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Genetic variability of genomic RNA 2 of four tobacco rattle tobravirus isolates from potato fields in the Northwestern United States

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Abstract

Sequence analysis of RNA 2 of four Tobacco rattle virus (TRV) isolates collected from potato fields in Oregon (OR2, Umt1), Washington (BM), and Colorado (Cot2) revealed significant homologies to the ORY isolate from North America. Phylogenetic analysis based on a comparison of nucleotide (nt) and amino acid (aa) sequences with other members of the genus Tobravirus indicates that the North American isolates cluster as a distinct group. All of the RNAs are predicted to contain open reading frames (ORFs) potentially encoding the coat protein (CP, ORF 2a) and 37.6 kDa (ORF 2b) ORFs. In addition, they all contain a region of similarity to the 3′ terminus of RNA 1 of ORY, including a truncated portion of the 16 kDa cistron from the 3′ end of RNA 1. Three of the isolates, which are nematode transmissible, OR2, BM, and Cot2, also contain a third putative ORF (ORF 2c) which encodes a protein of 33.6 kDa. The fourth isolate, Umt1, which is not nematode transmissible, is the most divergent of the isolates as it encodes a truncated version of ORF 2c. The ORF 2c deletion in Umt1 may contribute to its inability to be transmitted by the vector. The results reported in this article indicate again that the TRV genome is flexible. Interestingly, although both isolates Umt1 and Cot2 were mechanically transmitted to tobacco from potato, only Umt1 exhibits the deletion in RNA 2. TRV Isolate Umt1, therefore, appears to be another example of rapid adaptation of the TRV genome to non-field conditions.

Keywords: Tobravirus; Tobacco rattle virus; Variability; Phylogenetic analysis; Nematode; Vector transmission

1. Introduction

Tobacco rattle virus (TRV), type member of the Tobravirus group, has a single-stranded RNA genome consisting of two positive-sense RNA molecules encapsidated separately. The larger genomic segment, RNA 1, is encapsidated into the structurally "longer" nucleoprotein particles, and is approximately 7000 nucleotides (nt) in length. The sequence of TRV RNA 1 is 99% identical between the few isolates which have been sequenced to date (MacFarlane, 1999; Sudarshana and Berger, 1998). The smaller genomic segment, RNA 2, is encapsidated into the "shorter" nucleoprotein particles and varies in length from about 1900–3800 nt, depending upon the isolate. In contrast to the sequences of RNA 1, the sequence of RNA 2 shows considerable variability among virus isolates (Harrison and Robinson, 1986; MacFarlane, 1999).

The RNA 1 of tobraviruses contains either three or four open reading frames (ORFs) (MacFarlane, 1999). The largest of these is postulated to be involved in RNA replication, consisting of polymerase, helicase, and methyl transferase domains. Additionally, RNA 1 possesses an ORF coding for the movement protein (Hamilton et al., 1987) and the "16K" ORF, whose function is not well understood. The presence of a fourth ORF, for which the function has not been determined, was postulated by Angenent et al. (1989). Tobravirus RNA 2 codes for the capsid protein (CP)

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and one or two nonstructural genes are involved in nematode transmission (MacFarlane et al., 1996; Ploeg et al., 1993; Vassilakos et al., 2001; Vellios et al., 2002). The size and genomic organization of RNA 2 varies considerably among tobravirus isolates. Isolates that lack transmission genes can arise by repeated mechanical inoculation or by vegetative propagation (Hernandez et al., 1996; Visser et al., 1999). Recombination between tobravirus RNAs has been demonstrated (Goulden et al., 1991; Hernandez et al., 1996).

The RNA sequences of several tobraviruses from Europe have been published (for review, see MacFarlane, 1999), yet very little is known about the molecular characteristics of TRV isolates from the United States. The virus is widespread in the Pacific Northwest (Mojtahedi and Santo, 1999; Mojtahedi et al., 2001a,b) and Sudarshana and Berger (1998) have published the sequence of both genomic RNAs of isolate ORY (''Oregon yellow'', ATCC accession PV72; RNA1, GenBank Accession No. AF034622 and RNA2, GenBank Accession No. AF034621). Recently, Perez et al. (2000) reported the sequence of 463 bp of the 16K ORF of a TRV isolate from Florida. In this paper we report complete sequences and document sequence variability in RNA 2 of four TRV isolates obtained from different areas of the United States.

2. Materials and methods

2.1. Virus isolates

Four TRV isolates from the western United States were investigated. Isolate BM was isolated from a Samsun NN tobacco plant which had been planted in soil from a field near Pasco, WA known to be heavily infested with viruliferous nematodes (Crosslin et al., 1999). Isolate Umt1 was mechanically transmitted to tobacco from tissue of a corky ringspot syndrome (CRS)-symptomatic potato tuber of breeding clone A85519.6 grown in a field near Umatilla, OR (Crosslin et al., 1999). Isolate OR2 was obtained from a tobacco plant grown in soil from a similar site near Umatilla, OR. Isolate Cot2 was mechanically transmitted from a CRS-symptomatic potato tuber grown in Colorado. The BM, OR2, and Umt1 isolates had been maintained for about 2 years, and the Cot1 isolate for about a year, by approximately monthly mechanical inoculations to Samsun NN tobacco before being used for nucleic acid extraction and cloning.

All isolates were identified as TRV based on symptomatology on host plants, indirect enzyme-linked immunosorbent assay (ELISA), and reverse-transcription polymerase chain reaction (RT-PCR) with TRV-specific primers (Robinson, 1992; Crosslin et al., 1999). In transmission tests with Samsun NN tobacco, *Paratrichodorus allius*, the only known TRV vector species in the Columbia River basin of Washington and Oregon (Mojtahedi and Santo, 1999), transmitted the BM, Cot2, and OR2 isolates, but not the Umt1 isolate (H. Mojtahedi, unpublished data).

Symptoms of isolates BM, OR2, Umt1, and Cot2 on Samsun NN tobacco were similar. All produced systemic mottle, leaf distortion, necrotic lesions, and brown petiole and stem lesions. Controlled nematode inoculations to potato were limited to isolates OR2 and BM, which correspond to isolates Umatilla, and Pasco, respectively, of Mojtahedi et al. (2001a,b). On Norkotah Russet potato, the BM isolate usually produced more severe tuber symptoms than did the OR2 isolate (Mojtahedi et al., 2001a,b).

2.2. Reverse transcription-polymerase chain reaction (RT-PCR) and cloning of RNA 2

Total nucleic acid extracts were prepared from leaf disks of systemically infected Samsun NN tobacco as described (Presting et al., 1995). Oligonucleotide primers, based on the upstream (179) and downstream (180) primer sequences of MacFarlane (1996), were synthesized by Sigma–Genosys (Houston, TX). Some minor changes were made in the primer sequences. The triple degeneracy included by MacFarlane (1996) at nt 23 of the upstream (viral; sense) primer was changed to a T. The sequence used was 5′ ATAAAACATTGCACC[A/T][A/T]TGGTGTTGC 3′. The sequence of the downstream primer (complementary) was changed to remove the SmaI site at the 5′ end and to increase the length of the primer by three nts. The sequence was 5′ CGTAATAACGCTTACGTAGGCGAG 3′. The downstream primer was used to prepare the first-strand cDNA from the nucleic acid extracts as previously described (Crosslin and Thomas, 1995). PCR amplification was carried out similarly, using 20 pmol each of downstream and upstream primers in a 50 μl reaction. Cycling conditions were those reported by MacFarlane (1996) using a PTC-200 thermal cycler (MJ Research, Watertown, MA).

Amplification products were resolved by electrophoresis in 0.8% agarose gels stained with ethidium bromide. Bands were visualized under UV light and excised using Gene Capsule extractors (Geno Technology, St. Louis, MO) and purified by electroelution. The purified products ranged in size from approximately 2500–3500 base pairs (bp). One to 4 μl of the purified PCR products were ligated into the plasmid vector pCR2.1-Topo (Invitrogen, Carlsbad, CA) according to the manufacturers' instructions and transformed into *E. coli* Top 10 cells (Invitrogen). Plasmids containing inserts corresponding to the size of the PCR products were selected for sequence analysis.

2.3. Sequence analysis

Plasmid inserts were sequenced using M13 forward and reverse primers and internal primers designed from prior sequence information by the dideoxy chain termination method at the sequencing facility at Washington State University, Pullman, WA and at the University of Maryland Center for Agricultural Biotechnology Sequencing Facility using an automated DNA sequencer (Applied Biosystems International model 377, Perkin–Elmer/Applied Biosystems, Norwalk, CT). Sequence data were analyzed using LASERGENE software and CLUSTAL W or PROTEAN programs (DNASTAR, Inc., Madison, WI). Cladistic analysis was performed with the program phylogenetic analysis using parsimony (PAUP) version 4.0b10 written by Swofford (1993). Uninformative characters were excluded from the analysis and the phylogenetic tree was constructed using a branch and bound search, using random stepwise addition of sequences. Bootstrap analyses (1000 replicates) were performed to estimate support for the inferred phylogeny (Felsenstein, 1987).

3. Results

3.1. Genome organization and sequence analysis of cloned RNA 2

The complete sequences of RNA 2 of isolates OR2, Cot2, Umt1, and BM were obtained from a full-length cDNA clone of each isolate using both external, plasmid-specific primers and internal primers. Both strands were sequenced. The sequences have been deposited at GenBank and their accession numbers are listed in Table 1. Detailed descriptions of each isolate are shown in Table 2. A schematic representation of the deduced genome organization of the four tobravirus RNA 2 molecules is shown in Fig. 1.

To briefly summarize our results, sequence analysis of RNA 2 of four tobravirus isolates collected from potato fields in Oregon (OR2, Umt1), Washington (BM), and Colorado (Cot2) revealed significant homologies to the ORY isolate from North America (Sudarshana and Berger, 1998). Computer analysis revealed that all of the RNAs are predicted to contain ORFs which potentially encode the coat protein (CP, ORF 2a) and a 37.6 kDa protein (ORF 2b) (Fig. 1). In addition, they all contain a region that is proposed to originate from the 3′ terminus of RNA 1 of TRV (Sudarshana and Berger, 1998), including a truncated portion of the 16 kDa cistron from the 3′ end of RNA 1. Three of the isolates which are nematode transmissible, isolates OR2, BM, and Cot2, also contain a third putative ORF (ORF 2c) which encodes a protein of 33.6 kDa. The fourth isolate, Umt1, which is not nematode transmissible, contains the smallest and most divergent RNA 2 of the isolates as it encodes only a truncated version of ORF 2c (5′ 59 amino acids (aa) of 2c) (Fig. 1). The two incomplete ORFs (2c and 1b) present in the 3′ region of isolate Umt1 are encoded in separate and overlapping reading frames.

3.2. Comparison of RNA 2 with other tobravirus isolates

RNA 2 of the newly described isolates share 92–98% nt sequence identity among themselves, and with the previously sequenced isolate from Oregon, ORY. Isolate ORY shares the highest nt sequence identity (98%) to isolates OR2 and Umt1 (excluding the deletion in Umt1), also collected in Oregon. However, the isolates share only 30–50% sequence identity with TRV isolates ON, Pay4, Ppk20, Rostock, and Tp01, collected in Europe (Table 1). A phylogenetic tree, constructed by

Table 1
Tobravirus sequences used for comparisons

Virus isolate designation	Virus[b]	GenBank accession	Genome segment	Size (nts)	Reference
ORY (PV72[a])	TRV	AF034622	RNA 1	6790	Sudarshana and Berger, 1998
ORY (PV72[a])	TRV	AF034621	RNA 2	3261	Sudarshana and Berger, 1998
PpK-20	TRV	Z36974	RNA 2	3856	Hernandez et al., 1995
ON	TRV	Z97357	RNA 2	3357	Uhde et al., 1998
TCM	PepRSV	X03955	RNA 2	3389	Angenent et al., 1986
SP5	PEBV	X51828	RNA 2	3374	Goulden et al., 1990
Pay4	TRV	AJ250488	RNA 2	3926	Vassilakos et al., 2001
Tp01	TRV	AJ009833	RNA 2	3216	MacFarlane et al., 1999
Rostock	TRV	AJ272198	RNA 2	2014	Heinze et al., 2000
BM	TRV	AY166663	RNA 2	3536	this paper
OR2	TRV	AY166661	RNA 2	3379	this paper
Cot2	TRV	AY166662	RNA 2	3536	this paper
Umt1	TRV	AY166660	RNA 2	2905	this paper

[a] American Type Culture Collection (ATCC) accession.
[b] TRV, Tobacco rattle virus; PepRSV, Pepper ringspot virus; PEBV, Pea early browning virus.

Table 2
Detailed sequence information of isolates described in this study

Virus isolate designation	Length, nt	5′ NTR, 3′ NTR, nt	ORF's, kDa	Region proposed to originate from RNA 1[a]
OR2	3679	523, 565	22.3 (CP), 37.6 (2b), 34.6 (2c)	3139–3679
BM	3536	523, 421	22.2 (CP), 37.6 (2b), 33.6 (2c)	3120–3536
Cot2	3536	523, 421	22.3 (CP), 37.6 (2b) 33.5 (2c)	3120–3536
Umt1	2905	523, 703	22.3 (CP) 37.6 (2b), (5′ 59 aa's of 2c) (Δ2c)	2411–2905

[a] Contains a truncated ORF of the 16 kDa (ORF 1b) cistron from the 3′ region of RNA 1.

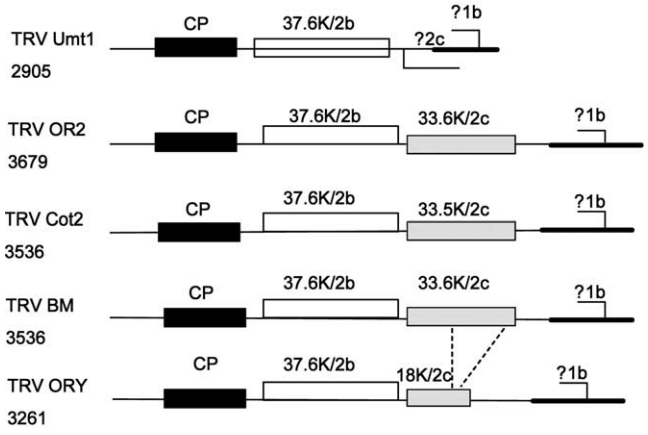

Fig. 1. Schematic representation of the genome organization of RNA 2 of the Umt1, OR2, Cot2, BM, and ORY isolates. CP, coat protein. Open-ended boxes denote truncated ORFs. The bold line indicates sequence homology to the 3′ terminus of RNA1 of ORY. Vertical dashed lines between isolates BM and ORY denote a deletion of 140 aas in ORF 2c of ORY.

comparison of the complete RNA 2 nt sequences of several tobravirus isolates, including TRV, Pea early browning virus (PEBV), and Pepper ringspot virus (PepRSV), revealed that the North American isolates form a phylogenetic cluster that is distinct from the other isolates (Fig. 2). The North American isolates also form a distinct cluster when the analysis is performed using alignment of aas encoded in the CP, 2b, and 2c ORFs (data not shown).

Examination of the pairwise alignments of the proteins encoded in the RNA 2 ORFs was made. Within the North American cluster, the aa identity in the CP is 97–99%. Although the North American and European isolates share only up to 45% aa identity in their respective CP's, there are blocks of conserved aas, as was noted by Sudarshana and Berger (1998) (data not shown). It should be noted that the European isolates share only 38–70% identity among themselves.

aa alignment of ORF 2b, one of the proteins found to be associated with nematode-transmissibility, revealed a broad range of sequence identity among all the TRV isolates analyzed, from 11 to 98%, with most variability again found between the European and North American isolates. TRV isolate PAY4 shares only 10–13% sequence identity with the North American isolates, but shares 95% sequence identity with the ON isolate. There are very few (9) aas that are conserved in ORF 2b across all of the isolates. Within the North American cluster, however, identities were high and ranged from 96 to 98%. The Umt 1 isolate, which in not transmissible by nematodes, differs from the nematode-transmissible isolates characterized in this study at four aa positions in ORF 2b.

Similar analysis of ORF 2c revealed a range of 4–98% sequence identity among all the isolates. This is the most divergent of the RNA 2 ORF's as there are no aa residues conserved across all of the TRV isolates sequenced to date (data not shown). The range of

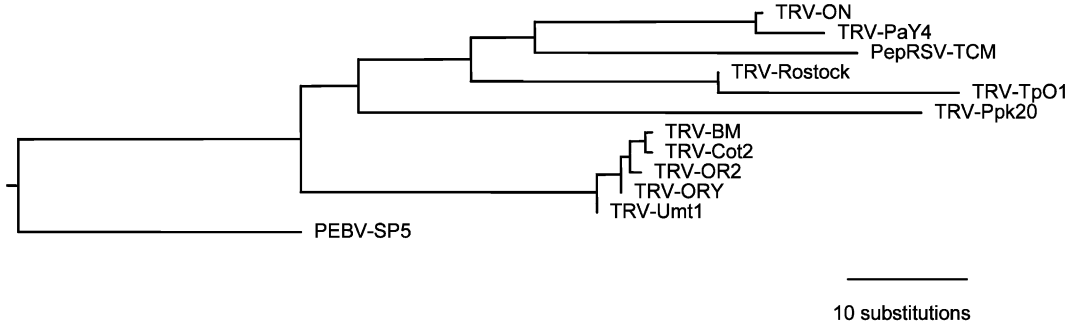

Fig. 2. Phylogram of the complete nt sequences of RNA 2 of isolates listed in Table 1. Nt sequences were aligned using the CLUSTAL W program. The single most parsimonius tree is also based on PAUP analysis using the branch and bound search and stepwise addition. The percentages of replication (bootstrap 50% majority-rule) that support each major branch are shown above the line. Below the tree is a scale bar indicating the number of nt substitutions.

sequence identities among the North American isolates is 73–98%. The wider range of sequence identity can also be accounted for by the observation that isolates ORY and Umt1 isolates lack 141 (47%) and 218 (72%) aas, respectively, of ORF 2c (as shown schematically in Fig. 1).

The presence and location in RNA 2 of the truncated 1b ORF and 3′NTR of RNA 1 are consistent with what has been found with other tobravirus isolates (MacFarlane, 1999) (Fig. 1). The 1b ORF encodes a 16 kDa cysteine-rich protein that plays a role in seed transmission of PEBV (Wang et al., 1997); no biological function has been attributed to this protein in TRV (Angenent et al., 1989; Guilford et al., 1991). The RNA 1-derived region has been proposed to arise by strand-switching during virus replication at A–U rich sequences that resemble the 5′- terminus of RNAs 1 and 2, or by stem-loop structures that form from direct repeats (Angenent et al., 1986; Hernandez et al., 1996; Swanson and MacFarlane, 1999). A region with the sequence AUUUUAAA occurs after ORF 2c in the region of nt 2880 in RNA 2 of isolates BM, Cot2, and OR2. Isolate Umt1 contains the sequence AUUUUACUUUU near nt position 2400 at the site where the 2c ORF is truncated. It is not known whether these sequences serve as switch points for the polymerase.

4. Discussion

Sequence analysis of RNA 2 of four TRV field isolates revealed similar genome organizations to other characterized tobravirus isolates (MacFarlane, 1999) and sequence conservation among the North American isolates. Interestingly, although isolates BM and Cot2 were collected from potato fields in two different geographic areas in the Northwestern region of the US, the sequences of RNA 2 are 95% identical to one another. This phenomenon has also been reported for the TRV Rostock isolate, collected in Germany, and the TRV TpO1 isolate collected in the United Kingdom (MacFarlane et al., 1999; Heinze et al., 2000). These two isolates are 98% identical at the 5′ terminal 1300 nts; however, Rostock RNA 2 is a recombinant with an RNA 1-derived region at its 3′ terminus while TpO1 is not a recombinant.

Most of the genetic variability among the North American isolates was found to be localized to ORF 2c, where Umt1 was found to have an extensive deletion in the coding region. Isolate ORY also contains a truncated, although larger, version of ORF 2c (Sudarshana and Berger, 1998; Fig. 1).

Tobraviruses are transmitted as intact virions by soil-inhabiting, root-feeding nematodes. Tobraviruses persist in the field only if their ability to be transmitted by the nematode vector is maintained. Isolates can lose their ability to be nematode transmitted by repeated mechanical inoculation or by vegetative propagation of infected crop plants (Harrison and Robinson, 1986; Hernandez et al., 1996). Two of the isolates described in this study, isolates BM (Washington) and OR2 (Oregon) were obtained from tobacco bait plants grown in soil collected from potato fields known to be heavily infested with viruliferous nematodes. Isolates Umt1 (Oregon) and Cot2 (Colorado) were both mechanically transmitted to tobacco from CRS-symptomatic potato tubers. In subsequent nematode transmission tests, only isolate Umt1 was not transmissible.

Although the molecular mechanisms underlying the transmission process are unknown, it has been shown that the genes encoded by RNA 2 determine the transmission of tobraviruses by their nematode vectors (MacFarlane and Brown, 1995; MacFarlane et al., 1996; Hernandez et al., 1997). Mutagenesis studies with infectious cDNA clones of the TRV isolate Ppk20 revealed that the protein product of ORF 2b gene, and not ORF 2c, was required for transmission by its nematode vector, *Paratrichodorus pachydermus* (Hernandez et al., 1995, 1997). In contrast, mutagenesis studies of infectious cDNA clones of the isolate TPA56 of PEBV revealed that the four proteins—CP, 2b, 2c, and a fourth, 9 kDa protein-all contribute to nematode transmission by *Trichodorus primtivus* (MacFarlane et al., 1996; Schmitt et al., 1998). Although the contribution of each RNA 2 ORF to vector transmission may be specific to the virus and vector species, the commonly speculated roles for RNA 2-encoded proteins in nematode transmission are, among others, recognition and interaction between the virus and receptors in the vector that may affect transmission efficiency and/or vector specificity (MacFarlane et al., 1999; Visser et al., 1999).

A closer examination of the RNA-2 encoded proteins of the nematode transmissible and non-transmissible North American TRV isolates was performed to gain a better understanding of the inability of isolate Umt1 to be transmitted by *P. allius*. Comparison of the CPs show that they share 97–99% sequence identity. Isolate Umt1 differs from OR2, its closest relative that is nematode transmissible, at one only position, with an aspartate to valine change at position 45.

The second ORF (2b) shares 96–99% sequence identity among the North American isolates, with Umt 1 carrying four aa differences when compared with the nematode transmissible OR2 isolate. Interestingly, a leucine to arginine change at residue 113 in Umt1 results in a localized protein conformational change that appears as a new potential antigenic determinant on the surface of the folded protein (as revealed by using the PROTEAN program of DNASTAR; data not shown). In another study, a single aa change (glycine to arginine) at position 177 in ORF 2b of PEBV prevented nematode transmission (Vellios et al., 2002). The region surround-

ing this aa was predicted to form a coiled-coil domain that may be important for interaction of the 2b protein with the virus coat protein or the nematode vector.

As stated earlier, most sequence divergence among the North American isolates occurs in ORF 2c. Although isolate Umt1 shares 98% sequence identity in the amino terminal portion of the 2c protein with the other isolates, it is lacking 218 of 299 aas of ORF 2c. The North American isolate ORY also encodes a truncated version of 2c (Fig. 1). ORY was obtained from the American Type Culture Collection and was maintained by mechanical transmission; it is not known if this isolate is transmissible by nematodes (P. Berger, personal communication). In light of the earlier reports on the role of ORF 2c in vector transmission, the ORF 2c deletion in Umt1 may contribute to its inability to be transmitted by its nematode vector.

The results reported in this article confirm the flexibility of the TRV genome. Interestingly, although both isolates Umt1 and Cot2 were mechanically transmitted to tobacco from potato, only Umt1 exhibits the deletion in RNA 2. TRV Isolate Umt1, therefore, appears to be another example of rapid adaptation of the TRV genome to non-field conditions and changes in selection pressure. It has been suggested that the high frequency of loss of genes required for vector transmission during replication has resulted in the virus developing a strategy using vector transmission as a bottleneck to eliminate so-called 'defective', less fit, species from the population (Pirone and Blanc, 1996; Visser et al., 1999).

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