreaks in a late-planted processing tomato field in Kern Co in 2015. This isolate was recovered from field samples and used to generate the infectious clones and agroinoculation system (agroclone) that was used to fulfill Koch's postulates and confirm this was an outbreak of Tomato necrotic dwarf disease (ToNDD). The sequence analyses of the infectious RNA 1 and RNA 2 clones of ToNDV-K15 revealed the typical organization of a torradovirus, with RNA 1 encoding for the polyprotein that will be cleaved into the proteins involved in replication, and the RNA 2 encoding for the protein ORF1 (20kDa protein) and the ORF 2, which encodes for the polyprotein that is cleaved into MP and the 3 CPs.

The sequence compasirons and current ICTV species demarcation values for torradovirus, i.e., CP with < 80% amino acid sequence identity and the ProPol with < 75%. My comparisons performed with ToNDV-K15 sequences revealed CP identities with ToMarV were above the species threshold (79%), whereas values for the ProPol were below the threshold (78%). This makes it difficult to classify ToNDV as a species or a strain of ToMarV as it has been suggested (van der Vlugt et al., 2015). Thus, these results indicate a need to re-examine species criteria for torradoviruses, especially for close cases such as ToMarV and ToNDV. This case is further complicated by the fact that ToNDD was the first torradovirus disease recognized, although the causal virus was not identified as a torradovirus. It would be interesting to perform pseudo recombination experiments between RNA 1 and RNA 2 of ToNDV and ToMarV to help determine

if these are divergent strains (PRs are infectious) or distinct species (PRs are not infectious). Thus, these experiments will demonstrate if the RNA 1 of ToMarV can replicate RNA2 of ToNDV and viceversa, and if proteins encoded by RNA 2 are compatible. In my research it was established that virus (virions) derived from the infectious RNA 1 and RNA 2 cloes of ToNDV-K15 were transmitted by the whitefly species Trialeurodes vaporiarorum and T. abutilonea. The next experiments would be to test transmission by Bemisia tabaci, as this is the whitefly species that poses an economic threat to California, including the Central Valley. Moreover, if processing tomato production expanded to the Imperial Valley or other desert regions, it is likely ToNDD will reemerge.

The infectious clones of ToNDV-K15 also allowed me to determine a partial host range of the virus, which is limited to Solanaceous species. It would be interesting to determine the role that weeds play in the persistence of ToNDV in the Imperial Valley as well as the titer of the virus in any weed hosts and efficiency of acquisition by the whiteflies. To determine how weed hosts contribute to the infection cycle especially when cucurbits are absent, surveys could be conducted to determine incidence of ToNDV-K15 infection in prevalent weed species. These results could be useful for management of ToNDV especially if only a few solanaceous weed hosts are identified.

The infectious clone also allowed me to screen tomato cultivars for resistance to infection by ToNDV. High disease incidence of infection in susceptible tomato cultivars and N. benthamiana plants were obtained with the ToNDV agroclone. It would be interesting to screen tomato cultuvars that are resistant to ToNDV with other tomato-infecting torradoviruses, such as ToTV, ToChSV and ToMarV to determine if the resistance is broad spectrum.

Chapter 3. Genetic and functional analyses of the genes and proteins of a new isolated of the torradovirus tomato necrotic dwarf virus (ToNDV) from Kern Co California, USA

In Chapter 3, I investigated the functional properties of proteins encoded by RNA 2 of ToNDV-K-15. I compared amino acid sequences of all the available torradovirus sequences of RNA 2-encoded proteins and determined highly conserved regions and functional motifs for mutagenesis, mostly using alanine replacement. The mutational analysis showed that, although there were very conserved amino acids in several regions of these proteins, they were not all essential for infection. I identified five mutants that were not infectious: (i) the knock-out of the ORF1, (ii) the LDF amino acids in ORF1, (iii) amino acid 210 (F) in the movement protein, (iv) a myristoilation domain in the largest CP, Vp35 and (v) a positively charged region in the smallest CP, Vp24. Moreover, all of these mutants were replication-competent, and impaired in cell-to-cell movement. This is only an initial genetic analysis of the sequences/proteins. Further work should involve mutating other conserved regions or, in case the regions are close to each other, mutating all of these in order to rule out redundancy of function. Also, complementation experiments could be performed to see if the infectious phenotype can be rescued with wild-type proteins of ToNDV-K15 or those of other viruses, e.g., luteoviruses.

I focused my functional studies on ORF1 and MP. I determined the subcellular localization of ORF1 and MP is mostly in the plasma membrane and they both localize to cell wall structures that are possibly plasmodesmata (PD), based on co-localization with the known PD marker, TMV-MP. Notably, ORF1 only transiently localized to PD ans was mobile via numerous vesicles that may transport viral RNA to PD. However, further studies are needed to purify these vesicles and examine the contents. It is also critical to determine if ORF1 and MP interact and, if so, to understand hoy they function to mediate cell-to-cell movement. Additionally, for the mutants that

do not move cell-to-cell, it would be interesting to tag these mutant proteins with GFP and observe their localization in comparison with wild-type proteins. Assays such as BiFC, yeast-two hybrid and Co-IP for membrane-bound proteins should be performed to address interaction between ORF1 and MP. Interactions of these viral proteins with host proteins is another area that is wide open for investigation. Co-immunoprecipitation experiments coupled with mass spectroscopy can show host proteins bound by the viral proteins to manipulate the plants.

I established that ORF1 protein binds ssRNA, consistent with playing a role in RNA transport. Additional binding studies should be performed, especially with MP. The binding of the Arm sequence of Vp24 with ssRNa should also be performed. Ultimately, important questions to be answered is the mechanism, i.e., via tubules or more novel 2-MP systems such as used by luteoviruses. Finally, it is also still unknown which of the torradovirus protein (s) serve as a silencing suppressor. Thus, long-distance movement and local silencing suppression experiments will need to be performed.

Chapter 4. Analysis of the complete genome of an isolate of *Squash vein yellowing virus* from California (SqVYV-CA) reveals a recombination event between genera that facilitated development of a specific RT-PCR test

In Chapter 4, I completed and analyzed the sequence of a new strain or highly divergent isolate of SqVYV, referred to as SqVYV-CA. I showed that SqVYV-CA is a member of the genus *Ipomovirus* and, is most closely related to the SqVYV-IL. However, SqVYV-CA has a recombinant event in the P1a gene, in which the 5' half is from the P1 gene of a yet to be identified potyvirus. In terms of protein function, the evidence for CVYV indicates P1b is also a suppressor of gene silencing, but not P1a (Valli et al., 2006). Thus, it would be of interest to test the SqVYV-CA hybrid P1/P1a and P1b for silencing suppressor and subcellular localization. Finally, the

function(s) of the ipomovirus P1a and potyvirus P1 proteins are not clear. Notably, the lack of an P1a gene/protein in the CBSV genome. This, it would be interesting to conduct mutagenesis and exchanges with other P1a, P1 of other sequences to gain insight into protein function. However, these mutagenesis experiments require a stable infectious clone of SqVYV-CA. Finally, my findings as well as other reports of recombination and genetic diversity in the P1a/P1 genes suggest these genes may be recombination hotspots in the family *Potyviridae*. In this regard, it would be interesting to determine if the SqVYV-CA P1a is under positive selection and is a disordered protein. I used the discovery of the recombinant SqVYV-CA P1/P1a gene to develop a primer pair for the specific diagnostic of the SqVYV-CA. The primers were validated for specificity and tested with field samples. Thus, it should also be possible to develop a SqVYV-CA specific RT-PCR test, to allow for high throughput testing and determination of viral titer in different cultivars and host species, e.g., weeds.

Finally, SqVYV-CA was first detected in the Imperial Valley in California in 2014 and it was detected in Fresno Co. in 2021 and 2022. It is likely that viruliferous whiteflies migrated from desert regions so it will be important to follow the whitefly populations and develop a test for detection of SqVYV-CA in whiteflies. The whitefly monitoring coupled with the knowledge of the persistence in crop or weed hosts will be useful in managing outbreaks of SqVYV-CA in the future.

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Valli, A., Martín-Hernández, A. M., López-Moya, J. J., & García, J. A. (2006). RNA Silencing

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