

Complete genomic sequence of a *Tobacco rattle virus* isolate from Michigan-grown potatoes

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Abstract Tobacco rattle virus (TRV) causes stem mottle on potato leaves and necrotic arcs and rings in potato tubers, known as corky ringspot disease. Recently, TRV was reported in Michigan potato tubers cv. FL1879 exhibiting corky ringspot disease. Sequence analysis of the RNA-1-encoded 16-kDa gene of the Michigan isolate, designated MI-1, revealed homology to TRV isolates from Florida and Washington. Here, we report the complete genomic sequence of RNA-1 (6,791 nt) and RNA-2 (3,685 nt) of TRV MI-1. RNA-1 is predicted to contain four open reading frames, and the genome structure and phylogenetic analyses of the RNA-1 nucleotide sequence revealed significant homologies to the known sequences of other TRV-1 isolates. The relationships based on the full-length nucleotide sequence were different from those based on the 16-kDa gene encoded on genomic RNA-1 and reflect sequence variation within a 20–25-aa residue region of the 16-kDa protein. MI-1 RNA-2 is predicted to contain three ORFs, encoding the coat protein (CP), a 37.6-kDa protein (ORF 2b), and a 33.6-kDa protein (ORF 2c). In addition, it contains a region of similarity to the 3' terminus of RNA-1, including a truncated portion of the 16-kDa

cistron. Phylogenetic analysis of RNA-2, based on a comparison of nucleotide sequences with other members of the genus *Tobravirus*, indicates that TRV MI-1 and other North American isolates cluster as a distinct group. TRV MI-1 is only the second North American isolate for which there is a complete sequence of the genome, and it is distinct from the North American isolate TRV ORY. The relationship of the TRV MI-1 isolate to other tobnavirus isolates is discussed.

Tobacco rattle virus (TRV) is the type species of the genus *Tobravirus* (family *Virgaviridae*) which includes *Pea early browning virus* (PEBV) and *Pepper ringspot virus* (PepRSV). TRV virions contain a single-stranded, bipartite RNA genome of two positive-sense RNA molecules that are encapsidated separately into tubular, rigid, rod-shaped particles of two predominant lengths, 190 nm and 50–115 nm [12]. TRV is transmitted by stubby root nematodes in the genera *Trichodorus* and *Paratrichodorus* [22]. When TRV is transmitted to developing potato (*Solanum tuberosum* L.), symptoms including arcs, rings, and dark blotches are produced in tubers. Collectively, the disease is called corky ringspot (CRS) in the US and spraing in Europe [13]. CRS has been known to occur in the Pacific Northwest for some time [4, 20], and the occurrence of TRV in potato has been reported in California, Colorado, and Florida [5, 21, 25]. Recently, and for the first time, CRS symptoms were associated with TRV infection in potatoes grown in Michigan (isolate MI-1; 14) and in Minnesota and Wisconsin [9]. Systemic infections of TRV MI-1 in tobacco produced typical chlorotic, necrotic rings, necrotic lesions and mottling (Fig. 1a). Purified nucleoprotein preparations separated as two distinct bands in sucrose density gradients (Fig. 1b). The symptoms in

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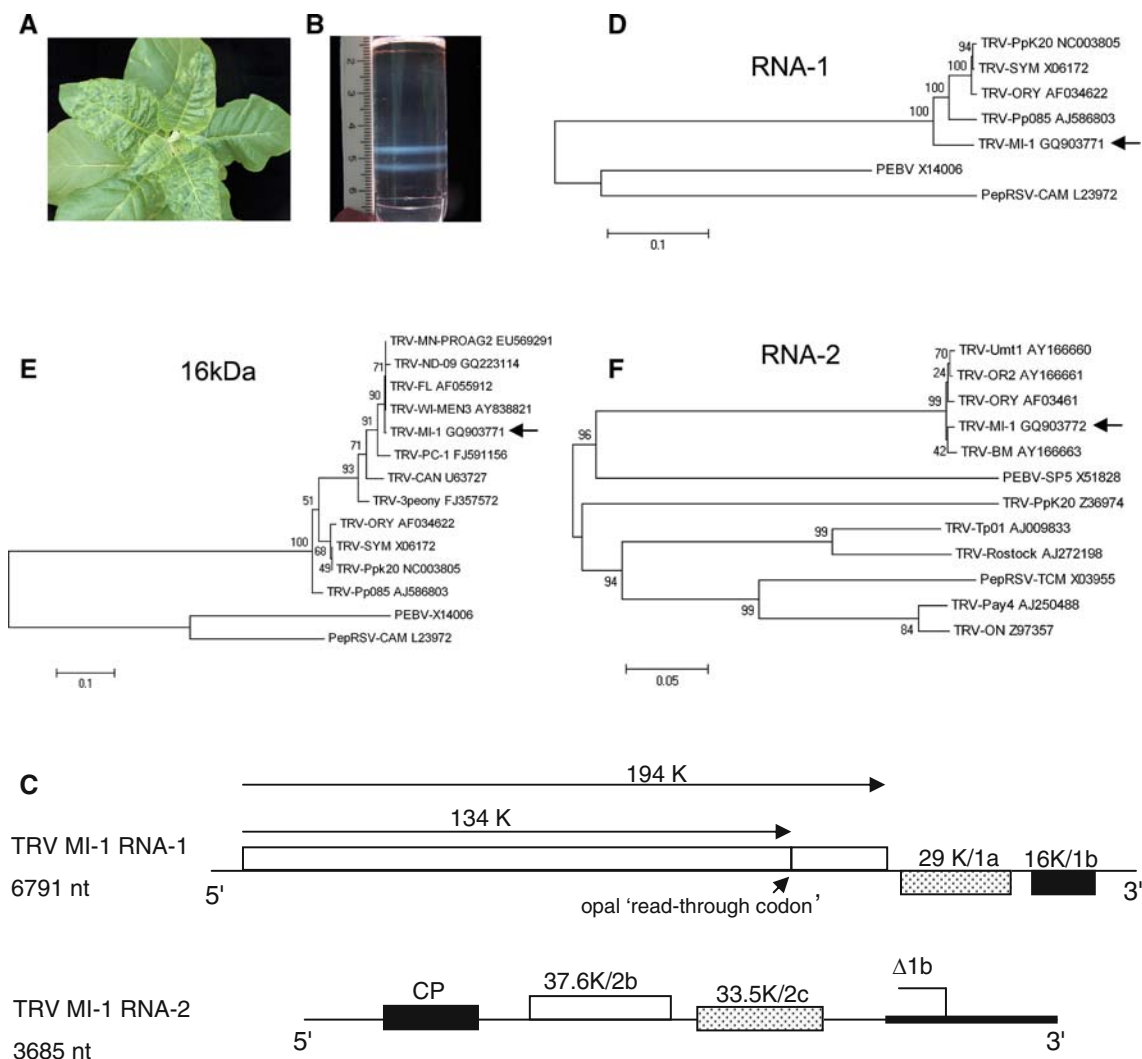
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tobacco and characteristics of the purified nucleoprotein preparations were similar to those of previously reported European and American isolates of TRV [5, 6, 11]. Here, we report the isolation and complete genomic sequence of TRV MI-1, obtained from CRS-symptomatic tuber tissue from potatoes grown in Michigan.

Potato tubers of cv. FL1867 exhibiting CRS symptoms were identified as described previously [14]. Approximately 1 g of symptomatic tuber tissue was ground with a mortar and pestle in 10 ml of cold 30 mM potassium phosphate buffer, pH 8.0, containing 10 mM sodium

diethyldithiocarbamate and 10 mM sodium thioglycollate (KDT buffer). Three carborundum-dusted leaves on each of two Samsun NN tobacco (*Nicotiana tabacum* L.) plants were rub-inoculated using a cotton swab. Plants were maintained in a greenhouse and observed for the mottling, necrotic lesions, and stem lesions characteristic of TRV infections [6, 11]. Symptomatic plants were tested by indirect enzyme-linked immunosorbent assay [3] using antiserum PVAS 820 obtained from the American Type Culture Collection (Manassas, VA, USA) to confirm the presence of TRV. Previously described TRV isolates served



of five TRV isolates, PEBV and PepRSV (**d**), the 16-kDa coding region of RNA-1 of 12 TRV isolates, PEBV and PepRSV (**e**), and the complete genome or RNA-2 of ten TRV isolates, PEBV and PepRSV (**f**). Multiple sequence alignments were obtained using the MEGA version 4 program, and the trees were constructed by the neighbor-joining method using 1,000 bootstrap replicates of the sequence data. The scale bar represents the number of nucleotide substitutions per site. Bootstrap confidence limits are shown at the nodes. The position of the TRV MI-1 isolate in each tree is indicated with an arrow

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as positive controls [5]. An isolate designated TRV MI-1 was selected for purification, cloning, and sequencing.

Nucleoproteins of TRV MI-1 were purified using a procedure modified from that of Bergh et al. [2]. Approximately 30 g of infected tobacco tissue was ground in 80 ml KDT buffer using a blender, and the slurry was strained through cheesecloth. The filtrate was centrifuged for 10 min at 15,000 rpm in a 42.1 rotor (Beckman, Palo Alto, CA, USA) at 4°C. The supernatant was adjusted to 4% (w/v) PEG (polyethylene glycol, MW6000-7000), 1.75% (w/v) sodium chloride was added, and the mixture was stirred on ice for 3–4 h followed by centrifugation for 15 min at 20,000 rpm in the same rotor at 4°C. The supernatant was discarded, and each of the large green pellets was resuspended in 0.5 ml of KDT overnight at 4°C. Vigorous pipetting aided resuspension. The following morning, the resuspended material was extracted with an equal volume of chloroform and centrifuged 3 min in a microcentrifuge to break the emulsion. The clarified supernatant was layered onto a 10–40% (w/v) sucrose gradient prepared the night before in 20 mM potassium phosphate buffer, pH 8.0 (PPB). The gradients were centrifuged for 2.25 h at 24,000 rpm in an SW25.1 rotor (Beckman) at 6°C. Virus bands were visualized by shining a dissecting light down through the gradients and were removed with a pipet. The collected materials were diluted with PPB and centrifuged for 2.25 h at 30,000 rpm in the 42.1 rotor at 4°C. The nucleoprotein pellets were resuspended in 0.5 ml of Tris-HCl, pH 8, overnight at 4°C. The virus concentration was determined by UV absorbance at 260 nm, assuming an extinction coefficient of 3 [15].

Viral RNA was extracted by incubating nucleoprotein preparations (50–100 µg/ml) in 1% sodium dodecyl sulfate and 100 µg/ml proteinase K for 1 h at 37°C and 10 min at 65°C. Preparations were then extracted with an equal volume of phenol-chloroform and centrifuged for 3 min. RNA was precipitated from the aqueous phase by adding 0.1 volume of 3 M sodium acetate (pH 5.6) and 2.5 volumes of 95% ethanol, incubating at –20°C for 1 h and centrifuging for 10 min in a microcentrifuge. The pelleted RNA was resuspended in 100 µl of sterile distilled water. Recovery of the RNA was verified by agarose gel electrophoresis of 5–10 µl of the preparations.

Full-length RNA-2 cDNA was prepared by RT-PCR as described previously using primers R2-4 (5'-ATAAAA CATTGCACCWWTGGTGGTTGC-3') and R2-3 (5'-CGTA ATAACGCTTACGTAGGCGAG-3') [5] and cloned into the pCRII-Topo vector (Invitrogen, Carlsbad, CA, USA). Full-length cDNA to RNA-1 was amplified from purified RNA preparations using primers r1-2 (5'-ATAAAACATT TCAATCCTTTGAACG-3') and r1-1 (5'-GGGCGTAATA ACGCTTACGTAG-3'). An aliquot of the reaction was evaluated by electrophoresis in a 1% agarose gel and

visualized by ethidium bromide staining and ultraviolet illumination. Initial attempts to clone the approximately 7,000-bp RNA-1 amplicon from ethidium-bromide-visualized material were unsuccessful. Instead, the amplicons were visualized with crystal violet and cloned using the pCR-XL-Topo kit (Invitrogen) as described by the manufacturer. Insert size was confirmed by *EcoRI* digestion and agarose gel electrophoresis. The full-length RNA-1 and RNA-2 cDNA plasmid clones were sequenced in both directions by ACGT, Inc. (Wheeling, IL, USA). The full-length sequences were deposited in GenBank as accession GQ903771 for TRV MI-1 RNA-1 and GQ903772 for TRV MI-1 RNA-2. Protein coding regions and sequence distances were computed using MapDraw and MegAlign programs in the DNASTAR package (DNASTAR, Inc., Madison, WI, USA). Phylogenetic and evolutionary analyses were conducted using the program *MEGA* version 4 [24].

TRV MI-1 RNA-1 consists of 6,791 nt, and the genome organization is similar to RNA-1 described for other TRV isolates [10, 18, 23]. RNA-1 encodes four putative open reading frames (ORFs) of predicted molecular weights 134, 194, 29 kDa (ORF 1a), and 16 kDa (ORF1b) and 5' and 3' non-coding regions (NCR) of 202 and 255 nt, respectively (Fig. 1c) (The ORF designation follows that in MacFarlane [18].) The first AUG begins at nucleotide 203 and encodes the first ORF (134 kDa) to a UGA (opal) stop codon. The ORF continues through this stop codon for a further 519 amino acid residues to encode the 194-kDa protein. The 134-kDa ORF encodes the amino terminal portion of the complete replicase of the 194-kDa protein and contains the helicase and nucleotide-binding motifs. The 29-kDa ORF encodes a putative movement protein [26], and the 16-kDa ORF is a small, cysteine-rich protein shown to act as a silencing suppressor protein and pathogenicity determinant [7, 16]. Phylogenetic analysis of the complete nucleotide sequence of TRV MI-1 RNA-1 with four reported full-length sequences of TRV RNA-1 revealed that TRV MI-1 is located on a branch separate from the other isolates (Fig. 1d). At the nucleotide level, there is 94–99% identity within the TRV cluster including TRV ORY; this cluster shares 92% sequence identity with TRV MI-1. The TRV isolates share only 60–62% identity at the nt level with PEBV and PepRSV. A closer examination of the RNA-1-encoded ORFs at the nt level revealed 97–100% identity among all TRV isolates in the 134/194-kDa ORF, 96–99% identity for ORF1a, and 85% identity between TRV MI-1 and the other TRV isolates in ORF 1b (the 16-kDa protein), while isolates within the TRV ORY cluster share 93–100% identity among themselves (data not shown). Therefore, the 16-kDa cysteine-rich protein (ORF 1b) appears to be less conserved than the other coding regions in RNA-1. Phylogenetic analysis of the nucleotide sequence of this region

among several of these isolates revealed the clustering of isolates into two main groups—one containing the European isolates (Pp085, SYM, PpK20) and the American (Oregon-ORY) isolate, while the other contains isolates from North America. Within the group containing TRV MI-1, there is 97–98% sequence identity, and this group shares 83–88% identity with the remaining isolates (Fig. 1e). The tobnaviruses PEBV and PepRSV are grouped on another branch of the tree and reflect the lack of 36 aa residues within 16-kDa coding region, resulting in translation of a 12-kDa cysteine-rich protein.

TRV MI-1 RNA-2 is 3,685 nt in length—computer prediction indicated three putative ORFs—and contains 5' and 3' NCRs of 522 and 571 nt, respectively. The first ORF, CP, encodes the 22.4-kDa putative coat protein, ORF2b encodes a 37.6-kDa protein, and ORF 2c encodes a protein of 33.6 kDa (Fig. 1c). Lastly, RNA-2 contains a region that is proposed to originate from the 3' terminus of RNA 1 of TRV, including a truncated portion (lacking the 5' 40 amino acids) of the 16-kDa cistron from the 3' end of RNA-1 [23]. Examination of pairwise alignments of RNA-2 revealed 92–98% identity among the distinct cluster of North American isolates, including MI-1, while these isolates share only 30–52% identity with European TRV isolates clustering in the second grouping (containing e.g. TRV-Rostock) (Fig. 1f). One or all of the proteins encoded in RNA-2 are speculated to play a role in transmission of tobnaviruses by their nematode vectors [19]. For example, a deletion in ORF 2c in the TRV-Umt1 isolate may contribute to its inability to be nematode-transmitted [5]. TRV MI-1 contains the complete ORF 2c coding region, leading to the prediction that it is nematode-transmissible.

In contrast to the relatively conserved genome organization of RNA-1 among TRV isolates, RNA-2 sequences differ widely in length and genome organization [18], which is reflected in the diversity revealed in the phylogenetic tree shown in Fig. 1f and in a report by Crosslin et al. [5]. The genome organization of TRV MI-1 RNA-2 most closely resembles that of TRV ORY and related isolates from North America [5]. The isolates that are less related, such as PEBV-SP5, PpK20, and others shown in the phylogenetic tree (Fig. 1f) either have truncated 2b and 2c ORFs, genome rearrangements, or additional ORFs [18]. Several of the isolates also contain the truncated 1b ORF at the 3' terminus of RNA 1, which may be the result of RNA recombination, contributing to the flexibility of the TRV genome.

As noted above, sequence differences in the 16-kDa region of RNA-1 are primarily responsible for the phylogenetic trees generated for RNA-1 and the 16-kDa gene (Fig. 1d, e). These nucleotide differences result in amino acid changes in a previously noted variable region (aa 83–117; absent in PEBV and PepRSV) of the 16-kDa protein.

This region has been shown to be tolerant of pentapeptide insertions without considerable reduction of the silencing suppressor function of the 16-kDa protein [7]. The North American isolate TRV ORY is more similar to the European isolates in this region than to the other North American isolates. European isolates, including TRV PpK20, and TRV ORY contain the region ⁸³REIWKQIRRNQAENMSA [T/A][A/T]KKSHNSKTSKKKFKED¹¹⁷, while TRV MI-1 and the remaining North American isolates contain the region ⁸³REIWKQIERVRAEKAFTTVKKSRSKPSKKI FKER¹¹⁷ (numbers indicate the location of the aa residue in the 16-kDa protein, and underlined residues represent differences between the two groups). It is not known if the residue differences in TRV MI-1 and related North American isolates affect the silencing suppression properties of the TRV PpK20 16-kDa protein studied by Ghazala et al. [7]. Alternatively, as the 16-kDa protein has been shown to be a determinant of pathogenicity, the amino acid differences may result in altered pathogenicity of these isolates.

Several questions arising from our study warrant additional research, such as (1) do the amino acid differences in the variable region of the 16-kDa protein between the TRV MI-1 (and related North American isolates) and the European isolates affect the silencing suppressor function of the protein? (2) What, if any, is the functional or evolutionary significance of the sequence similarity of the North American TRV ORY isolate with European isolates in genomic RNA-1, while TRV ORY RNA-2 clusters with other North American isolates? It is known that natural recombinants of RNA-1 and RNA-2 of tobnaviruses occur and that pseudo-recombinants between tobnaviral RNAs are stable; TRV ORY may reflect the generation of such a recombinant [1, 17]. (3) What is the significance of the retention of a truncated version of the 16-kDa gene on genomic RNA-2, and does it contribute to pathogenicity/silencing suppression? It has been proposed that a recombination event between RNA-1 and RNA-2 led to the presence of the C-terminal portion of the RNA-1-encoded 16-kDa protein on RNA-2 [1, 8]; however, it is retained in many TRV isolates in both Europe and North America.

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