IDENTIFICATION, TRANSMISSION, AND GENOMIC CHARACTERIZATION OF FOUR NEW VIRUSES OF CULTIVATED ROSES

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Abstract

Between the period 2005 and 2008 four previously undescribed viruses infecting cultivated roses were identified and fully characterized in Minnesota. These four viruses were transmitted by grafting from infected to healthy roses and found to be the likely causal agents of the diseases that they were associated with. Viruses were provisionally named after the characteristic symptoms in infected plants as follows: Rose yellow vein virus (RYVV), Rose yellow mosaic virus (RoYMV), Rosa rugosa leaf distortion virus (RrLDV), and Rose yellow leaf virus (RoYLV). Based on virion and genome properties it was determined that RYVV is a member of the family *Caulimoviridae*, RoYMV is a member of the family *Potyviridae*, and RrLDV and RoYLV are members of the family *Tombusviridae*. Phylogenetic analyses suggest that these four viruses belong to distinct new genera in their respective taxonomic families. The whole genomic sequence of each virus was deposited in GenBank under the accession numbers: RYVV JX028536; RoYMV NC_019031; RrLDV KC166238; and RoYLV, KC166239. Reliable diagnostic protocols were developed for each virus by PCR for RYVV detection, RT-PCR, immunosorbent electron microscopy (ISEM), and indirect enzyme-linked immunosorbent assay (ELISA) for RoYMV detection, and RT-PCR for both RrLDV and RoYMV detection.

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Introduction

Rose (*Rosa* spp.) is arguably one of the most important ornamental crops used in landscapes and in the cut flower industry worldwide. A number of viral diseases affect rose production and may reduce plant vigor, cut flower quality, or landscape aesthetic value.

Until recently viruses reported to infect roses were from two genera: *Ilarvirus* (*Prunus necrotic ringspot virus, Apple mosaic virus, and Tobacco streak virus*) and *Nepovirus* (*Arabis mosaic virus, Strawberry latent ringspot virus, Tobacco ringspot virus,* and *Tomato ringspot virus*) (Horst and Cloyd, 2007). Additionally *Blackberry chlorotic ringspot virus*, also an *Ilarvirus* has been reported from rose (Tzanetakis et al., 2006). Recently the tospoviruses, *Impatiens necrotic spot virus* (Shahraeen, Ghotbi et al. 2002) and *Tomato spotted wilt virus* (Ghotbi et al., 2005) have been reported in roses in Iran. A luteovirus, *Rose spring dwarf-associated virus*, has been reported in the USA (Salem et al., 2007) and South America (Rivera and Engel, 2010) and the *Alphacryptovirus*, *Rose cryptic virus-1* (Martin and Tzanetakis, 2008; Sabanadzovic and Sabanadzovic, 2008) (synonymies, Rosa multiflora cryptic virus (Salem et al., 2008) and Rose transient mosaic virus (Lockhart et al., 2011)) has been described in different regions of the USA. In 2011 the long speculated causal agent of rose rosette disease, an *Emaravirus*, was characterized (Laney et al., 2011).

The scope of this research was to characterize four of six newly described viruses that affect roses (Lockhart et al., 2011). One of these new viruses belongs to the family

Caulimoviridae (Mollov et al., 2012b), one belongs to *Potyviridae*, and two belong to *Tombusviridae*.

Unlike the ilarviruses and the nepoviruses that only show symptoms and aree detected early in the growing season, these new viruses exhibit symptoms throughout the season and can be detected readily during the entire year. Currently, roses for commerce between states and international trade, as well as these used for commercial propagation require virus indexing to ensure virus free material. Unfortunately virus-indexing standards only screen for pathogens that are known using available diagnostic techniques. In this research four novel virus pathogens were described and reliable detection methods were developed that can be utilized in the industry to screen rose germplasm and implement better control measures in the trade and production industries.

The objectives of this research were to: 1) identify these four viruses; 2) determine their transmission and establish the etiology of the diseases they cause; 3) develop diagnostic protocols for detection; 4) provide genomic characterization; and 5) establish their taxonomic relationship to known viruses of their respective taxa.

Chapter I

Complete nucleotide sequence of Rose yellow vein virus, a member of the family *Caulimoviridae* having a novel genome organization.

This report describes the complete nucleotide sequence and genome organization of Rose yellow vein virus (RYVV), a proposed new member of the family *Caulimoviridae.* The RYVV genome is 9314 bp in size, and contains eight open reading frames (ORFs). ORFs 1, 2, 3 have 22-38% amino acid sequence similarity to known members of the family *Caulimoviridae*. The remaining ORFs have no significant amino acid sequence similarity to known viruses. Based on differences in genome organization, low sequence similarity to known members of the family *Caulimoviridae* and phylogenetic analysis. RYVV appears to be a distinct new member of this family.

Introduction

Rose yellow vein virus (RYVV) is a caulimo-like virus causing one of a series of newly-described foliar disease of cultivated roses in the USA (Lockhart et al., 2011). Virions of RYVV are icosahedral, 42-45 nm in diameter and contain a circular noncovalently closed ds DNA genome, characteristics shared with members of the family *Caulimoviridae*. The virus, and the disease, were transmissible by grafting, but not by aphid or mechanical transmission, and only to rose (*Rosa* spp.) (Lockhart et al., 2011). This report is part of a larger study on the complete characterization of RYVV, and covers the aspects of

genome properties and organization and possible relationships to other members of the family *Caulimoviridae*.

Virus source, virion purification, DNA extraction

Virus used for genomic DNA characterization in this study was obtained from naturally-infected plants of the rose cv. Dr. Merkeley in Minnesota, USA. Source plants were propagated by rooted cuttings and maintained in an insect-proof greenhouse. Virions were purified from symptomatic infected leaf tissue, and genomic DNA was extracted and characterized as described previously (Lockhart, 1990; Lockhart et al., 2000).

Genome cloning

Genomic DNA extracted from purified virions was digested with *XbaI*, ligated into *XbaI*-digested pBluescript KS(+) (Stratagene) and cloned in *E. coli*. A pair of outward primers, RYVV-NL (5'-TGGCTGATGCCCTTACCAGA) and RYVV-NR (5'-CGATTTCACAACCGAGGAAGTTG) were designed from the sequence of a 3.1 kb cloned genome segment that had most significant nucleotide and amino acid sequence similarity to the RT-RNase H region of known caulimoviruses (Table 1). The outward primers were used with LA Taq DNA polymerase (Takara Bio. Inc.) and virion DNA to generate an ~8 kb amplicon corresponding to the genomic sequence between the primers. The ~8 kb fragment was cloned using a pGem-Teasy cloning kit (Promega). Four clones were selected and sequenced by primer walking. The gap between the outward primers was amplified from virion DNA using a pair of primers, RT1c (5' - TGGGCTTTCAGGGAATTTTGG) and RT2c (5'- AACCCAGCTCGGGAAACTCC)

and Phusion High Fidelity polymerase (New England Biolabs). The resulting 1835 bp amplicon was cloned using a TOPO Zero Blunt cloning kit (Invitrogen), and six clones sequenced. Sequences of RYVV genomic fragments generated by outward and gap primers were assembled in Sequencher (Sequencher® version 4.10.1 sequence analysis software, Gene Codes Corporation, Ann Arbor, MI USA). Significant amino acid sequence similarities to known viral sequences were determined using Blastx (Altschul et al., 1997). The complete RYVV genome sequence was deposited in the GenBank database under the accession number JX028536.

Sequence analysis and genome organization

The complete sequence of RYVV is 9314 bp in length, including a 5' untranslated region (UTR) of 1027 bp. Sequence analysis using the NCBI ORF Finder predicted the presence of 8 ORFs in the RYVV genomic sequence. The genome positions of these ORFs, the predicted sizes of the proteins they encode and their assumed functions, and most significant amino acid (aa) sequence similarities to known viruses are listed in Table 1. The ORF arrangement of RYVV in comparison to those of the type members of the genera *Caulimovirus, Soymovirus, Cavemovirus* and *Petuvirus* are represented diagrammatically in Fig. 1. Rose yellow vein virus has the basic genome composition (monopartite, circular noncovalently closed ds DNA) typical of known members of the family *Caulimoviridae*, and the virion structure (icosahedral, 42-45 nm in diameter) characteristic of the genera *Caulimovirus*, *Soymovirus*, *Cavemovirus* and Petuvirus (Hull et al., 2004). Consistent with RYVV being a member of the family *Caulimoviridae*, the largest ORF (ORF 3) encodes a putative polyprotein consisting of aspartic protease (AP),

RT and RNase H domains. The three features that distinguish RYVV from known members of the latter three genera are genome size, sequence similarity and genome arrangement. The RYVV genome, 9314 bp in size is the largest of known members of the family *Caulimoviridae*, slightly exceeding that of *Cycad leaf necrosis virus* (CLNV) (NC_011097.1, 9205 bp). As illustrated in Table 1, only three of the eight RYVV ORFs (ORFs 1, 2, 3) have significant amino acid sequence identity to corresponding genomic regions of known members of the family *Caulimoviridae*, and the levels of sequence identity (29-33%) are well below those currently designated for defining species demarcation in this family (Hull et al., 2004). Further evidence of the low level of genomic sequence similarity between RYVV and known members of the family *Caulimoviridae* was provided by the observation that while a standard Blastx search identified the relationship between the RYVV RT and RNase domains of ORF 3 and those of members of the family *Caulimoviridae*, no similar sequence recognition was found for the AP domain. However, changing the search parameters by using the Blastp Domain Enhanced Lookup Time Accelerated (DELTA) Blast with the PAM30 matrix and 5,000 hits, revealed levels of amino acid sequence similarity of 29, 27, and 18% between the first 300 amino acids of the RYVV ORF 3 sequence and the aspartic protease domains of *Figwort mosaic virus, Casava vein mosaic virus* and *Petunia vein-clearing virus*, respectively. On the basis of significant sequence similarity it was concluded that RYVV is unrelated taxonomically to any known species in the *Caulimoviridae*. There are also significant differences in genome organization between RYVV and other *Caulimoviridae*. As illustrated in Fig. 1, RYVV is unique in having overlapping ORFs 1, 2 and 3, and therefore, unlike *Caulimovirus* and *Soymovirus*, no

ORFs occurring between the MP and CP. The data summarized in Table 1 and Fig. 1 support the conclusions that RYVV is a previously undescribed species in the family *Caulimoviridae*, and has a genome arrangement not similar to that of any known genus in this family.

Phylogenetic analysis

The phylogenetic relationship of RYVV to representative species in the six extant genera (*Badnavirus, Caulimovirus, Cavemovirus, Petuvirus, Soymovirus* and *Tungrovirus*) in the family *Caulimoviridae* (Hull et al., 2004) was deduced using the amino acid sequences of the conserved region of the RT domain. The representative virus species and the accession numbers from which the RT sequences were derived are listed in Fig. 2. Alignment of amino acid sequences was done using MegAlign (DNAStar Software) and Clustal W (Larkin et al., 2007). The dendrogram was generated by the neighbor-joining method using PAUP version 4.0 (Swofford, 2003). The data presented in Fig. 2 illustrate that RYVV does not align with any of the six known genera in the family *Caulimoviridae*, and supports the conclusion, mentioned above, that RYVV is a distinct new member of this family.

Table 1. Rose yellow vein virus (RYVV) ORF genome position, predicted function, frame, protein size and significant amino acid (aa) sequence identity.

ORF	Function	Frame	Genome Position (nt)	Protein size		% aa sequence identity, virus and GenBank accession number
				aa	kDa	
1	Movement Protein	1	2689-3615	308	35	33%, ORF I *Soybean chlorotic mottle virus* NP_044299
2	Coat Protein	2	3602-5098	498	57	29%, ORF I *Cassava vein mosaic virus* AAA79871
3	Replicase	1	5095-7554	819	95	37%, ORF V *Strawberry vein banding virus* NP_043933
4	Unknown	3	7644-9314	556	63	No significant similarity
5	Unknown	2	1028-1288	86	10	No significant similarity
6	Unknown	1	1288-1554	88	10	No significant similarity
7	Unknown	1	1555-2088	177	21	No significant similarity
8	Unknown	2	2192-2692	166	19	No significant similarity

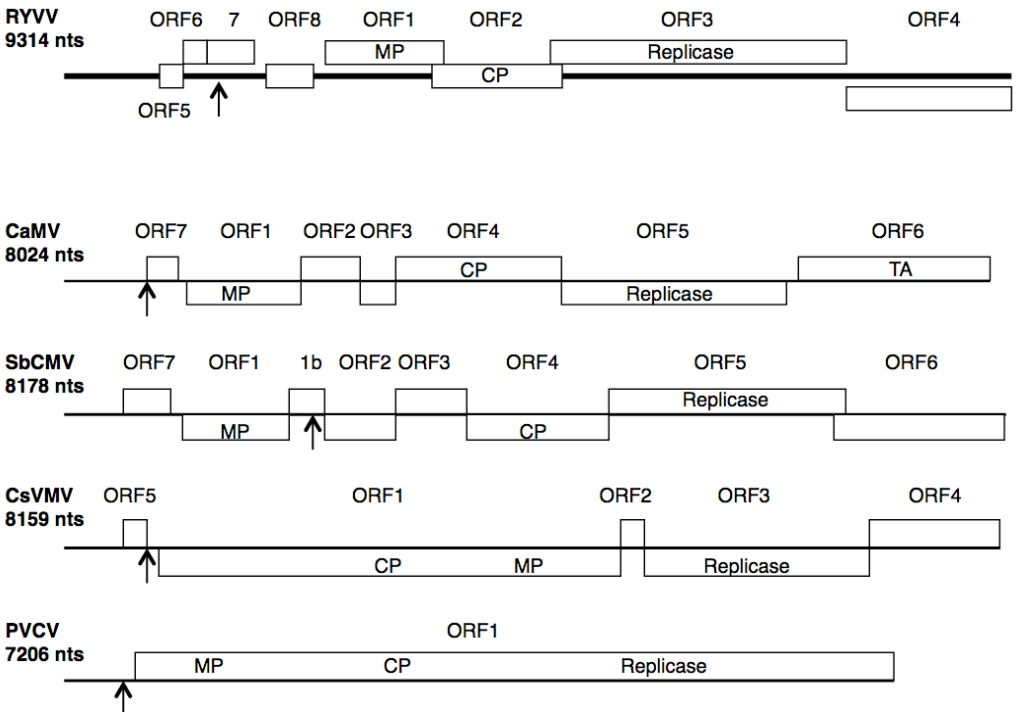


Figure 1. Diagrammatic representation of the ORF arrangement of Rose yellow vein virus (RYVV) relative to those of type members of the genera *Caulimovirus* (*Cauliflower mosaic virus*, CaMV), *Soymovirus* (*Soybean chlorotic mottle virus*, SbCMV), *Cavemovirus* (*Cassava vein mosaic virus*, CsVMV), *Petuvirus* (*Petunia vein clearing virus*, PVCV). MP = Movement protein, CP = Coat protein, arrow indicates origin of DNA replication.

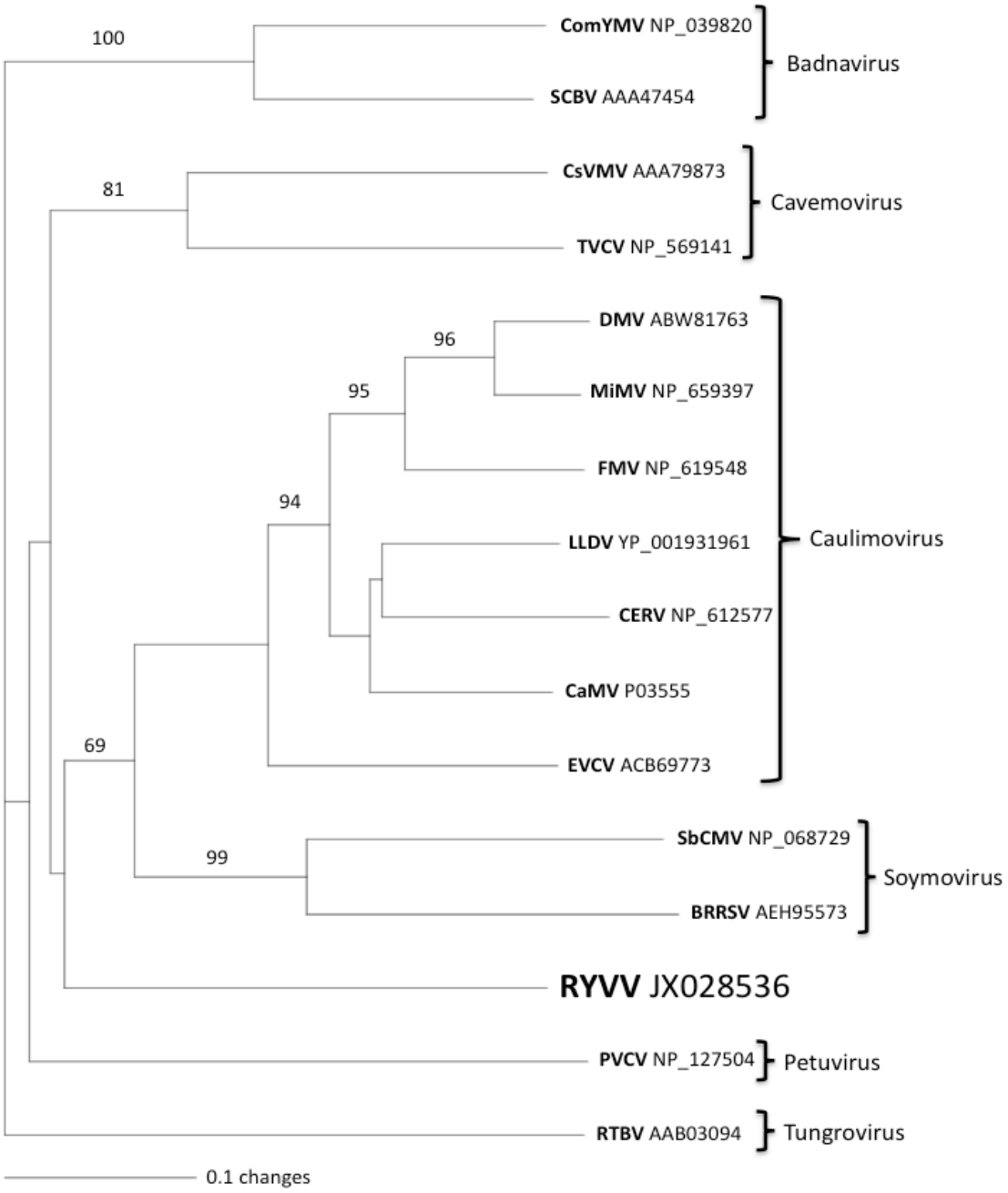


Figure 2. Phylogenetic relationship of Rose yellow vein virus (RYVV) to the following species in the six current genera in the family *Caulimoviridae*. *Blueberry red ringspot virus* (BRRSV), *Cauliflower mosaic virus* (CaMV), *Carnation etched ring virus* (CERV), *Commelina yellow mottle virus* (ComYMV), *Cassava vein mosaic virus* (CsVMV),

Dahlia mosaic virus (DMV), *Eupatorium vein clearing virus* (EVCV), *Figwort mosaic virus* (FMV), *Lamium leaf distortion associated virus* (LLDV), *Mirabilis mosaic virus* (MiMV), *Petunia vein clearing virus* (PVCV), *Rice tungro bacilliform virus* (RTBV), *Soybean chlorotic mottle virus* (SbCMV), *Sugarcane bacilliform virus* (SCBV) and *Tobacco vein clearing virus* (TVCV). The dendrogram was generated using the neighbor-joining method and was based on the amino acid sequences of the conserved reverse transcriptase (RT) region. Numbers represent the percentages (≥50%) of replicate trees in which the associated cluster was joined together in the bootstrap test (1000 replicates).

Chapter II

Identification, transmission and detection of Rose yellow vein virus, a previously undescribed member of the family *Caulimoviridae*

A previously undescribed virus with spherical 48-50 nm particles containing a circular ds DNA genome was associated with vein-yellowing and chlorotic spot symptoms in rose plants from cultivar Dr. Merkeley in Minnesota and in the cultivars Madame Pierre Oger, Mozart, Prosperity, and Schneezwerg in New York. The virus also occurred in *Rosa rugosa rubra* in Minnesota showing leaf yellowing and distortion. This virus was provisionally named Rose yellow vein virus (RYVV). It was transmitted by grafting to healthy plants of the cv. George Vancouver in which characteristic vein-yellowing symptoms developed and the presence of RYVV was confirmed, but was not transmitted by the aphid *Macrosiphum euphorbiae*. Based on virion and genome properties and organization RYVV falls within the family *Caulimoviridae* but is distinct from known members of this family. One recent report indicates that RYVV occurs outside of New York and Minnesota. A PCR method was developed for reliable detection of RYVV in roses and this method can be used to screen propagation stock for its presence.

Introduction

Previously unreported virus-like symptoms were observed in roses in New York and Minnesota during 2005-2010. A variety of symptoms including vein-yellowing and chlorotic spotting were observed in cvs. Madame Pierre Oger, Mozart, Prosperity, and

Schneezwerg in the New York Botanical Garden, Bronx, NY and in the cv. Dr. Merkeley in St. Paul, Minnesota. Symptoms of stunting, leaf distortion and yellow sectoring were observed in *Rosa rugosa rubra* in Minnesota. These symptoms differed from those associated with infection by known viruses of rose (*Rose mosaic virus* and *Prunus necrotic ringspot virus*) (Horst and Cloyd, 2007) and also varied markedly in persisting in new growth throughout the summer. Spherical virus-like particles 48-50 nm in diameter, resembling those of caulimoviruses, were detected by transmission electron microscopy in negatively stained partially purified leaf tissue extracts from symptomatic plants. The presence of these particles was consistently associated with disease symptoms. No other virus-like particles were detected in these extracts and none were detected in asymptomatic plants. In preliminary studies the particles were shown to contain circular ds DNA about 9 kb in size. The objectives of this study were to identify and characterize the virus associated with this new disease of roses, to determine its role and etiology of the disease, and to develop protocols for reliable RYVV detection in rose germplasm.

Virus source and propagation

The virus isolate used in this study was obtained from the rose cv. Dr. Merkeley growing in St. Paul, MN. This plant had foliar symptoms (Fig. 1A) and caulimovirus-like particles (Fig. 2) were readily detected by transmission electron microscopy (TEM) in negatively-stained partially purified leaf tissue extracts as described below. No other virus-like particles were observed in such preparations at any time during the year. This virus isolate was maintained by vegetative propagation from the source plant. Infected plants were grown in an insect-proof greenhouse at 18-24 ^{O}C.

Virus and disease transmission tests

Graft transmission tests were done by whip and tongue grafting of scions from infected cv. Dr. Merkeley plants on two healthy cv. George Vancouver plants showing no foliar symptoms and containing no caulimo-like virions detectable by TEM using partially purified leaf tissue extracts as described above. Graft-inoculated test plants were kept in the greenhouse at 18-24 ^{O}C and were pruned to stimulate axillary shoot development and observed for foliar symptom development. Inoculated plants were tested subsequently for presence of virus by TEM and PCR using primer pair RYVV-CPF and RYVV-CPR designed from RYVV genomic sequences as described below. Total DNA extracted from infected leaf tissue using a Qiagen DNeasy kit was used as template for PCR. Aphid transmission was done using *Macrosiphum euphorbiae* apterae raised on roses. Aphids were allowed to feed on infected cv. Dr. Merkeley for 24 hours and transferred in groups of 25 to each of four healthy cv. RADrazz (KnockOut®) rose test plants. After an inoculation feeding period of 48 hours aphids were killed by insecticide application. Inoculated plants were kept for 12 months, observed for symptom development, and tested for the presence of virus by TEM.

Virus detection

Detection of RYVV in rose was done by TEM and by PCR. For TEM detection partially purified leaf tissue extracts were prepared by extraction in 500 mM sodium potassium phosphate, pH 7.5, containing 1 M urea, 5% (w/v) polyvinylpyrrolidone (average molecular weight 40,000) and 0.5% (v/v) 2- mercaptoethanol. The initial extract was

filtered, centrifuged at 19,800 g_{max} for 15 minutes and the pellet discarded. After addition of Triton X-100 to final (v/v) of 5% the supernatant was centrifuged at 109,000 g_{max} for 70 minutes at 10 ^{O}C. The resulting pellets were resuspended in 10 mM sodium phosphate, pH 7.0, emulsified with an equal volume of chloroform and centrifuged at 19,800 g_{max} for 10 minutes. The upper aqueous phase was used for TEM examination following negative staining with 2% sodium phosphotungstate pH 7.0 (PTA) or aqueous uranyl acetate (UA).

PCR was done using two pairs of primers designed from the complete RYVV genomic sequence. The primer pairs were based on the sequences of the putative movement protein (MP) and coat protein (CP) regions, respectively, of the viral genome (Mollov et al., 2012b). The movement protein primers were RYVV-MPF, 5'-CAGGAAGGTCAAGCCTGGTGA, RYVV-MPR, 5'-TCCAATGGAATGGGCTTTGG and the coat protein primers were RYVV-CPF, 5'-GCCCAATCTCGGAATCTGACC, RYVV-CPR, 5'-CATTAAACGGCGGTGGCAGT. Using these primers and total DNA extracted with a Qiagen DNeasy ® kit, amplicons of 683 bp and 796 bp, respectively (Fig. 3) were generated using the following protocol: 95 ^{O}C for 2 min (1 cycle), 94 ^{O}C for 30 sec, 55 ^{O}C for 30 sec, 72 ^{O}C for 45 sec (35 cycles), 72 ^{O}C for 10 min (1 cycle). PCR products were cloned using the Topo TA cloning kit (Invitrogen) and sequenced.

Virus and disease incidence and transmission

The two healthy plants of the rose cv. George Vancouver that were graft-inoculated with RYVV-infected scions of cv. Dr. Merkeley developed systemic vein-yellowing symptoms (Fig. 1B) after 6-7 months.

Using the RYVV-MPF and RYVV-MPR primers it was verified that RYVV was transmitted by grafting from infected to healthy roses, and that this infection resulted in disease symptoms, thus establishing the role of RYVV in the etiology of the disease. This diagnostic PCR test also showed that the caulimovirus-like particles associated with disease symptoms in *R. rugosa rubra* (Fig. 1C) were those of RYVV. Although disease symptoms associated with infection by a caulimo-like virus were first observed in the cvs. Madame Pierre Oger, Mozart, Prosperity, and Schneezwerg in New York, these plants were destroyed before the diagnostic PCR primers were available. It can therefore be assumed, but not confirmed that RYVV was the causal agent of the disease observed in these cultivars. Recently, RYVV was detected in symptomatic roses in New Zealand by PCR using the RYVV MP and CP primers (Milleza et al.). This indicates that the virus and disease associated with its infection may occur more widely than is currently documented.

Summary and conclusions

The new virus described in this report and named Rose yellow vein virus (RYVV) was demonstrated by graft transmission to be the likely causal agent of a previously undescribed disease occurring naturally in *Rosa* spp. On the basis of virion and genome properties, and genome organization and amino acid sequence similarities, RYVV clearly falls into the family *Caulimoviridae*. However, RYVV has limited amino acid sequence identity to known members of this family, and differs in genome organization from currently recognized genera (*Caulimovirus, Petuvirus, Soymovirus,* and *Cavemovirus*) (King et al., 2012) in the family. From these observations it is concluded that RYVV is a

new member of the family *Caulimoviridae*, that it is unrelated to known members of this family, and appears to represent a new genus within the *Caulimoviridae* (Mollov et al., 2012b). The occurrence of RYVV in Minnesota and recently in New Zealand has been verified by PCR indexing using RYVV-specific primers (Milleza et al.; Perez-Egusquiza et al.). It appears likely that caulimovirus-like virions associated with disease symptoms in New York were those of RYVV, although this remains to be substantiated. If evidence of a wider distribution of RYVV were to be discovered, it would depend on several factors. The first would be the presence of virus-like foliar symptoms in roses testing negative for presence of the most commonly occurring viruses such as RMV and PNRSV. The second would be the persistence of foliar symptoms in new growth throughout the year. The third would be testing for presence of RYVV using the PCR primers and protocol described above, as was done recently in New Zealand (Milleza et al.; Perez-Egusquiza et al.). With knowledge of these observations and procedures it would be possible to detect RYVV in rose propagation stock and eliminate infected stock plants from the production chain.

A B C

Figure 1. Foliar symptoms observed in roses infected with Rose yellow vein virus (RYVV). A) Vein-yellowing and chlorosis naturally occurring in cv. Dr. Merkeley; B) Vein-yellowing after graft inoculation of cv. George Vancouver; C) Leaf distortion naturally occurring in *Rosa rugosa rubra* (two infected leaves, left and one healthy leaf, right).

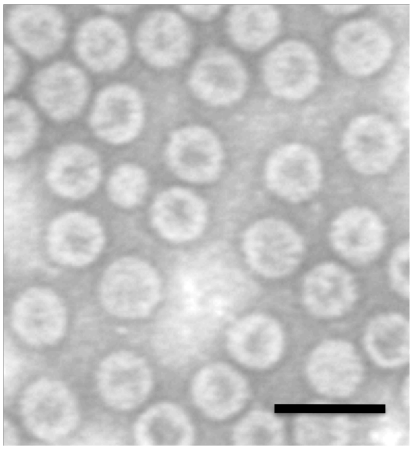


Figure 2. Virions of Rose yellow vein virus (RYVV). Purified preparation negatively stained with uranyl acetate. Scale bar equals 100 nm.

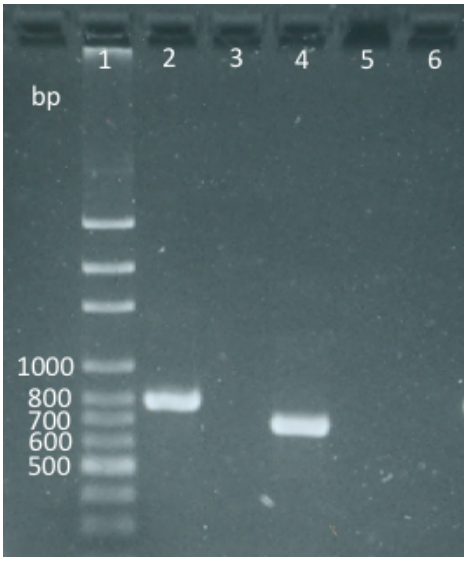


Figure 3. Detection of Rose yellow vein virus (RYVV) by PCR using RYVV-MPF+ RYVV-MPR and RYVV-CPF+ RYVV-CPR primer pairs. Lanes 1: 100 bp ladder, lane 2: MP positive, lane 3: MP negative, lane 4: CP positive, lane 5: CP negative, lane 6: Water template

Chapter III

Complete nucleotide sequence of Rose yellow mosaic virus, a novel member of the family *Potyviridae*.

The complete genomic sequence of Rose yellow mosaic virus (RoYMV) was determined and found to have all the characteristic features of the family *Potyviridae*. The RoYMV genome is 9508 nucleotides long excluding the 3'-poly-(A) tail and contains a single open reading frame encoding a polyprotein of 3067 amino acids. The RoYMV P3 and CI cistrons are shorter than those of other members of the family *Potyviridae*, and the 6K1 cistron is completely absent. Comparative sequence analyses revealed that RoYMV had highest amino acid homology across the entire genome sequence to *Brome streak mosaic virus* (33%) and to *Turnip mosaic virus* (30%) at the coat protein level. Based on low sequence similarity to known members of the family *Potyviridae* and phylogenetic analyses, RoYMV appears to be a distinct new member of this family.

Introduction

Rose (*Rosa hybrida* L.) is arguably the most widely grown ornamental plant worldwide. Until 2007 (Horst and Cloyd, 2007) the only known viruses reported to infect rose were the ilarviruses *Prunus necrotic ringspot virus, Apple mosaic virus, and Tobacco streak virus* and the nepoviruses *Arabis mosaic virus, Strawberry latent ringspot virus, Tobacco ringspot virus,* and *Tomato ringspot virus*. Recently a luteovirus (Rivera and Engel, 2010; Salem et al., 2007); a *Partitiviridae* cryptic virus, Rose cryptic virus-1, (Martin and

Tzanetakis, 2008; Sabanadzovic and Sabanadzovic, 2008; Salem et al., 2008); and an *Emaravirus* (Laney et al., 2011) have been reported to occur in roses. We have described previously a virus with filamentous 720-750 nm particles associated with a disease of roses characterized by yellow mosaic, premature leaf senescence and necrotic stem lesions (Lockhart et al., 2011). The objectives of this study were to characterize the RoYMV genome and to determine its taxonomic status and phylogenetic relationships.

Materials and methods

Virus source and virion purification and characterization

The RoYMV isolate used in this study (Minnesota-1) was obtained from naturally infected plants of the rose cv. Ballerina. Source plants were kept in an insect proof greenhouse under standard growing conditions (18 to 24 ^{O}C). Virions were purified from 200 grams of symptomatic leaf tissue. The tissue was ground in liquid nitrogen and suspended in 4 volumes (w/v) of 500 mM sodium phosphate buffer, pH 7.5 containing 1 M urea, 5% (w/v) polyvinylpyrrolidone (average molecular weight 40,000) and 0.5% (v/v) 2-mercaptoethanol. The initial filtered extract was centrifuged at 19,800 g_{max} for 15 minutes. After addition of 5% (v/v) Triton X-100 the supernatant was centrifuged at 109,000 g_{max} for 2 hours at 10 ^{O}C. The pellets were resuspended in 10 mM sodium phosphate buffer, (pH 7.0) and shaken with an equal volume of chloroform. The resulting emulsion was then centrifuged at 13,800 g_{max} for 10 minutes and the upper aqueous phase was layered over 5 ml of 30% (w/v) sucrose in dH_2O followed by centrifugation at 109,000 g_{max} for 2 hours at 10 ^{O}C. The final pellets were resuspended in 400 µl 1X Turbo DNase reaction buffer (Ambion) and incubated with 50 U Turbo DNase at 37 ^{O}C for 30

minutes. Five µl of RNase (50 µg/ml) was then added and the reaction incubated at room temperature for 5 minutes. Virion nucleic acid was extracted with SDS-phenol and chloroform and precipitated with an equal volume of isopropanol. Precipitated nucleic acid was collected by centrifugation, rinsed with 70% ethanol and dried under vacuum. The purified suspension from which genomic RNA was extracted was used to estimate *Mr* of virion coat protein. The molecular mass of virion capsid subunits was estimated by discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) using a Benchmark™ 10-220 kDa protein ladder (Invitrogen) as size markers and TMV as a reference capsid protein with a known *Mr* of 18 kDa.

Genomic cloning, sequencing and sequence analysis

A genomic library was prepared from RoYMV virion RNA using the method described by Froussard (Froussard, 1992). The resulting genomic segments were cloned using a pGem T-easy kit (Promega, Madison WI). Clones were screened by PCR using universal primers and 24 clones were selected. Plasmid DNA was isolated from overnight cultures using a Zippy® Plasmid DNA extraction kit (Zymo Research). The cDNA clones were sequenced in both directions. The sequence was assembled using Sequencher 4.10.1 (Gene Codes Corporation, Ann Arbor, MI). Nine pairs of primers (Table 1) were designed from the contig to generate overlapping amplicons covering the entire sequence. These amplicons were generated from purified virion RNA template by RT-PCR using Ready-to-Go™ RT-PCR Beads (GE Healthcare). The amplicons from each reaction were then cloned and two to four clones per ligation were selected and sequenced as described. The 5' terminal sequence of the genome was obtained using a RoYMV specific primer

RoYMV5, (5'-TGACCCATTCCTTGCGGTCT-3') and the RACE second-generation kit (Roche Applied Science). The resulting 800 bp amplicon was ligated into pGem T-easy and four clones were selected and sequenced in both directions to obtain and assemble the 5' terminal sequence. The 3' sequence of the genome was obtained by RT-PCR as described above, using a RoYMV specific primer RoYMV3 (5'-ATGTGGCGGCACAAATTTCA-3') and a $6NT_{18}$ primer. An amplicon of approximately 1,280 bp was cloned and sequenced as described above.

The complete sequence of the RoYMV was assembled using Sequncher 4.10.1 (Gene Codes Corporation, Ann Arbor, MI). Putative cleavage sites were determined by comparison to known conserved sequences of members of *Potyviridae* (Adams et al., 2005a).

The polyprotein sequences of the following 32 viruses were selected from NCBI BLASTP (Altschul et al., 1997) results and used for multiple sequence alignment (Combet et al., 2000) to confirm conserved regions and to determine putative cleavage sites: *Algerian watermelon mosaic virus* (AWMV, NC_010736), *Banana bract mosaic* (BBrMV, NC_009745), *Barley mild mosaic virus RNA 1*, (BaMMV, NC_003483), *Barley yellow mosaic virus RNA 1* (BaYMV, NC_002990), *Bean common mosaic virus* (BCMV, NC_003397), *Beet mosaic virus* (BtMV, NC_005304), *Brome streak mosaic virus* (BStMV, NC_003501), *Blackberry virus Y* (BVY, NC_008558), *Caladenia virus A* (CalVA, NC_018572), *Cassava brown streak virus* (CBSV, NC_012698), *Chinese yam necrotic mosaic virus* (CYNMV, NC_018455), *Cocksfoot streak virus* (CSV, NC_003742), *Cucumber vein yellowing virus* (CVYV, NC_006941), *Hordeum mosaic virus* (HoMV, NC_005904), *Johnsongrass mosaic virus* (JGMV, NC_003606), *Lupine*

mosaic virus (LuMV, NC_014898), *Oat necrotic mottle virus* (ONMV, NC_005136), *Peace lily mosaic virus* (PeLMV, ABI34613), *Peru tomato mosaic virus* (PTMV, NC_004573), *Plum pox virus* (PPV, NC_001445), *Potato virus Y* (PVY, NC_001616), *Ryegrass mosaic virus* (RgMV, NC_001814), *Squash vein yellowing virus* (SqVYV, NC_010521), *Sugarcane streak mosaic virus* (SCSMV, AET14222), *Sweet potato mild mottle virus* (SPMMV, NC_003797), *Tobacco etch virus* (TEV, NP_062908), *Tomato mild mottle virus* (TomMMoV, CCD57807), *Triticum mosaic virus* (TriMV, NC_012799), *Turnip mosaic virus* (TuMV, NC_002509), *Wheat eqlid mosaic virus* (WEqMV, NC_009805), *Wheat streak mosaic virus* (WSMV, NC_001886), *Wild potato mosaic virus* (WPMV, NC_004426).

Phylogenetic analysis

Phylogenetic analyses were performed using both whole genome and coat protein amino acid sequences using ClustalW (Thompson et al., 1994). A neighbor-joining algorithm with a boostrap value of 1000 replicates was carried out and phylogenetic trees were generated with the MEGA 4.1 software package (Tamura et al., 2007). Amino acid sequence accession numbers used for the whole genome phylogenetic analysis were the same as described above. Accession numbers used for the CP phylogenetic analysis were: *Algerian watermelon mosaic virus* (AWMV, YP_001936196), *Araujia mosaic virus* (AjMV, ABS76430), *Banana bract mosaic virus* (BBrMV, YP_001718531), *Barley mild mosaic virus* (BaMMV, NP_734300), *Barley yellow mosaic virus* (BaYMV, NP_734308), *Bean common mosaic virus* (BCMV, NP_734122), *Beet mosaic virus* (BtMV, NP_954628), *Brome streak mosaic virus* (BStMV, NP_734262), *Caladenia virus*

A (CalVA, YP_006666647), *Cassava brown streak virus* (CBSV, YP_002905061), *Chinese yam necrotic mosaic virus* (CYNMV, YP_006590102), *Cocksfoot streak virus* (CSV, NP_734398), *Cucumber vein yellowing virus* (CVYV, YP_308886), *Hordeum mosaic virus* (HoMV, YP_063395), *Johnsongrass mosaic virus* (JGMV, NP_734407), *Lupine mosaic virus* (LuMV, YP_004123940), *Malva vein clearing virus* (MVCV, ACJ72226), *Oat necrotic mottle virus* (ONMV, NP_940831), *Papaya ringspot virus* (PRSV, ABG33856), *Peace lily mosaic virus* (PeLMV, ABI34613), *Pepper veinal mottle virus* (PVMV, ACO55445), *Peru tomato mosaic virus* (PTMV, NP_787946), *Plum pox virus* (PPV, NP_734348), *Potato virus Y* (PVY, NP_734250), *Rhopalanthe virus Y* (RVY, AAF00518), *Ryegrass mosaic virus* (RgMV, NP_734328), *Squash vein yellowing virus* (SqVYV, YP_001789001), *Sugarcane streak mosaic virus* (SCSMV, YP_006423942), *Sweet potato mild mottle virus* (SPMMV, NP_734288), *Tobacco etch virus* (TEV, NP_734206), *Triticum mosaic virus* (TriMV, YP_002956096), *Tulip mosaic virus* (TulMV, CAA45178), *Turnip mosaic virus* (TuMV, BAC02834), *Wheat eqlid mosaic virus* (WEqMV, YP_001468096), *Verbena canadensis potyvirus* (VerCPV, AAX37310), *Wheat streak mosaic virus* (WSMV, NP_734274), *Wild potato mosaic virus* (WPMV, NP_741977), *Yam mild mosaic virus* (YMMV, AAQ12332).

Results

Flexuous filamentous rods, ranging from 720-750 nm in length were observed by transmission electron microscopy (TEM) in negatively stained purified RoYMV suspensions from which genomic RNA was recovered (Fig. 1A). No other virus-like particles were observed. SDS-PAGE analysis (Fig. 1B) revealed the presence of a single structural (capsid) protein approximately 35-36 kDa in size. The complete nucleotide sequence of the RoYMV genome was obtained from multiple overlapping cDNA clones obtained from virion RNA extracted from the infected rose cv. Ballerina. The sequence of the RoYMV genomic RNA is 9508 nt excluding the 3' poly-A end. The complete sequence was deposited GenBank (Accession number NC_019031). Molar percentages of nucleotides on the whole genome level are: G23.6; A30; C19.9; and U26.5. The 5' UTR is 185 nucleotides long and it is AT rich (65%).

The RoYMV genomic sequence consists of a single large ORF starting at the first AUG (186-188 nt) and ending at the ochre stop codon UAA (9387-9389 nt). This ORF is 9204 nt (3067 amino acids) in length and encodes a polyprotein of 350 kDa. The polyprotein is predicted to be cleaved (Table 2) and processed into nine mature peptides: P1, HC-Pro, P3, CI, 6K2, VPg, NIa-Pro, Nib, and CP (Fig. 2). The P1 protease is 424 aa long with a calculated *Mr* of 49 kDa. The protein identity to other *Potyviridae* orthologs is 25-31%. It contains the conserved C-terminal sequence H-10X-D-31X-VKPGWSG-21X-CVIQG-19X-MEFY/S that represent the active site of the serine protease and the P1/HC-Pro cleavage site (Adams et al., 2005a). The RoYMV HC-Pro is 465 aa long and has *Mr* of 53 kDa. Its identity to HC-Pro orthologs is 29-40% and contains a conserved aa sequence at the C-terminus: GYCY-30X-LGPWP—36X-HF-36X-YQVG/G. The RoYMV P3 (31

kDa) protein is 262 aa long and is 21-28% identical to corresponding orthologs. The 70 kDa CI peptide is 625 aa long and has 31-39% similarity with *Potyviridae* CI orthologs. BLASTP analysis of the CI protein has revealed conserved HrpA helicase motifs DEXDc and HELICc. The RoYMV polyprotein aa sequences GCGKS at 1226-1230 and DECH at 1311-1314 are present in the CI cistron. The underlined residues have been identified as RNA helicase motifs (Kadare and Haenni, 1997).

Downstream of the CI protein a 50 aa, 6 kDa mature peptide is predicted. This peptide has no aa sequence similarities with 6K2 *Potyviridae* orthologs. The 22 kDa, 196 aa VPg has 34% aa sequence similarity to the VPg of both *Potato virus Y* and *Lupine mosaic virus*. A nuclear localization signal (NLS) consisting of 34 aa, KRDKRGTMSFYHNHDSEFADDFGSAYDTRSKKNK, has been predicted by PredictProtein (www.predictprotein.org) in the NIa-VPg N-terminus at polyprotein residues 1840- 1873. The NIa-Pro proteinase has *Mr* of 27 kDa and is 241 aa with similarity to orthologs ranging from 27% to 34%. It has the conserved motif H-39X-DVAVF-64X-GSCGSLLVAVNDHHVVGIH-9X-W (Adams et al., 2005a) with underlined residues present in the RoYMV polyprotein between positions 2064 and 2201. The replicase NIb (57 kDa) is 495 aa long. The CP is 309 aa and is estimated to have *Mr* of 35 kDa, consistent with the SDS-PAGE analysis (Figure 2b). The 3' UTR is 119 nt long excluding the poly-A tail.

Phylogenetic analyses using whole polyprotein (Fig. 3A) and coat protein (Fig. 3B) from *Potyviridae* members represented by all eight genera in both tests failed to cluster RoYMV within any existing genera.

Discussion

This report describes the genomic characterization of RoYMV, a previously undescribed filamentous virus associated with a mosaic disease of cultivated roses. Based on virion morphology, genome structure and organization and amino acid sequence similarity, RoYMV is concluded to be a member of the family *Potyviridae*. The RoYMV amino acid sequence shows less than 35% similarity to any virus species in the family. The family *Potyviridae* is the largest plant virus family ((2011) containing 175 viruses described to date organized in eight genera: *Potyvirus*, *Bymovirus*, *Macluravirus*, *Ipomovirus*, *Tritimovirus*, *Rymovirus*, *Poacevirus*, and *Brambyvirus*. Most members of the family with the exception of *Bymovirus* have a monopartite genome consisting of ten mature proteins P1, HC-Pro, P3, 6K1, CI, 6K2, NIa-VPg, NIa-Pro, Nib, and CP (Adams et al., 2005a; Adams et al., 2005b).

The P1 protease of the RoYMV lacks the N-terminus of a typical P1 potyvirus. BLASTP comparisons of RoYMV P1 only aligns the C-terminus to the P1 proteins of *Cassava brown streak virus,* an *Ipomovirus* (28% identity), *Sugarcane streak mosaic virus*, a *Poacevirus* (27% identity*)*, *Brome streak mosaic virus*, a *Tritimovirus* (28% identity), and *Cucumber vein yellowing virus*, an *Ipomovirus* (31% identity).

Similarly, only about a third of the C-terminus of the RoYMV HC-Pro has homology to the HC-Pro of *Algerian watermelon mosaic virus* (41% identity), *Cocksfoot streak virus* (34% identity) and *Johnsongrass mosaic virus* (29% identity), all Potyviruses. Potyviruses are known to be aphid transmitted (Gibbs et al., 2008) and the transmission is mediated by the HC-Pro conserved motifs. None of the KITC, IGN, CCC, and PTK

motifs (Huet et al., 1994; Urcuqui-Inchima et al., 2001) are present in the RoYMV HC-Pro. Instead, a putative C-2x-C mite transmission motif (Xu et al., 2010) is found at 48-51 aa residues at the N-terminus of the protein and suggesting that the virus could be mite transmitted. The common rose viruses *Apple mosaic virus* (ApMV) and *Prunus necrotic ring spot virus* (PNRSV) usually express symptoms early in the season with the plants becoming asymptomatic later with no possibility of detecting the viruses. In contrast the symptoms caused by RoYMV (Lockhart et al., 2011) persist throughout the growing season and the virus can be detected season long as well. One possible explanation could be the suppression of posttranscriptional gene silencing (Vionnet et al., 1999) that is reportedly manifested by the HC-Pro peptide from members of the family *Potyviridae* (Brigneti et al., 1998; Takeshita et al., 2012).

The RoYMV P3 cistron is most similar to *Cassava brown streak virus,* an *Ipomovirus* (22% identity). Sequence analysis of the RoYMV did not predict the existence of the small 6K1 cistron. The 6K1 role is unclear and thought to be bound to P3 (Urcuqui-Inchima et al., 2001) and, therefore is not expressed as a separate protein in the RoYMV genome.

A BLASTP evaluation of the CI protein of the RoYMV revealed 90% or higher overlap with the CI peptides from members of the genus *Tritimovirus*. The 6K2 small peptide has not been identified by BLAST analysis to have homology to any viral protein. The role of the 6K2 is yet to be established (Urcuqui-Inchima et al., 2001) but is believed to be connected to intracellular localization. Downstream of the P1 residue Glu of the 6K2 C-terminus, at the N-terminus of the VPg a NLS motif is predicted. The predicted NLS

motif consists of about 25% positively charged Lys and Arg residues. Between 90 and 98% overlap was detected of the RoYMV VPg and the VPg proteins of selected members of the genus *Potyvirus*.

The amino acid sequence identity of the NIa-Pro was between 28-34% similar with 90-99% coverage to NIa-Pro peptides from members of the genera *Potyvirus*, *Tritimovirus*, and *Ipomovirus*.

The NIb replicase and the CP are the most conserved proteins. The RoYMV NIb BLAST results show an overlap of 90% or higher coverage and 38-50% identity to viruses represented in almost all eight genera of the family *Potyviridae*. The CP BLAST results suggest highest similarity to viruses of the genus *Potyvirus* (70-90% coverage and 28-32% identity).

As mentioned above, the RoYMV HC-Pro is missing aphid transmission motifs. Similarly, the DAG aphid transmission motif, found at the N terminus of the CP peptide (Lopez-Moya et al., 1999), is missing from the RoYMV CP. Both HC-Pro and CP mediate the aphid transmission in the genus *Potyvirus*. As discussed above the other peptides have similarities to viruses from almost all the genera within the *Potyviridae*, the HC-Pro and the CP amino acid homology is exclusively to *Potyvirus,* a genus known to be aphid transmitted (Gibbs et al., 2008). For phenotypic uniformity of rose cultivars, roses are typically clonally propagated (Krüssmann, 1981). It is possible that RoYMV originated with the ability to be aphid transmitted, and because of the vegetative nature of propagation of its host, it lost the ability to be transmitted by aphids. Despite the fact that the RoYMV CP BLASTP analyses suggest highest identity to Potyvirus, CP phylogenetic

analysis (Fig. 2 B) failed to group RoYMV within this or with any known genera in the family.

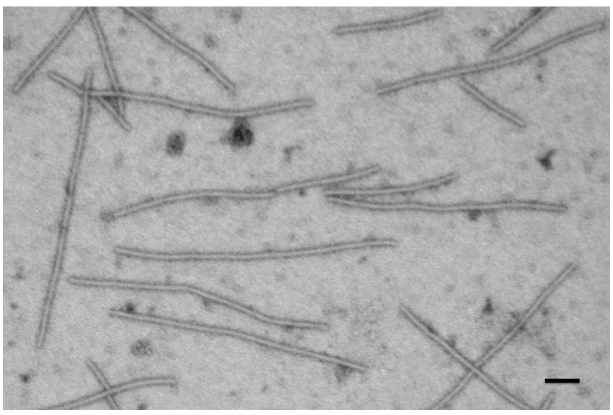
Based on these observations and the molecular criteria for species demarcation (Adams et al., 2005b; King et al., 2012) it is concluded that RoYMV is a new member of the family *Potyviridae*. RoYMV is unrelated to currently known members of the family, and appears to represent a new genus within *Potyviridae*. To our knowledge this is the first report of a virus from the family *Potyviridae* that infects rose.

Table 1. Overlapping primers used for Rose yellow virus genome sequence assembly and genome sequence conformation. Nucleotide positions correspond to the first nucleotide at the 5' position.

Primer	Sequence	Position
bw1f	CGAGCCAACAATGACTGGTGA	686
bw1r	GAGCACATGCGGCGTCATAG	1646
bw2f	GGAGTGCTTGTCCACCGTGA	1308
bw2rb	TCACGGTGGACAAGCACTCC	2963
bw3f	GCCTGCGTCAGATCCCTCAT	2622
bw3r	TGCCGACGACCAAAAACTCA	3836
bw4f	TGATCGCGCCAGAAACGATA	3357
bw4r	TTGCGCTATTCGGCTTAGGG	5107
bw5f	GCAAGCAAGTTGTTCCATCA	4260
bw5r	ATTGGCCCATCTTGCCTGAA	6162
bw6f	GTCAATCCGTTGGGGCAAAA	5817
bw6r	GAATGGGTCGTGCGGAAGTC	6698
bw7f	TAAACGGGTTCGGCTTTGGA	6334
bw7r	CGATCGCCCATTGATGTTCA	7094
bw8f	TAATGCGGCTGCCGAAAGAA	6928
bw8r	TTGCCGAAACGTTCGCTACA	7959
bw9f	CAAGGGTGGAACAGGCTTGC	7644
bw9r	TCCAGGTTCATACCCAGCGA	8491

Table 2. Location of putative cleavage sites of Rose yellow mosaic virus (RoYMV) polyprotein. Letters in bold represent conserved amino acid residues.

Cleavage site	Amino acid sequence (P6-P1/P'1-P'3)	Position of amino acid (P1/P'1)
P1/Hc-Pro	DI**M**EF**Y**/**S**GP	424/425
Hc-Pro/P3	AD**Y**Q**V**G/**G**LI	889/890
P3/CI	LTRGKG/SRS	1151/1152
CI/6K2	QC**V**LFE/GAI	1776/1777
6K2/VPg	HY**E**V**V**E/FEA	1826/1827
VPg/Pro	ES**V**DF**E**/QRG	2022/2023
Pro/NIb	FQMI**HQ**/**G**DK	2263/2264
NIb/CP	EAVYF**Q**/MAT	2758/2759



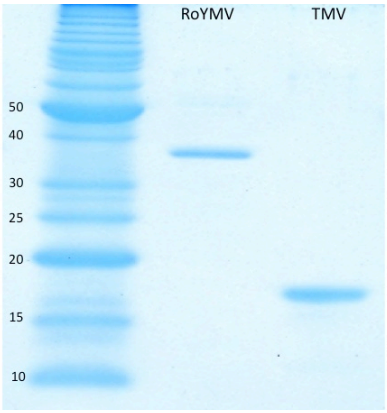


Fig. 1A Fig1B

Figure 1. Virions of Rose yellow mosaic virus (RoYMV) and RoYMV capsid protein size estimation.

Fig 1A. Virions of RoYMV in purified preparation negatively stained with 2% sodium phosphotungstate pH 7.0. Scale bar equals 100 nm. Fig 1B. Estimation of RoYMV capsid protein size by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Lane 1: Benchmark^{TM} Protein Ladder; Lane 2: RoYMV capsid protein; Lane 3: *Tobacco mosaic virus* capsid protein.

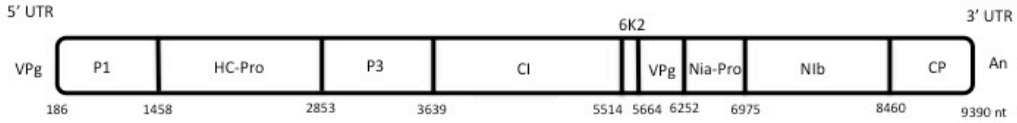


Figure 2. Schematic representation of the Rose yellow mosaic virus (RoYMV) genome. Numbers represent the first nucleotide of each mature peptide and the poly A tail.

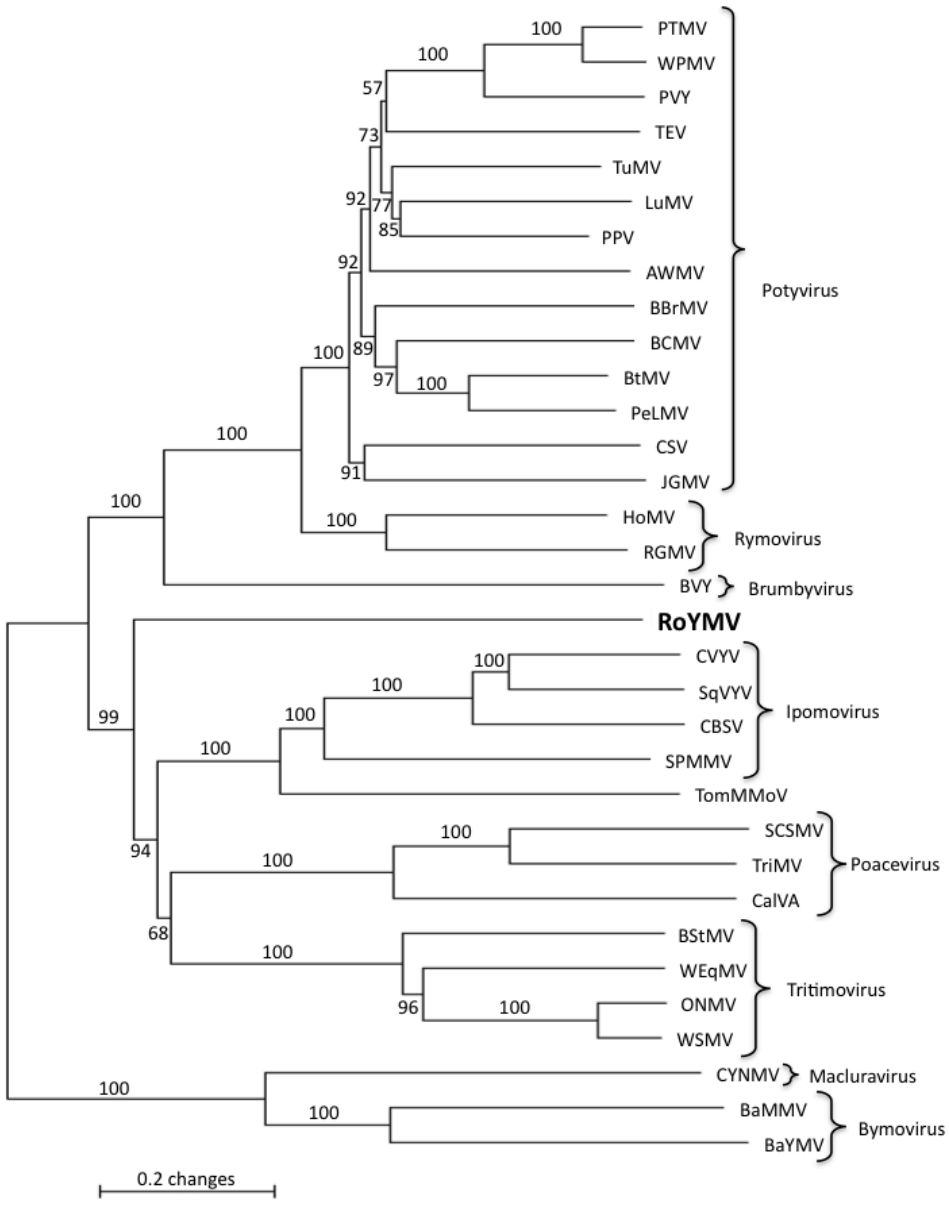

PTMV
WPMV
PVY
TEV
TuMV
LuMV
PPV
AWMV
BBrMV
BCMV
BtMV
PeLMV
CSV
JGMV
Potyvirus
HoMV
RGMV
Rymovirus
BVY
Brumbyvirus
RoYMV
CVYV
SqVYV
CBSV
SPMMV
Ipomovirus
TomMMoV
SCSMV
TriMV
CalVA
Poacevirus
BStMV
WEqMV
ONMV
WSMV
Tritimovirus
CYNMV
Macluravirus
BaMMV
BaYMV
Bymovirus
0.2 changes

Fig. 3A

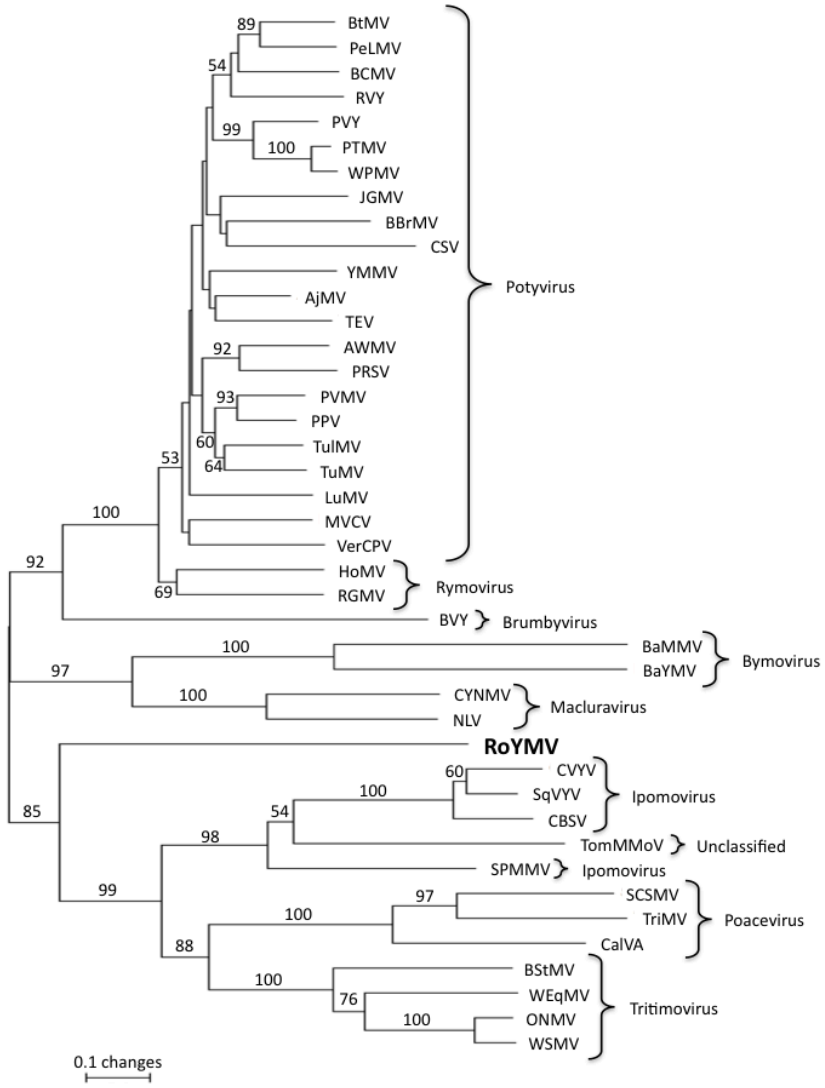


Fig. 3B

Figure 3. Unrooted phylogenetic trees of the (A) whole polyprotein amino acid sequences and (B) the coat protein of *Potyviridae* constructed by the neighbor joining method. Virus names and accession numbers are mentioned in text. The numerical values represent the bootstrap (1000 replicates) numbers above 50%. The scale bars correspond to substitutions per amino acid site.

Chapter IV

Identification, transmission and detection of Rose yellow mosaic virus, a previously undescribed member of the family *Potyviridae*

A previously undescribed virus with filamentous 720-750 nm particles was associated with mosaic symptoms and premature defoliation in the rose cvs. June Bride and Captain Harry Stebbings in Minnesota and in the rose cvs. Ballerina, Buff Beauty, Mozart, Cornelia, Nastarana, Dorothy Perkins, and Sir Thomas Lipton in New York. The virus was provisionally named Rose yellow mosaic virus (RoYMV). The virus was graft transmitted to healthy plants of cvs. June Bride, BAIpeace (Love and Peace^{TM}), George Vancouver, Tropicana, and 95-1 which developed foliar symptoms and in which the presence of the virus was confirmed. The virus was not transmitted by mechanical inoculation or by *Macrosiphum euphorbiae*. Based on virion morphology and genome properties, RoYMV appears to belong to the family *Potyviridae* but has substantial differences from known viruses of this family. Serological and RT-PCR assays were developed for reliable detection of RoYMV in roses.

Introduction

Previously unreported virus-like symptoms were observed in roses in New York and Minnesota. These symptoms include yellow mosaic and premature leaf senescence in cvs. June Bride, Captain Harry Stebbings, Buff Beauty, Mozart, Cornelia, Nastarana, Dorothy Perkins, and Sir Thomas Lipton. The rose cv. Ballerina developed severe

necrotic cane lesions in addition to characteristic foliar symptoms. The necrotic symptoms were similar to those described for rose streak disease (Secor et al., 1977) and appeared to be genotype specific. RoYMV was graft transmitted to 14 Ballerina x 95-1 (Zlesak et al., 2005) and to 14 95-1 x Ballerina crosses. All of the 28 Ballerina offspring developed yellow mosaic but cane necrosis was observed in 22 of the 28 genotypes. Filamentous virus particles were detected by transmission electron microscopy (TEM) in negatively stained partially purified leaf tissue extracts from symptomatic leaf tissue and the presence of these particles was consistently associated with disease symptoms. No other virus-particles were detected in these extracts and no virus particles were detected in asymptomatic plants. The objectives of this study were to identify and characterize the virus associated with this disease, to determine its role in the etiology of the disease, and to develop protocols for its detection in rose germplasm.

Virus source and propagation

The virus isolate used in this study was obtained from naturally infected rose plants of cv. Ballerina. These plants had foliar (Fig. 1A) and stem (Fig. 1B) symptoms and filamentous virus-like particles (Fig. 2) were readily detected by TEM in negatively stained partially purified symptomatic leaf extracts. The virus isolate was maintained in cv. Ballerina plants propagated by vegetative cuttings, and kept in an insect proof greenhouse under normal growing conditions (18-24 ^{O}C) and 16:8 hour day:night photoperiod.

Virus and disease transmission tests

Graft transmission tests were done by whip and tongue grafting of scions from infected cv. Ballerina plants to healthy cvs. June Bride, BAIpeace (Love and Peace^{TM}), George Vancouver, Tropicana, and Monica. Also the RoYMV was graft transmitted to 14 genotypes of each Ballerina x 95-1 and 95-1 x Ballerina (n = 28 genotypes). All plants that were subjected to grafting experiments exhibited no foliar symptoms and no filamentous virus-like particles were detected by TEM prior to grafting. Graft-inoculated plants were kept in the greenhouse until the presence of the virus was confirmed. Plants were tested for the presence of the virus by TEM and RT-PCR using primer pair RoYMVcF (5'-GCGATCAAGGCAGCAGGAGT-3') and RoYMVcR (5'-TGCACACATAAAGCGCCACA-3') designed from the RoYMV genomic sequence as described below. Total RNA extracted from infected plant material using a RNeasy ® plant mini kit (Qiagen) was used as template for the RT-PCR. Aphid transmission was done using the potato aphid *Macrosiphum euphorbiae* apterae. Aphids were allowed to feed on infected cv. Ballerina for 24 hours and were transferred to healthy rose plants of cv. June Bride. After an inoculation feeding period aphids were killed by an insecticide application. Inoculated plants were kept and observed for symptoms and tested for the presence of the virus as described above.

Virus detection

RoYMV was detected by transmission electron microscopy (TEM), immunosorbent electron microscopy (ISEM) (Lockhart et al., 1992) and by RT-PCR. For TEM detection partially purified leaf tissue extracts were prepared in 500 mM sodium phosphate buffer,

pH 7.5 containing 1 M urea, 5% (w/v) polyvinylpyrrolidone (average molecular weight 40,000) and 0.5% (v/v) 2- mercaptoethanol. The initial filtered extract was centrifuged at 19,800 gmax for 15 minutes. After addition of 5% (v/v) Triton X-100 the supernatant was centrifuged at 109,000 gmax for 2 hours at 10 ^{O}C. The pellets were resuspended in 10 mM sodium phosphate buffer, (pH 7.0) and shaken with an equal volume of chloroform. The resulting emulsion was then centrifuged at 13,800 gmax for 10 minutes and the upper aqueous phase was used for TEM examination. TEM grids were prepared by negative staining using 2% sodium phosphotungstate pH 7.0 (PTA) and uranyl acetate (UA). For antiserum production virions of RoYMV were extracted from infected leaf tissue of rose cv. Ballerina and purified by differential centrifugation and isopycnic density gradient centrifugation in Cs_2SO_4 as described (Currier and Lockhart, 1996). An antiserum against purified virions was raised in a New Zealand White rabbit using a schedule of subcutaneous immunizations with purified virion antigen emulsified in an equal volume of Titermax Gold adjuvant (Sigma-Aldrich, St. Louis) as described previously (Mollov et al., 2007). Antiserum obtained from blood samples collected 45 to 80 days after the initial immunization were used for detection of RoYMV by indirect enzyme-linked immunosorbent assay (ELISA) (Koenig and Paul, 1982) using crude leaf extracts, and by ISEM (Fig. 2) using partially purified leaf tissue extracts.

RT-PCR was done using primer pair RoYMVcF (5'-GCGATCAAGGCAGCAGGAGT-3') and RoYMVcR (5'-TGCACACATAAAGCGCCACA-3') and were based on the RoYMV genomic sequence (Mollov et al., 2012a). Using these primers and total RNA extracted with Qiagen RNeasy ® plant mini kit RT-PCR (Ready-to-Go^{TM} RT-PCR Beads, GE Healthcare) a product of 704 bp was generated (Fig. 3). Thermocycler

conditions were: 42 ^{O}C for 45 min (1 cycle), 94 ^{O}C for 2 minutes (1 cycle), 94 ^{O}C for 30 sec, 57 ^{O}C for 30 sec, 72 ^{O}C for 45 sec (35 cycles), 72 ^{O}C for 10 min (1 cycle).

Summary and conclusion

Based on the transmission by graft inoculation of both RoYMV and disease symptoms from infected cv. Ballerina to healthy plants of cvs. June Bride, Love and Peace, George Vancouver, Tropicana, and Monica it was concluded that RoYMV is the likely causal agent of the disease. Cane necrotic lesions similar to those described for rose streak disease (Secor et al., 1977) were observed only in cv. Ballerina and Ballerina-offspring. Detection protocols described above could be used to determine whether cane necrosis symptoms occurring in roses with a different genetic background may be associated with RoYMV infection. Based on virion and genome properties (Mollov et al., 2012a) RoYMV was identified as a new member of the family *Potyviridae*, many of which are transmitted by arthropod vectors. The potato aphid *M. euphorbiae*, which commonly colonizes roses, did not transmit RoYMV, and no other potential vectors were identified. It is possible that spread of RoYMV in roses occurs primarily by cutting propagation and grafting, therefore reliable detection methods are necessary for screening rose germplasm for the presence of this virus. The three detection protocols that were developed, indirect ELISA, ISEM, and RT-PCR, gave consistently reliable results and can be used for routine detection of RoYMV in roses.

Acknowledgements

The authors thank Drs. Stan Hokanson and Vance Whitaker for providing the twenty-eight Ballerina progeny used for grafting experiments in this study.

Fig. 1A Fig. 1B

Figure 1. Foliar and cane symptoms observed in rose cv. Ballerina infected with Rose yellow mosaic virus RoYMV.

Fig. 1A: Foliar symptoms observed in rose cv. Ballerina infected with RoYMV.

Fig. 1B: Cane symptoms observed in rose cv. Ballerina infected with RoYMV.

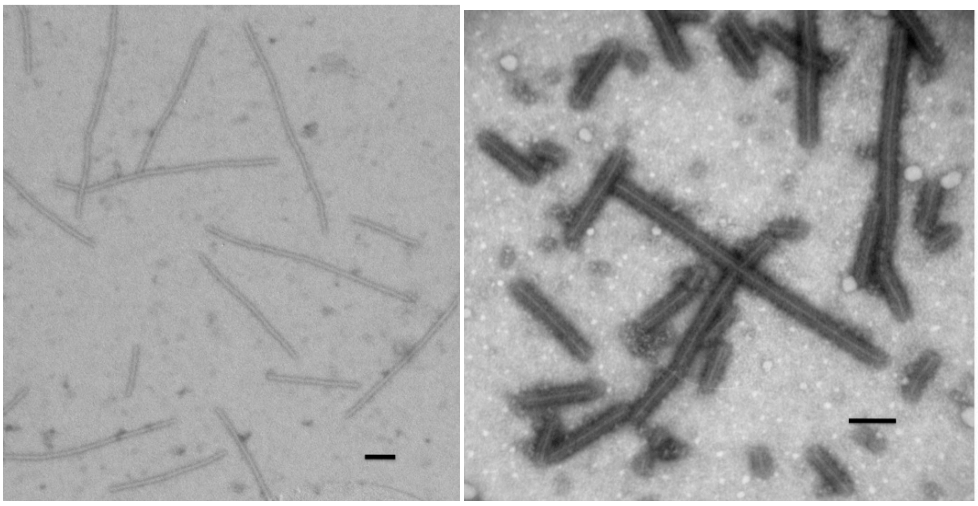


Fig. 2A Fig. 2B

Figure 2. Virions of Rose yellow mosaic virus (RoYMV), negatively stained with 2% sodium phosphotungstate (PTA) pH 7.0. Scale bars equal 100 nm.

Fig. 2A: Virions of RoYMV in purified preparation used for antiserum production.

Fig. 2B: Virions of RoYMV detected by Immunosorbent electron microscopy (ISEM) in a partially purified leaf tissue extract.

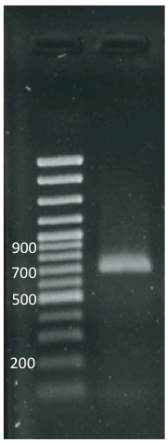


Figure 3. Detection of Rose yellow mosaic virus (RoYMV) by RT-PCR using the primer pair RoYMVcF and RoYMVcR. Lane 1: 100 bp DNA molecular ladder, lane 2: RoYMV positive reaction (704 bp product).

Chapter V

Complete nucleotide sequence of Rosa rugosa leaf distortion virus, a new member of the family *Tombusviridae*

This report describes the complete nucleotide sequence and genome organization of Rosa rugosa leaf distortion virus (RrLDV), the causal agent of a previously undescribed virus disease of *Rosa rugosa*. The RrLDV genome is a positive-sense ssRNA, 3971 nucleotides in length containing six open reading frames (ORFs). ORF1 encodes a 27 kDa peptide (p27). ORF2 shares a common start codon with ORF1 and continues through the amber stop codon of p27 to produce an 87 kDa protein (p87) with amino acid similarity to the RNA-dependent RNA polymerase (RdRp) of *Tombusviridae*. ORF3 encodes a protein of 8 kDa with no significant similarity to known viral sequences. ORF4 encodes a 6 kDa protein (p6) with similarity to the p13 movement proteins of *Tombusviridae*. ORF5 has no conventional start codon and overlaps with p6. A putative +1 frame shift mechanism allows p6 translation to continue through the stop codon and results in a 12 kDa protein with high homology to the carmovirus p13 movement protein. The 37 kDa protein encoded by ORF5 has amino acid sequence similarity to coat proteins (CP) of *Tombusviridae*. Phylogenetic analyses of the RdRp and CP amino acid sequences placed RrLDV in a subgroup close to the genus *Carmovirus* of the family *Tombusviridae*.

Introduction

Recently we described a virus with spherical particles (Lockhart et al., 2011) associated with one of several new foliar diseases of rose. The virus was named Rosa rugosa leaf distortion virus (RrLDV) after the typical symptoms associated with infection by this virus. Both the disease and the virus were graft transmitted from diseased to healthy *R. rugosa* suggesting that RrLDV is likely the causal agent of the disease (our unpublished results). The objectives of this study were to characterize the RrLDV genome and determine its taxonomic and phylogenetic relationship to known viruses.

Virus source, virion purification, genomic cloning and sequencing

The virus used in this study was obtained from naturally infected plants of *Rosa rugosa* 'Charles Albanel'. Virions were purified from symptomatic infected leaf tissue, and genomic RNA was extracted and characterized as described previously (Mollov et al., 2012a). A genomic library was created from virion RNA using the method described by Froussard (Froussard, 1992). The DNA library fragments were cloned using a pGem T-easy™ kit (Promega, Madison WI) according to manufacturers' instructions and clones were screened by PCR using M13 universal primers. Twelve clones were selected and plasmid DNA was extracted from overnight cultures using a Zippy™ Plasmid DNA extraction kit (Zymo Research) and sequenced in both directions. Sequences were assembled by Sequencher 4.10.1 software (Gene Codes Corporation, Ann Arbor, MI). Six pairs of primers were designed to produce overlapping amplicons covering the RrLDV genomic sequence except for the 5' and 3' termini. These primers, respective nucleotide position, and their sequences are: RrW1F, 93 (5'-

CCGCAACGATGAGATTGTGC-3'); RrW1R, 896 (5'-GACCCGCTCCATGAGCCCAC-3'); RrW2F, 778 (5'-TGGGTTAAGGAAGGCCGTGA-3'); RrW2R, 1460 (5'-GCTGAAGCACTCGCGGTACA-3'); RrW3F, 1426 (5'-GTTTCCGTGGACGCCTTGAG-3'); RrW3R, 2093 (5'-TTCACCCCTGTGCTGATCCA-3'); RrW4F, 1899 (5'-GCACTCAACCACCCCCTTTG-3'); RrW4R, 2446 (5'-GGCGCCAACCTCAAATCAAC-3'); RrW5F, 2294 (5'-CGTAGACCGCACCCAAAAGC-3'); RrW5R, 3091 (5'-CGGCCTCGTATCTGGGATTG-3'); RrW6F, 2790 (5'-ACGTTCGTCGTCGGAATGGT-3'); RrW6R, 3397 (5'-GGTAGGACCCTGACGCCTGA-3').

The overlapping genomic segments were generated by RT-PCR using Ready-to-Go™ RT-PCR Beads (GE Healthcare) and virion RNA as the template. The PCR products were cloned, sequenced, and assembled as described above. The 5' terminal sequence of the RrLDV genome was obtained using sequence-specific primer RrLDV5pSP1, (5'-GCTGCATCCAGCAGCTTTCA-3') and the RACE second-generation kit (Roche Applied Science). The resulting product was subjected to a second round of nested PCR using the 5' RACE anchor primer (5'-GACCACGCGTATCGATGTCGAC-3') and a second RrLDV5pSP2 (5'-GATGAGACCTGCCGGGTTTG-3') sequence-specific primer. The nested PCR amplicon of about 500 bp was ligated into pGem T-easy from which four clones were selected and sequenced in both directions to obtain and assemble the 5' terminal sequence.

To obtain the 3' terminus of the RrLDV genome virion RNA was A-tailed with Poly(A) Polymerase (Epicentre, Madison WI) using 1 mM dATP for 4 minutes at 37 ^{O}C. The resulting product was used as template for making cDNA using $6NT_{18}$ primer and SuperScript^{TM} III reverse transcriptase. Using the produced cDNA a first PCR was carried out using the RrLDV genome-specific RrLDV3p1 (5'-CGAGGCACATCAAAGGACCA-3') and $6NT_{18}$ primers. A nested PCR was performed using the first 3' RT-PCR amplicon with a second sequence-specific RrLDV3p1 (5'-TCATTCCAAGACGGGGTGGT-3') and $6NT_{18}$ primers with the DNA product cloned as described. Six clones were selected and sequenced in both directions. The complete sequence of the RrLDV genome was assembled using Sequencher 4.10.1 (Gene Codes Corporation, Ann Arbor, MI). Significant amino acid sequence similarities to known viral sequences were determined using BLASTP (Altschul et al., 1997). The complete RrLDV genome sequence was deposited into the NCBI GenBank under the accession number KC166238.

Sequence analysis and genome organization

The complete genomic sequence of the RrLDV was determined to be 3971 nucleotides in length. The genome contains six open reading frames (ORFs) (Fig. 1, Table 1). The 5' UTR sequence is only six nucleotides long (CAAACT) and is equal in length to the shortest 5' UTR previously described (Castano and Hernandez, 2005). The first ORF encodes a protein of 27 kDa. This protein has highest amino acid sequence similarity (46%) to the p27 protein of *Pelargonium line pattern virus* (PLPV). At position 718-720 this ORF has the amber stop codon UAG that is read through to produce the 87 kDa

protein. The RrLDV sequence 5'-AAA **UAG** GGG-3' (715-723 nt) surrounding the amber stop codon is consistent with the proposed relative efficiency of readthrough sequences (A/C/U)(A/U)A **UAG** (G/C)(G/A) (Skuzeski et al., 1990), and exact match with corresponding sequence occurring in *Maize chlorotic mottle virus* (Nutter et al., 1989) and *Carnation mottle virus* (Guilley et al., 1985). The RrLDV ORF 2 is mostly similar to PLPV and *Pelargonium chlorotic ring pattern virus* (PCRPV) at 61% identity to both viruses. Multiple sequence alignment of p87 proteins from 38 viruses (Combet et al., 2000) representing all eight genera of the family *Tombusviridae* revealed that no conserved domains were present in the p27 part of the proteins. Immediately after the readthrough stop codon a conserved Gly residue is present in 37 of the 38 viruses (RdRp sequences accession numbers are listed in Fig. 2A legend). In a comparison of the RrLDV RdRp sequence with those of all 37 viruses 100% highly conserved motifs were identified: R-(X3)-V-(X11)-P-(X42)-Y-(X21)-F-(X)-K-(X)-EK-(X8)-P-(X)-PR-(X)-I-(X)-PR-(X4)-N-(X7)-L-(X22)-G–(X23)-G-(X)-D-(X2)-RFDQH-(X)-S-(X2)-AL-(X2)-E-(X20)-Q-(X16)-Y–(X4)-R-(X6)-T-(X2)-GN-(X19)-L-(X)-N-(X)-GDD-(X30)-E-(X3)-FC-(X3)-P-(X13)-R-(X8)-D-(X31)-P, where the first residue R is in position 301 of the RrLDV p87 N-terminus and the last P is in position 728 of the C-terminus.

A putative ORF3 is predicted to encode an 8 kDa protein. This protein lacks significant similarity to proteins of known viruses. ORF4 encodes a protein p6 (6 kDa) similar to the movement proteins of PCRPV (62% identity) and PLPV (59% identity). A small ORF4 that overlaps p6 is encoded downstream without a conventional start codon and has no amino acid sequence similarity to known viral proteins. Evidence suggests (Brierley, 1995) that this ORF is expressed by a frameshift that would result in a 12 kDa fused

protein p12. The putative p12 protein has amino acid sequence similarity of 60% to PCRPV movement protein. The frameshift has been previously reported to occur in the translation of the PLPV movement protein (Castano and Hernandez, 2005). ORF6 is predicted to encode a 37 kDa protein similar to the CP of the *Tombusviridae* members: PCRPV (48%); PLPV (47%); *Elderberry latent virus* (45%).

Phylogenetic analysis

The taxonomic and phylogenetic relationship of RrLDV to selected virus species in the eight genera (*Aureusvirus*, *Avenavirus*, *Carmovirus*, *Dianthovirus*, *Machlomovirus*, *Necrovirus*, *Panicovirus*, and *Tombusvirus*) of *Tombusviridae* was deduced using the amino acid sequences of 38 p87 (replicase) and 29 coat proteins using ClustalW alignment (Larkin et al., 2007). The dendrograms were generated using MEGA 5.0 (Tamura et al., 2011) by the neighbor-joining method (Saitou and Nei, 1987).

Phylogenetic analyses of the RdRp and CP (Fig. 2) show that RrLDV groups with the unclassified PLPV and PCRPV. As illustrated in Fig. 1, RrLDV is unique in having genome organization similar to *Carmovirus* (King et al., 2012) with the exception of the p8 protein that overlaps with p87. The RdRp and CP phylogenetic groups formed by using amino acid sequences from selected *Tombusviridae* show taxonomic discrepancies (Fig. 2) within the family. The phylogenetic trees were generated by the neighbor joining method and results were similar when other algorithms were applied (maximum parsimony, unweighted pair group method with arithmetic mean, maximum likelihood; data not shown). These results were consistent with other studies showing multi-cluster

genera grouping of *Tombusviridae* (Gulati-Sakhuja and Liu, 2010; Gulati-Sakhuja et al., 2011). The data presented in Fig. 1 and Fig. 2 supports the conclusion that RrLDV is a previously uncharacterized virus in the family *Tombusviridae* and to our knowledge the first virus of this family infecting rose.

Table 1. Rosa rugosa leaf distortion virus (RrLDV) putative ORFs, protein product, function and nucleotide position.

ORF	Product	Function	Start	Stop
ORF1	p27	Replicase	7	720
ORF2	p87	Replicase	7	2292
ORF3	p8	Unknown function	2069	2287
ORF4	p6	Movement protein	2262	2444
ORF5	p8	Movement protein	2338	2610
ORF6	p37	Coat protein	2594	3613

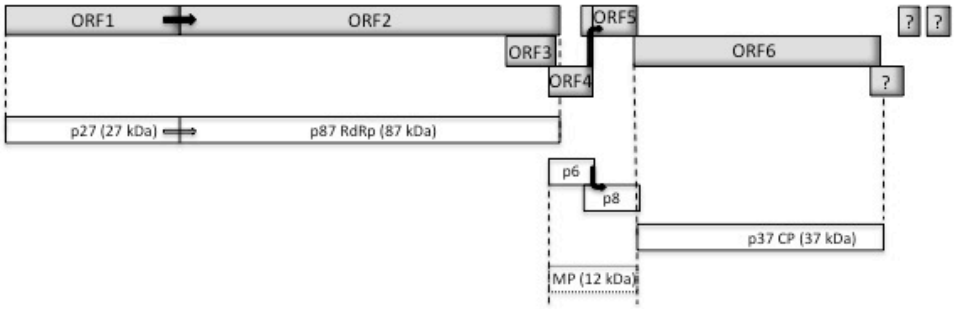


Figure 1. Schematic representation of Rosa rugosa leaf distortion (RrLDV) virus genome and predicted translation strategy. ORFs 1, 2, and 5 are in frame 1; ORFs 3 and 6 are in frame 2; and ORF 4 is in frame 3. Straight arrow depicts a readthrough of an amber stop codon. Curved arrow shows putative frameshift that results in the 12 kDa fused movement protein. Small boxes with question mark at the 3' terminus represent possible additional ORFs of unknown function.

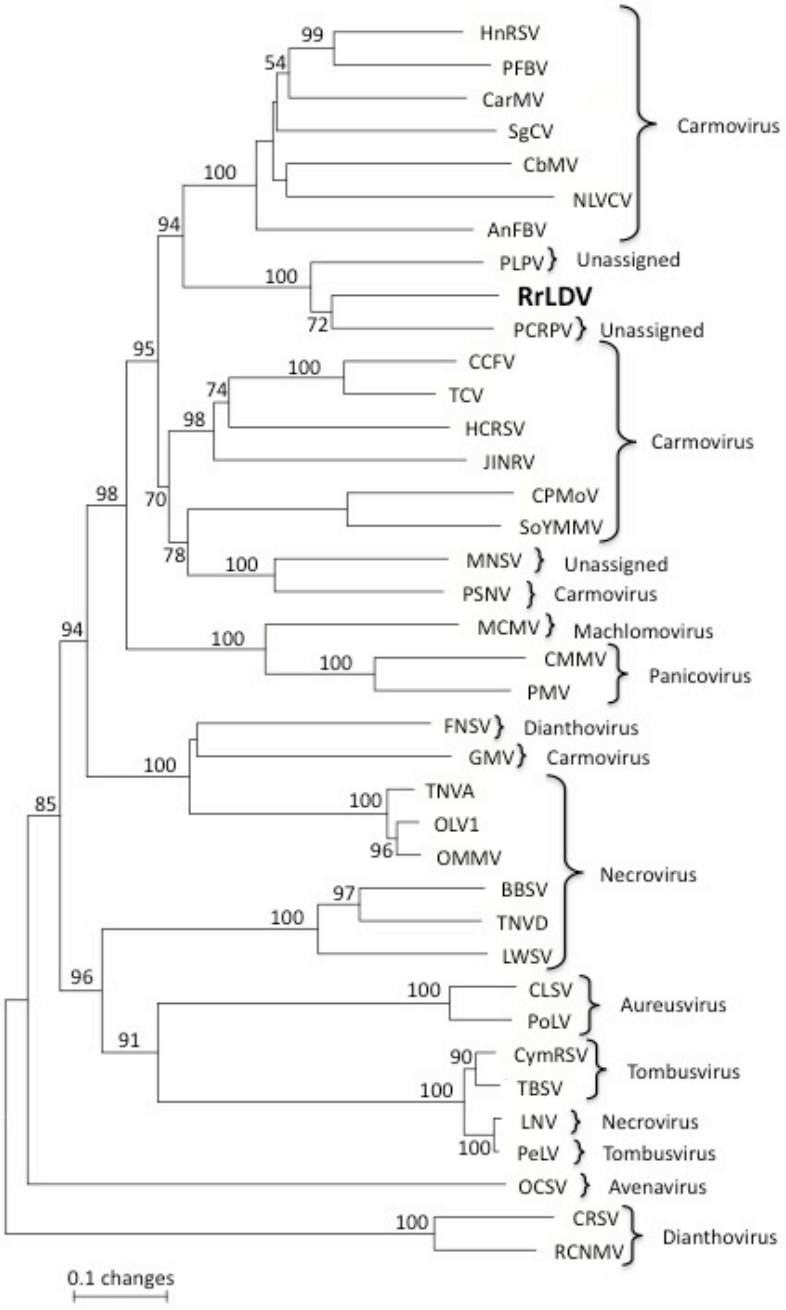

HnRSV
PFBV
CarMV
SgCV
CbMV
NLVCV
AnFBV
Carmovirus
PLPV
Unassigned
RrLDV
PCRPV
Unassigned
CCFV
TCV
HCRSV
JINRV
CPMoV
SoYMMV
Carmovirus
MNSV
Unassigned
PSNV
Carmovirus
MCMV
Machlomovirus
CMMV
PMV
Panicovirus
FNSV
Dianthovirus
GMV
Carmovirus
TNVA
OLV1
OMMV
BBSV
TNVD
LWSV
Necrovirus
CLSV
PoLV
Aureusvirus
CymRSV
TBSV
Tombusvirus
LNV
Necrovirus
PeLV
Tombusvirus
OCSV
Avenavirus
CRSV
RCNMV
Dianthovirus
0.1 changes

Fig. 2A

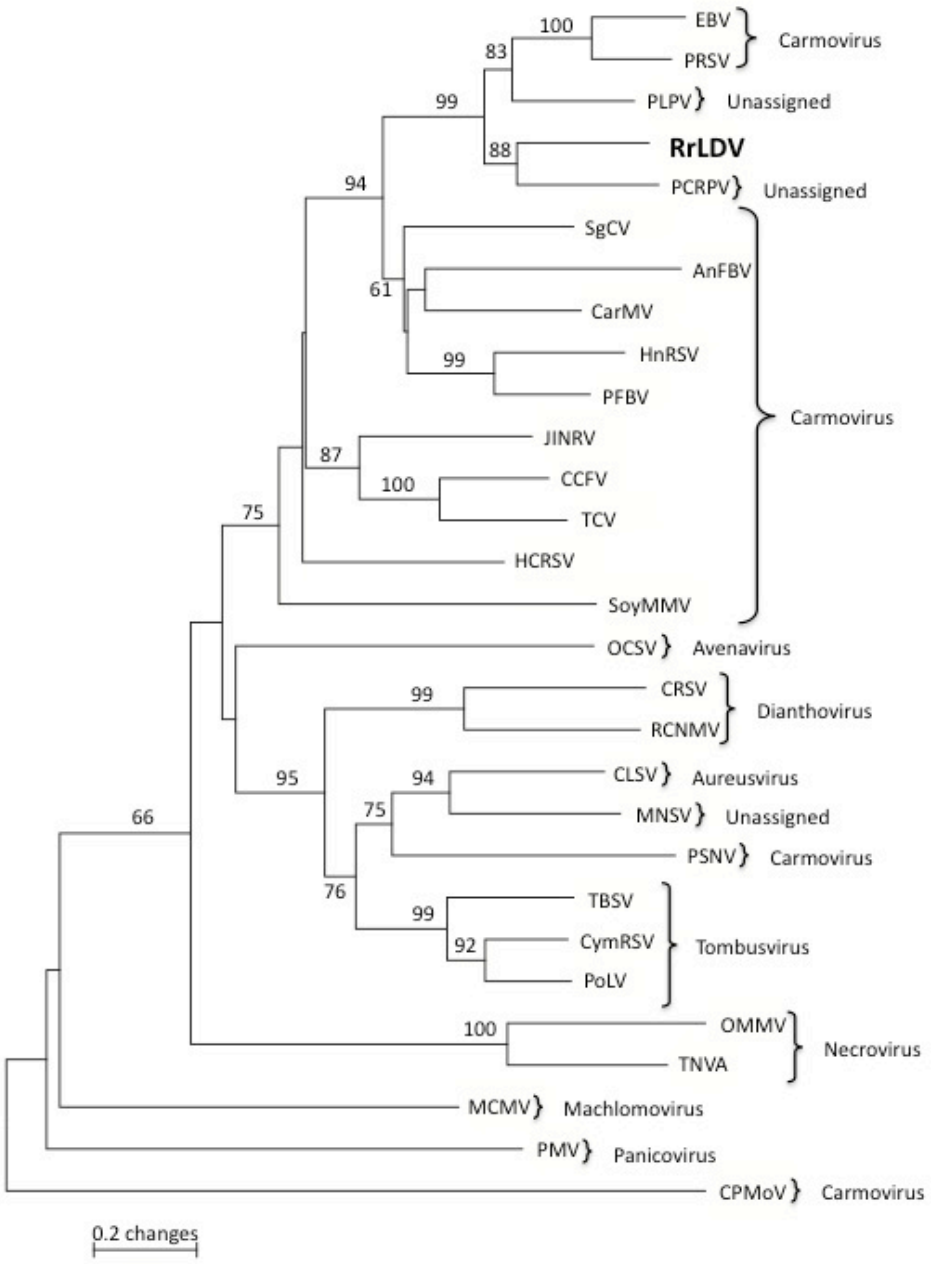


Fig. 2B

Figure 2. Phylogenetic analysis of Rosa rugosa leaf distortion virus (accession number KC1666238) and selected members of the family *Tombusviridae* based on the amino acid sequences of the replicase and the coat protein constructed by the neighbor joining

method. The numerical values represent the bootstrap (1000 replicates) numbers above 50%. The scale bars correspond to substitutions per amino acid site.

Fig. 2A. Unrooted phylogenetic trees of the replicase amino acid sequences of *Tombusviridae*. Replicase amino acid sequence accession numbers used in the phylogenetic analysis were: AnFBV (*Angelonia flower break virus* YP_459960.1); BBSV (*Beet black scorch virus*, CBH32825.1); CarMV (*Carnation mottle virus*, ADA69469.1); CbMV (*Calibrachoa mottle virus*, ACT36594.1); CCFV (*Cardamine chlorotic fleck virus*, NP_041884.1); CLSV (*Cucumber leaf spot virus*, ABV30916.1); CMMV (*Cocksfoot mild mosaic virus*, YP_002117834.1); CPMoV (*Cowpea mottle virus*, NP_619521.1); CRSV (*Carnation ringspot virus*, NP_619711.1); CymRSV (*Cymbidium ringspot virus*, NP_613260.1); FNSV (*Furcraea necrotic streak virus*, ACW84407.1); GMV (*Galinsoga mosaic virus*, NP_044732.1); HCRSV (*Hibiscus chlorotic ringspot virus*, NP_619671.1); HnRSV (*Honeysuckle ringspot virus*, YP_004191789.1); JINRV (*Japanese iris necrotic ring virus*, AFL55711.1); LNV (*Lisianthus necrosis virus*, YP_588430.1); LWSV (*Leek white stripe virus*, NP_044740.1); MCMV (*Maize chlorotic mottle virus*, AFV60461.1); MNSV (*Melon necrotic spot virus*, BAF47094.1); NLVCV (*Nootka lupine vein clearing virus*, YP_001039884.1); OCSV (*Oat chlorotic stunt virus*, NP_619751.1); OLV1 (*Olive latent virus*, 1 AAZ43259.1); OMMV (*Olive mild mosaic virus*, AEC50092.1); PCRPV (*Pelargonium chlorotic ring pattern virus*, YP_052925.1); PeLV (*Pear latent virus*, NP_835239.1); PFBV (*Pelargonium flower break virus,* NP_945123.1); PLPV (*Pelargonium line pattern virus*, YP_238475.1); PMV (*Panicum mosaic virus*, NP_068342.1); PoLV (*Pothos latent virus*, CAA60596.1); PSNV (*Pea stem*

necrosis virus, NP_862835.2) RCNMV (*Red clover necrotic mosaic virus*, NP_620523.1); SgCV (*Saguaro cactus virus*, NP_044382.1); SoYMMV (*Soybean yellow mottle mosaic virus*, ACN59473.1); TBSV (*Tomato bushy stunt virus*, NP_062897.1); TCV (*Turnip crinkle virus*, AAP78486.1); TNVA (*Tobacco necrosis virus A* ADE10194.1); TNVD (*Tobacco necrosis virus D* ACT31460.1).

Fig. 2B. Unrooted phylogenetic trees of the coat protein amino acid sequences of *Tombusviridae*.

Coat protein amino acid sequence accession numbers used in the phylogenetic analysis were: AnFBV (*Angelonia flower break virus*, YP_459964.1); CarMV (*Carnation mottle virus,* NP_051885.1); CCFV (*Cardamine chlorotic fleck virus*, NP_041887.1); CLSV (*Cucumber leaf spot virus*, YP_512365.1); CPMoV (*Cowpea mottle virus*, NP_613272.1); CRSV (*Carnation ringspot virus*, NP_613255.1); CymRSV (*Cymbidium ringspot virus*, NP_613262.1); EBV (*Elderberry latent virus*, AAK74061.1); HCRSV (*Hibiscus chlorotic ringspot virus*, NP_619676.1); HnRSV (*Honeysuckle ringspot virus*, YP_004191793.1); JINRV (*Japanese iris necrotic ring virus*, NP_038458.1); MCMV (*Maize chlorotic mottle virus,* NP_619722.1); MNSV (*Melon necrotic spot virus*, NP_041231.1); OCSV (*Oat chlorotic stunt virus*, NP_619753.1); OMMV (*Olive mild mosaic virus,* YP_224020.1); PCRPV (*Pelargonium chlorotic ring pattern virus*, YP_052929.1); PFBV (*Pelargonium flower break virus*, ABD93258.1); PLPV (*Pelargonium line pattern virus*, YP_238481.1); PMV (*Panicum mosaic virus*, NP_068346.1); PoLV (*Pothos latent virus*, CAA60597.1); PSNV (*Pea stem necrosis virus*, NP_862839.1); RCNMV (*Red clover necrotic mosaic virus*, NP_620526.1); SgCV

(*Saguaro cactus virus*, NP_044388.1); SoYMMV (*Soybean yellow mottle mosaic virus*, YP_002333479.1); TBSV (*Tomato bushy stunt virus*, NP_062899.1); TCV (*Turnip crinkle virus*, NP_620723.1); TNVA (*Tobacco necrosis virus A* NP_056828.1).

Chapter VI

Complete nucleotide sequence of Rose yellow leaf virus, a new member of the family *Tombusviridae*

The genome of the Rose yellow leaf virus (RoYLV) was been determined to be 3918 nucleotides in size. The RoYLV genome consists of seven open reading frames (ORFs). ORF1 encodes a 27 kDa peptide (p27). ORF2 shares a common start codon with ORF1 and continues through the amber stop codon of p27 to encode a 87 kDa (p87) protein that has amino acid similarity to the RNA dependent RNA polymerase (RdRp) of the *Tombusviridae*. ORFs 3 and 4 encode proteins of 8 and 7 kDa respectively that have no significant amino acid similarity to known viral sequences. ORF5 encodes a 6 kDa (p6) protein that has similarity to movement proteins of the *Tombusviridae*. ORF5A has no conventional start codon and overlaps with p6. A putative +1 frame shift mechanism allows p6 translation to continue through the stop codon and results in a 12 kDa protein that has high homology to the carmovirus p13 movement protein. The 37 kDa protein encoded by ORF6 has amino acid sequence similarity to coat proteins (CP) of *Tombusviridae*. ORF7 encodes a 7 kDa protein with no significant amino acid similarity to known viral proteins. Phylogenetic analyses of the RdRp and CP amino acid sequences placed RoYLV together with the unclassified Rosa rugosa leaf distortion virus (RrLDV), *Pelargonium line pattern virus* (PLPV), and *Pelargonium line pattern virus* (PLPV) in a subgroup of the family *Tombusviridae*.

Introduction

In a recent report describing a number of new foliar diseases of rose we described a virus with spherical particles associated with a yellow leaf disease (Lockhart et al., 2011) of the rose cv. Softee. The virus was named Rose yellow leaf virus (RoYLV) after the typical symptoms associated with infection by this virus. The symptoms associated with this virus are mosaic and yellowing of the leaf and premature senescence (Fig 1A). The disease and the virus were graft transmitted from diseased to healthy rose (Fig 1B) suggesting that RoYLV is the likely causal agent of the disease. The objectives of this study were to characterize the RoYLV genome and to determine its taxonomic and phylogenetic relationship to known viruses.

Virus source, virion purification, genomic cloning and sequencing

The isolate of the virus used in this study was obtained plants of the Rosa hybrid cv. Softee that were purchased from a commercial nursery. The plants were kept in a greenhouse at 18-24 ^{O}C and 16:8 hour day:night photoperiod.

Viral RNA was extracted from partially purified preparations of virions by phenol/chloroform extraction and precipitated with isopropanol as described previously (Mollov et al., 2012a). A genomic library was created from virion RNA using the method described by Froussard (Froussard, 1992). The DNA library fragments were cloned using a pGem T-easy^{TM} kit (Promega, Madison WI) according to manufacturers' protocol and clones were screened by PCR using M13 universal primers. Twelve clones were selected and plasmid DNA was extracted from overnight cultures using a Zippy^{TM} Plasmid DNA extraction kit (Zymo Research) and sequenced in both directions. Sequences were

assembled by Sequencher 4.10.1 software (Gene Codes Corporation, Ann Arbor, MI). Four pairs of primers were designed to produce overlapping amplicons (Table 1) covering the RoYLV genomic sequence except for the 5' and 3' termini. The overlapping genomic segments were generated by RT-PCR using Ready-to-Go^{TM} RT-PCR Beads (GE Healthcare) and virion RNA as template. The PCR products were cloned, sequenced, and assembled as described. The 5' terminal sequence of the RoYLV genome was obtained by using sequence specific primer Sf5P1 (Table 1) and the RACE second-generation kit (Roche Applied Science). The resulting product was subjected to a second round nested PCR using the 5' RACE anchor primer (5'-GACCACGCGTATCGATGTCGAC-3') and a second Sf5P2 sequence specific primer. The resulting amplicon was ligated into pGem T-easy and three clones were selected and sequenced in both directions to obtain and assemble the 5' terminal sequence.

To obtain the 3' terminus of the RoYLV genome 3' cDNA was produced as described in Chapter 5. Using the cDNA a first PCR was carried out using the RoYLV sequence specific primer Sf3P1 (Table 1) and $6NT_{18}$ primer. A nested PCR was performed using the first 3' PCR product and second sequence specific Sf3P2 and $6NT_{18}$ primers. The DNA product was cloned and sequenced as described. The complete sequence of the RoYLV genome was assembled using Sequencher 4.10.1 (Gene Codes Corporation, Ann Arbor, MI). Significant amino acid sequence similarities to known viral sequences were determined using BLASTP (Altschul et al., 1997). The complete RoYLV genome sequence was deposited into GenBank under the accession number KC166239.

Sequence analysis and genome organization

The complete genomic sequence of the RoYLV was determined to be 3918 nucleotides in length. The genome contains seven open reading frames (ORFs) (Figure 2, Table 1). The RoYLV 5' UTR is only six nucleotides long (CAAACC), similar to those of Rosa rugosa leaf distortion virus (RrLDV) (Chapter 5) and *Pelargonium line pattern virus* (PLPV) (Castano and Hernandez, 2005) and represent the shortest known plant viral 5' UTR (Castano and Hernandez, 2005). The RoYLV ORF1 encodes a protein of 27 kDa. This protein has highest amino acid sequence similarity to the p27 proteins of RrLDV (88%) and to PLPV (47%). At position 718-720 this ORF has the amber stop codon UAG that is read through to produce the 87 kDa protein. The RoYLV sequence 5'-AAA **UAG** GGG-3' (715-723 nt) surrounding the amber stop codon is consistent with the proposed relative efficiency of readthrough sequences (A/C/U)(A/U)A **UAG** (G/C)(G/A) (Skuzeski et al., 1990), and is an exact match with the corresponding sequence occurring in RrLDV (Chapter 5), *Maize chlorotic mottle virus* (Nutter et al., 1989) and *Carnation mottle virus* (Guilley et al., 1985). The RoYLV ORF2 amino acid sequence is 90% similar to RrLDV ORF2 and 61% similar to the p87 proteins of PLPV and *Pelargonium chlorotic ring pattern virus* (PCRPV). Multiple sequence alignment of p87 proteins from 39 viruses (Combet et al., 2000) representing all 8 genera of the family *Tombusviridae* revealed that no conserved domains were present in the p27 part of the proteins. Immediately after the readthrough stop codon a conserved Gly residue is present in 38 of the 39 viruses (RdRp sequences accession numbers are listed in the Fig. 3A legend).

ORF3 and ORF4 are predicted to encode 8 and 7 kDa proteins respectively. These proteins have no significant similarity to proteins of known viruses. ORF5 produces a

6kDa protein (p6) similar to the movement proteins of RrLDV (90% identity), PCRPV (62% identity), and PLPV (59% identity). A small ORF5A that overlaps p6 is encoded downstream without a conventional start codon. Evidence suggests (Brierley, 1995) that this ORF is expressed by a frameshift that would result in a 12 kDa fused protein p12. The putative p12 protein has amino acid sequence similarity of 90% to RrLDV and 60% to PCRPV movement proteins. The frameshift has been reported previously to occur in the translation of the PLPV movement protein (Castano and Hernandez, 2005). ORF6 is predicted to encode a 37 kDa protein similar to the CP of the *Tombusviridae* members: RrLDV (95%); PCRPV (48%); PLPV (47 %); and *Elderberry latent virus* (45%). ORF7 encodes a 7 kDa proteins that overlaps with the CP ORF and has no amino acid sequence similarity to known viral protein sequences.

Virus detection and disease transmission

Diagnostic primer pairs were designed from the RdRp (SfRF- SfRR) and the coat protein (SfCF- SfCR) regions (Table 1). Using total RNA (extracted by RNeasy ® Plant Mini Kit, Qiagen) from symptomatic plant material the primer pairs produce RT-PCR amplicon sizes of 863 and 402 bp, respectively. Despite the fact that the RrLDV nucleotide sequence has the highest similarity to the RoYLV with a whole genome nucleotide sequence of 86% identity, these primer pairs yielded no visible band when tested against RrLDV infected material (data not shown). The SfRF- SfRR and SfCF-SfCR diagnostic primers sequences were subjected to searches against the GenBank database and with the exception of the SfRR primer that was found to be completely

identical to a sequence from the RdRp region of RrLDV, no sequences were identified that were 100% identical to these primers.

Graft transmission experiments using scions of infected rose cv. Softee plants and healthy cv. Red Cascade rootstocks that contained no spherical virus particles detectable by TEM and tested negative by RT-PCR were used. Grafting was done by the whip and tongue method and infected plants of cv Red Cascade were kept in the greenhouse at 18-24 ^{O}C. After 4 months, infected plants developed typical symptoms of leaf yellowing and premature senescence (Fig. 1B). Total RNA was extracted from symptomatic leaves harvested from the grafted rose cv. Red Cascade and RT-PCR using SfCF and SfCR primers (Table 1) was performed. The product of expected size (402 bp) was sequenced, sequences compared to RoYLV, and found identical to RoYMV coat protein.

Phylogenetic analysis

The taxonomic and phylogenetic relationship of RoYLV to selected virus species in the eight genera (*Aureusvirus*, *Avenavirus*, *Carmovirus*, *Dianthovirus*, *Machlomovirus*, *Necrovirus*, *Panicovirus*, and *Tombusvirus*) of *Tombusviridae* was deduced using the amino acid sequences of 39 p87 (replicase) and 30 coat proteins using ClustalW alignment (Larkin et al., 2007). The dendrograms were generated using MEGA 5.0 (Tamura et al., 2011) by the neighbor-joining method (Saitou and Nei, 1987). Phylogenetic analyses of the RdRp and CP (Fig 3) show that RoYLV groups with the unclassified RrLDV, PLPV and PCRPV. As illustrated in Fig. 3, RoYLV is unique in having genome organization similar to *Carmovirus* (King et al., 2012) with the exception of the three small proteins that overlap with the replicase and the coat protein. The data

presented in Fig. 2 and Fig. 3 supports the conclusion that RoYLV is a previously uncharacterized virus in the family *Tombusviridae* and the second virus of this family infecting rose.

Table 1. Rose yellow leaf virus (RoYLV) primer sequences used for RT-PCR and RACE reactions in the genome assembly, genome sequence conformation, and for detection. Nucleotide positions correspond to the first nucleotide at the 5' position.

Primer	Position	Nucleotide sequence
Overlapping primers		
Sw1F	167	ACACAATCCCCACCGCTGAT
Sw1R	1015	GGGGAATGATTTCCGCACAA
Sw2F	721	GGGTGCCTCATGGAAACCTG
Sw2R	1899	CAAAGGGGGTGGTTGAGTGC
Sw3F	1899	GCACTCAACCACCCCCTTTG
Sw3R	2755	CACAAGGCGTGTGACCTTGG
Sw4F	2582	TCTCCGTAGGAAATGGCAGCA
Sw4R	3448	CCATGCCTGACTGGCTCAGA
5'end Primers		
Sf5P1	310	CGGACCCTCCTACGCACAAC
Sf5P2	167	ATCAGCGGTGGGGATTGTGT
3'end Primers		
Sf3p1	3358	GGTGCCCTCATTCCAAGACG
Sf3p2	3449	CTGAGCCAGTCAGGCATGGA
Diagnostic primers		
SfCF	3102	ACGAAGCCGTGGCCAGTAAA
SfCR	3484	CCTCTGTCGACGTGGTCGTG
SfRF	1056	TTTGTTGCATGAGGGCAGGA
SfRR	1899	CAAAGGGGGTGGTTGAGTGC

Table 2. Rose yellow leaf virus (RoYLV) putative ORFs, protein product, function and nucleotide position.

ORF	Product	Function	start	stop
ORF1	p27	Replicase	7	720
ORF2	p87	Replicase	7	2292
ORF3	p8	Unknown function	365	592
ORF4	p7	Unknown function	2081	2269
ORF5	p6	Movement protein	2262	2444
ORF5A	p12	Movement protein	2262	2610
ORF6	p37	Coat protein	2594	3613
ORF7	p7	Unknown function	3015	3224

Fig 1A

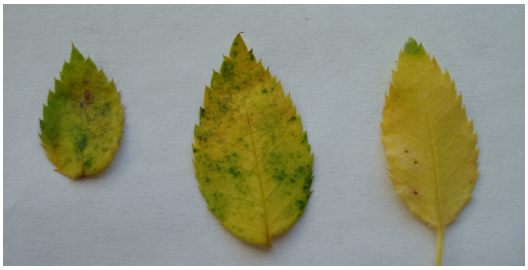


Fig 1B

Figure 1. Foliar symptoms observed in roses infected with Rose yellow leaf virus (RoYLV). Fig. 1A: Leaf yellowing naturally occurring in cv. Softee; Fig 1B: Leaf yellowing after graft inoculation of cv. Red Cascade.

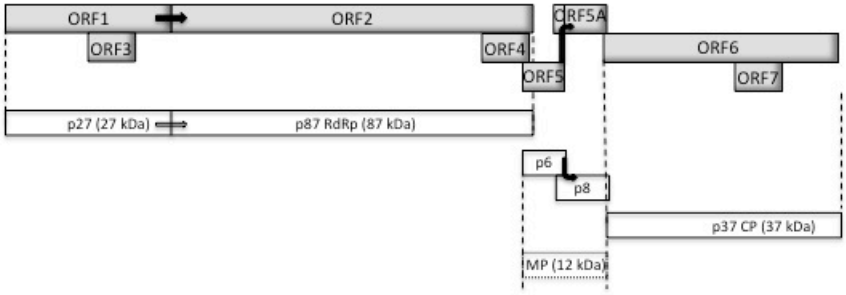


Figure 2. Schematic representation of Rose yellow leaf virus genome and predicted translation strategy.

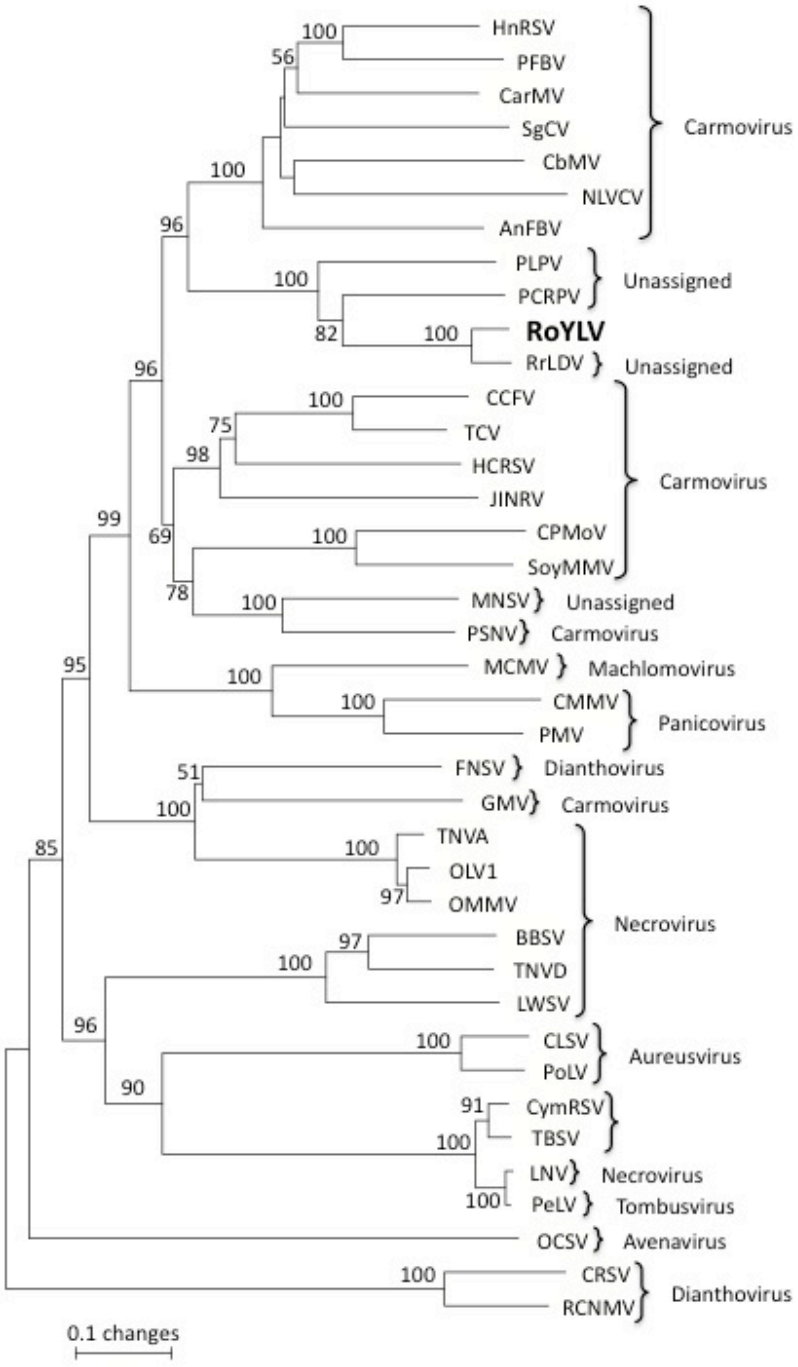

HnRSV
PFBV
CarMV
SgCV
CbMV
NLVCV
AnFBV
Carmovirus
PLPV
PCRPV
Unassigned
RoYLV
RrLDV
Unassigned
CCFV
TCV
HCRSV
JINRV
CPMoV
SoyMMV
Carmovirus
MNSV
Unassigned
PSNV
Carmovirus
MCMV
Machlomovirus
CMMV
PMV
Panicovirus
FNSV
Dianthovirus
GMV
Carmovirus
TNVA
OLV1
OMMV
BBSV
TNVD
LWSV
Necrovirus
CLSV
PoLV
Aureusvirus
CymRSV
TBSV
LNV
Necrovirus
PeLV
Tombusvirus
OCSV
Avenavirus
CRSV
RCNMV
Dianthovirus
0.1 changes

Fig. 3A

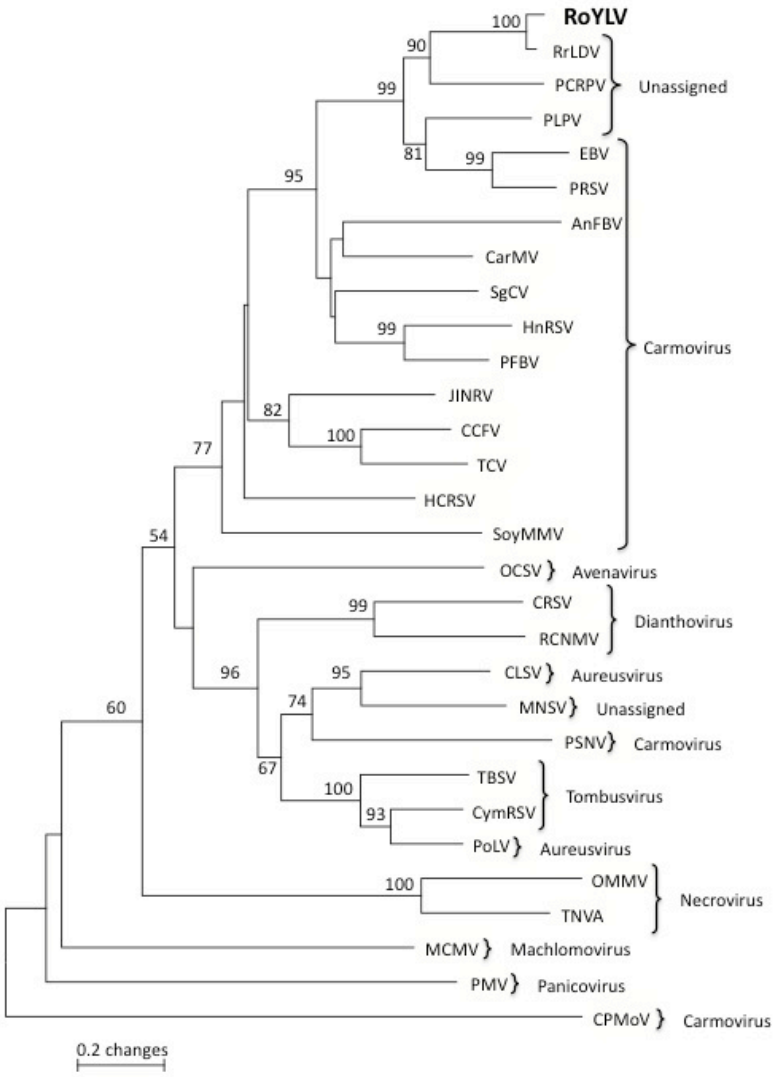


Fig. 3B

Figure 3. Phylogenetic analysis of Rose yellow leaf virus (accession number KC1666239) and selected members of the family *Tombusviridae* based on the amino acid sequences of the replicase and the coat protein constructed by the neighbor joining method. The numerical values represent the bootstrap (1000 replicates) numbers above 50%. The scale bars correspond to substitutions per amino acid site.

Fig. 3A. Unrooted phylogenetic trees of the replicase amino acid sequences of *Tombusviridae*. Replicase amino acid sequence accession numbers used in the phylogenetic analysis were: AnFBV, (*Angelonia flower break virus* YP_459960.1); BBSV (*Beet black scorch virus*, CBH32825.1); CarMV (*Carnation mottle virus*, ADA69469.1); CbMV (*Calibrachoa mottle virus*, ACT36594.1); CCFV (*Cardamine chlorotic fleck virus*, NP_041884.1); CLSV (*Cucumber leaf spot virus*, ABV30916.1); CMMV (*Cocksfoot mild mosaic virus*, YP_002117834.1); CPMoV (*Cowpea mottle virus*, NP_619521.1); CRSV (*Carnation ringspot virus*, NP_619711.1); CymRSV (*Cymbidium ringspot virus*, NP_613260.1); FNSV (*Furcraea necrotic streak virus*, ACW84407.1); GMV (*Galinsoga mosaic virus*, NP_044732.1); HCRSV (*Hibiscus chlorotic ringspot virus*, NP_619671.1); HnRSV (*Honeysuckle ringspot virus*, YP_004191789.1); JINRV (*Japanese iris necrotic ring virus*, AFL55711.1); LNV (*Lisianthus necrosis virus*, YP_588430.1); LWSV (*Leek white stripe virus*, NP_044740.1); MCMV (*Maize chlorotic mottle virus*, AFV60461.1); MNSV (*Melon necrotic spot virus*, BAF47094.1); NLVCV (*Nootka lupine vein clearing virus*, YP_001039884.1); OCSV (*Oat chlorotic stunt virus*, NP_619751.1); OLV1 (*Olive latent virus*, 1 AAZ43259.1); OMMV (*Olive mild mosaic virus*, AEC50092.1); PCRPV (*Pelargonium chlorotic ring pattern virus*, YP_052925.1); PeLV (*Pear latent virus*, NP_835239.1); PFBV (*Pelargonium flower break virus,* NP_945123.1); PLPV (*Pelargonium line pattern virus*, YP_238475.1); PMV (*Panicum mosaic virus*, NP_068342.1); PoLV (*Pothos latent virus*, CAA60596.1); PSNV (*Pea stem necrosis virus*, NP_862835.2) RCNMV (*Red clover necrotic mosaic virus*, NP_620523.1); RrLDV (Rosa rugosa leaf distortion virus KC1666238); SgCV (*Saguaro cactus virus*, NP_044382.1); SoYMMV (*Soybean yellow mottle mosaic virus*,

ACN59473.1); TBSV (*Tomato bushy stunt virus*, NP_062897.1); TCV (*Turnip crinkle virus*, AAP78486.1); TNVA (*Tobacco necrosis virus A* ADE10194.1); TNVD (*Tobacco necrosis virus D* ACT31460.1).

Fig. 3B. Unrooted phylogenetic trees of the coat protein amino acid sequences of *Tombusviridae*.

Coat protein amino acid sequence accession numbers used in the phylogenetic analysis were: AnFBV (*Angelonia flower break virus*, YP_459964.1); CarMV (*Carnation mottle virus,* NP_051885.1); CCFV (*Cardamine chlorotic fleck virus*, NP_041887.1); CLSV (*Cucumber leaf spot virus*, YP_512365.1); CPMoV (*Cowpea mottle virus*, NP_613272.1); CRSV (*Carnation ringspot virus*, NP_613255.1); CymRSV (*Cymbidium ringspot virus*, NP_613262.1); EBV (*Elderberry latent virus*, AAK74061.1); HCRSV (*Hibiscus chlorotic ringspot virus*, NP_619676.1); HnRSV (*Honeysuckle ringspot virus*, YP_004191793.1); JINRV (*Japanese iris necrotic ring virus*, NP_038458.1); MCMV (*Maize chlorotic mottle virus,* NP_619722.1); MNSV (*Melon necrotic spot virus*, NP_041231.1); OCSV (*Oat chlorotic stunt virus*, NP_619753.1); OMMV (*Olive mild mosaic virus,* YP_224020.1); PCRPV (*Pelargonium chlorotic ring pattern virus*, YP_052929.1); PFBV (*Pelargonium flower break virus*, ABD93258.1); PLPV (*Pelargonium line pattern virus*, YP_238481.1); PMV (*Panicum mosaic virus*, NP_068346.1); PoLV (*Pothos latent virus*, CAA60597.1); PSNV (*Pea stem necrosis virus*, NP_862839.1); RCNMV (*Red clover necrotic mosaic virus*, NP_620526.1); RrLDV (Rosa rugosa leaf distortion virus KC1666238); SgCV (*Saguaro cactus virus*,

NP_044388.1); SoYMMV (*Soybean yellow mottle mosaic virus*, YP_002333479.1); TBSV (*Tomato bushy stunt virus*, NP_062899.1); TCV (*Turnip crinkle virus*, NP_620723.1); TNVA (*Tobacco necrosis virus A* NP_056828.1).

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