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ScienceDirect
Virus Research 121 (2006) 199–204
Virus Research
www.elsevier.com/locate/virusres

A virus between families: nucleotide sequence and evolution of *Strawberry latent ringspot virus*

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Received 7 January 2005; received in revised form 31 May 2006; accepted 2 June 2006
Available online 11 July 2006

Abstract

Several clones of golden ginger mint (*Mentha* × *gracilis*, 'Variegata') were found infected with *Strawberry latent ringspot virus* (SLRSV). The virus was purified and cloned and the complete nucleotide sequence of a mint isolate was obtained. RNA 1 consists of 7496 nucleotides excluding the poly-A tail and encodes a polyprotein with signature enzymatic motifs found in other picorna-like plant viruses. RNA 2 consists of 3842 nucleotides excluding the poly-A tail, encoding a polyprotein that is processed to a putative movement protein and the two coat proteins of the virus. A satellite RNA of 1117 nucleotides was associated with this isolate encoding for a putative protein of 31 kDa. Phylogenetic analysis revealed that SLRSV shares characteristics with members of the *Cheravirus, Fabavirus, Comovirus* and *Sadwavirus* genera indicative of the uniqueness of SLRSV. The close relationship of SLRSV with these genera led to the examination of aphid and beetle transmission of the virus with, however, negative results.

Keywords: *Comovirus*; *Cheravirus*; Nucleotide sequence; Phylogenetic analysis

1. Introduction

Strawberry latent ringspot virus (SLRSV) was first identified more than 40 years ago (Lister, 1964), and its host range exceeds 126 species belonging to 27 families (Schmelzer, 1969). SLRSV is transmitted by *Xiphinema diversicaudatum* and *X. coxi* (Murant, 1974), and this is the reason that the virus was originally classified as a nepovirus. The virus RNA 2 encodes the structural proteins and sequence analysis indicated that these proteins are not related to any member of the *Comoviridae* family (Everett et al., 1994; Kreiah et al., 1994). Since these reports, no additional SLRSV sequence data have been obtained.

SLRSV was recently found in commercial strawberry fields in the western coast of the United States, where the nematode vectors are not known to exist and in *Mentha* × *gracilis*, 'Variegata' obtained from mail-in nurseries in Maryland, Ohio and Nebraska (Martin et al., 2004; Postman et al., 2004). These were unexpected findings given the nematode elimination strategies used in strawberry production and nursery industry.

This communication presents the complete nucleotide sequence of a mint isolate of SLRSV termed SLRSV-M. RNA 1 encodes the viral non-structural proteins and has genome organization similar to that of picorna-like plant viruses. RNA 2 encodes a polyprotein that is processed releasing two coat proteins (CP) and a peptide predicted to be the movement protein (Kreiah et al., 1994). SLRSV-M was accompanied by a satellite RNA for which the sequence was also determined. Sequence analysis indicated a close relationship of the SLRSV replication-related proteins with orthologous proteins of the *Fabavirus* and *Comovirus* genera while the CP, often a determinant of vector transmission (Harrison et al., 1974; Hohnle et al., 2001; Andret-Link et al., 2004; Lee et al., 2005), shared no obvious homology with other CPs in these genera. These findings led to the hypothesis that SLRSV may be transmitted by an aphid or a beetle vector in addition to nematodes, although there are no reports of viruses being transmitted by more than one type of vector.

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doi:10.1016/j.virusres.2006.06.001

2. Materials and methods

2.1. Virus purification, cDNA synthesis and genome amplification

The National Clonal Germplasm Repository (NCGR) variegated mint clone MEN 454.001 was used as a source of SLRSV in this study. The virus was transferred from mint to *N. occidentalis* by mechanical inoculation and was purified according to the method of MacDonald et al. (1991). Reverse-transcription (RT) of the viral single-stranded RNA and cDNA cloning was conducted as described by Tzanetakis et al. (2005a). Alignment with related viruses in GenBank (Altschul et al., 1997) indicated that most of the genome was successfully cloned. The missing regions were acquired after reverse transcription-polymerase chain reaction (RT-PCR) using procedures described by Tzanetakis et al. (2005c) while the 5′ termini were obtained after performing 5′ RACE (Invitrogen, Carlsbad, CA) according to manufacturer's instructions.

2.2. Genome and phylogenetic analysis

Sequencing was performed at the facilities of Macrogen Corp. (Seoul, South Korea) using an ABI 3730 XL automatic DNA sequencer. The consensus sequence was obtained after aligning at least four clones obtained either by shotgun cloning, cloned RT-PCR products or directly sequenced PCR products. Genome assembly was done with the CAP3 software (Huang, 1996). The sequences have been deposited in the GenBank under the accession numbers AY860978, AY860979 and AY860980 for RNA 1, RNA 2 and the satellite RNA, respectively.

The cleavage sites of the RNA 1 polyprotein were identified after aligning the protein sequences of SLRSV, *Apple latent spherical virus* (ALSV) and *Cherry rasp leaf virus* (CRLV) with ClustalW (Thompson et al., 1994), together with the predicted protein secondary structure using the SSpro software (Pollastri et al., 2002). The amino acid (aa) identity and similarity of SLRSV proteins with orthologous proteins of related viruses were calculated using MatGat (Campanella et al., 2003). Phylogenetic analysis of the conserved helicase and polymerase motifs was performed using ClustalW (Thompson et al., 1994) and trees were visualized using TreeView (Page, 1996).

2.3. Immunocapture reverse transcription-polymerase chain reaction (IC-RT-PCR)

IC-RT-PCR was performed as described elsewhere (Wetzel et al., 1992). Three sources of SLRSV antibodies, two commercial sources (Agdia, Elkhart, IN and STA Laboratories, Longmont, CO) and one from Dr. Stace-Smith (Agriculture and Agri-Food Canada, Vancouver, BC) in addition to antibodies against *Tomato ringspot virus* and *Tobacco ringspot virus* from our antibody collection were utilized to capture virions onto Maxisorb microtiter plates (NUNC, Rochester, NY). The RT reaction was carried out in a microtiter plate, then an aliquot of that reaction was used for the PCR reaction (Tzanetakis et al., 2004) using oligonucleotide primers F (5′ CAGGTGGAGCCAACAGGTGATCTTCT 3′) and R (5′ TTCAGGGGTAAGGGAGCAGGGGCTATC 3′) that amplify a 1664 nucleotide (nt) region of RNA 1.

2.4. Vector transmission studies

Two potential insects were tested for their ability to transmit SLRSV. *Ovatus crataegarius* (mint aphid), the dominant aphid species colonizing mint, were given a 72 h acquisition access period on the mint clone, MEN 454.001, prior to transfering 10 aphids to each of 30 healthy mint plants for a 72 h inoculation access period. Thirty days post inoculation, test plants were examined for the presence of SLRSV using RT-PCR as described (Postman et al., 2004).

Diabrotica undecimpunctata howardii (spotted cucumber beetle), a known vector of many comoviruses (Gergerich and Scott, 1996), was evaluated for transmission of SLRSV. SLRSV-M was transferred to cucumber as the virus that reaches high titers in the plant. Field-collected adult spotted cucumber beetles, were held in captivity on detached pinto bean (*Phaseolus vulgaris*) leaves for 2 days. Single beetles were placed on SLRSV-M infected cucumber leaves, in Petri dishes overnight, and five beetles that had fed on the acquisition tissue were given access to individual cucumber seedlings for 16 h. After a 3-week incubation period in the greenhouse, the cucumber plants were evaluated for virus infection using ELISA (Edwards and Cooper, 1985).

3. Results

The complete nucleotide sequence of SLRSV-M was determined. The 5′ untranslated regions (UTR) are 252 and 312 nt for RNA 1 and 2, respectively, with 85% sequence identity in the first 111 nt. The 3′ UTR of RNA 1 and 2 are both 560 nt long excluding the poly-A tail with 81% nt sequence identity. While RNA 1 and 2 share high nucleotide identity in both UTRs, verification that RNA 1 belongs to SLRSV was accomplished by IC-RT-PCR. Amplicons were obtained only when plates were coated with anti-SLRSV antibodies (Fig. 1). All RNA 1 and 2 polyprotein cleavage sites are predicted to reside between Ser-Gly**.** This cleavage site has been experimentally determined for RNA 2 by protein sequencing (Everett et al., 1994; Kreiah et

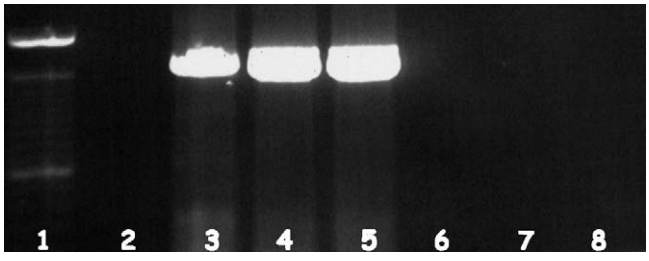

Fig. 1. Immunocapture reverse transcription-polymerase chain reaction for verification of the identity of *Strawberry latent ringspot virus* (SLRSV) RNA 1. Lane 1: 100 base-pairs marker (Invitrogen, Carlsbad, CA); lane 2: blank; lanes 3–5: 1664 bps amplicons obtained after capture of virions using three different sources of SLRSV antibodies; lanes 6–7: *Tomato ringspot virus*- and *Tobacco ringspot virus*-antibody coated wells; lane 8: well coated with blocking agent.

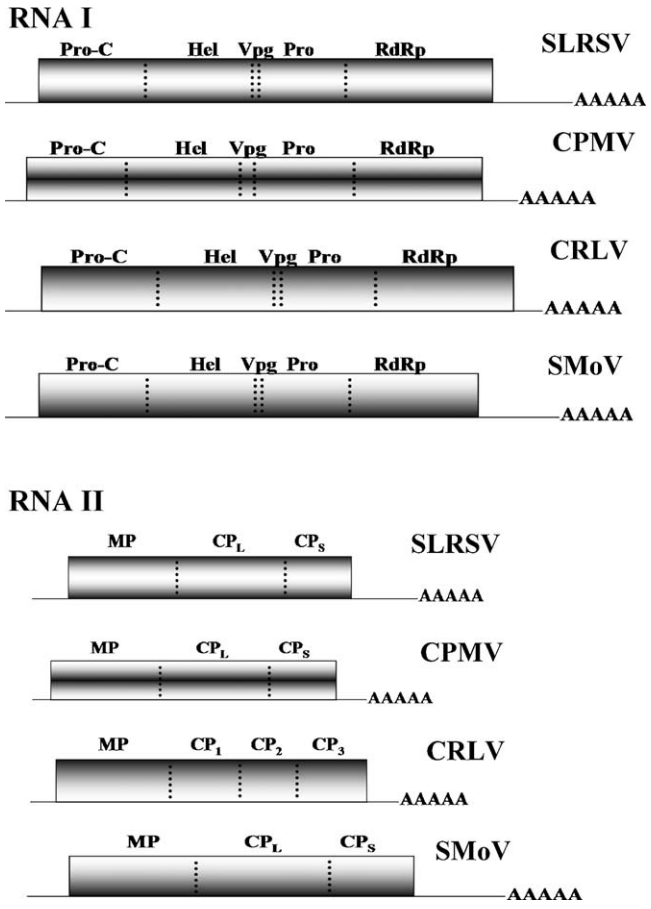

Fig. 2. Genomic organization comparison of *Strawberry latent ringspot virus* (SLRSV) and representative species in genera of other picorna-like plant viruses. *Abbreviations*: *Cherry rasp leaf virus*, CRLV (*Cheravirus*); *Cowpea mosaic virus*, CPMV (*Comovirus*); *Strawberry mottle virus*, SMoV (*Sadwavirus*); protease cofactor (Pro-C); helicase (Hel); viral genome-linked protein (Vpg); protease (Pro); RNA-dependent RNA polymerase (RdRp); movement protein (MP); large coat protein (CP_L); small coat protein (CP_S).

al., 1994). The glycine N-terminus of mature proteins has been shown to have a long turn-around time in cells (Bachmair et al., 1986), a predicted trait of viral proteins.

RNA 1 is 7496 nt excluding the poly-A tail and encodes a polyprotein of 2227 aa with a predicted mass of 250 kDa (Fig. 2). The putative start codon at 253–255 nt is in excellent context for translation (Kozak, 1986). The N-terminus of the polyprotein is predicted to encode the protease cofactor, which serves as a regulator of the viral cysteine protease (Peters et al., 1992). The 81 kDa putative protease cofactor did not share significant homology with other viral proteins found in GenBank other than the protease cofactor motif found in como- and nepoviruses. The F-X_{27}-W-X_{11}-L-X_{21}-L-X-E motif (X can be any amino acid) (Ritzenthaler et al., 1991) is modified in SLRSV with Met instead of Trp, and Val instead of Leu (modified aa underlined in the motif). The second region of the polyprotein encodes a putative RNA helicase (Fig. 2). The 61 kDa putative protein is found between residues 720 and 1276. Helicase conserved motifs were identified between 68 and 266 aa of the mature peptide (Marchler-Bauer et al., 2003) while a transmembrane domain motif was found between residues 505 and 527. The viral genome-linked protein (Vpg) located downstream of the helicase was 28 aa long and did not contain the *Comoviridae* family conserved motif (Mayo and Fritsch, 1994), nor the modified motif proposed by Thompson et al. (2002). A putative cysteine 3C-like protease was located immediately downstream the Vpg (Fig. 2). The 29 kDa predicted protein has a conserved His-Asp-Cys catalytic triad at positions 1346, 1383 and 1484, respectively, and was in agreement with the analogous motifs identified previously (Rott et al., 1995; Someya et al., 2002). The 76 kDa polymerase, found at the C-terminus of the polyprotein, contained the eight motifs characteristic of RNA dependent RNA polymerases (Koonin, 1991) between 1752 and 2013 aa (Fig. 2).

SLRSV-M RNA 2 encodes a 109 kDa polyprotein with 82% nt and 93% aa sequence identity (98% similarity) with a cherry isolate from New Zealand (SLRSV-NZ) (Everett et al., 1994), as well as 84% nt identity over the length of the molecule and 94% aa identity (96% similarity) for the first 833 aa of the polyprotein of a strawberry isolate (SLRSV-H) from the United Kingdom (Kreiah et al., 1994). The SLRSV-M RNA 2 polyprotein is predicted to be processed to three mature proteins, including two CPs and a 366 aa peptide at the N-terminus of the polyprotein, having a Leu-Pro-Leu motif (184–186 aa) found in *Comovirus* and other viral movement proteins (Fig. 2) (Koonin et al., 1991). The 40 kDa polypeptide is hypothesized to function as the viral movement protein (MP). Based on the proposed cleavage sites, the first aa in the two CPs of SLRSV-M are identical to those of SLRSV-NZ and -H isolates. The sizes of the CPs are in agreement with what has been reported previously, with the large CP being 43 kDa and the small CP found downstream of its counterpart being 26 kDa (Fig. 2) (Everett et al., 1994; Kreiah et al., 1994).

The satellite RNA associated with SLRSV-M is 1117 nt and shared 88% nucleotide sequence identity with the satellite of SLRSV-H (Kreiah et al., 1993). The molecule has a single open reading frame initiating at 42 nt and terminating at position 905. The start codon of the putative 31 kDa protein is in good translational context and the predicted polypeptide did not have significant similarity with other proteins in GenBank.

All 30 plants used for transmission of SLRSV-M with the mint aphid, tested negative for the virus by RT-PCR 30 days post inoculation, unlike the case of Mint vein banding associated virus and Mint virus-1 transmissions (Tzanetakis et al., 2005b,c). Thirty-two plants used in the transmission tests with the spotted cucumber beetle also tested negative for the presence of the virus, while it was successfully used for transmission of *Cowpea mosaic virus* among others (Gergerich et al., 1991).

4. Discussion

SLRSV is an economically important virus due to its extensive host range and the yield losses it can cause. SLRSV was considered a quarantine virus in the United States until its recent identification in both strawberry and mint (Martin et al., 2004; Postman et al., 2004). The use of methyl bromide as a soil disinfectant should eliminate the nematode vectors of the virus. Because SLRSV is now known to be geographically widespread

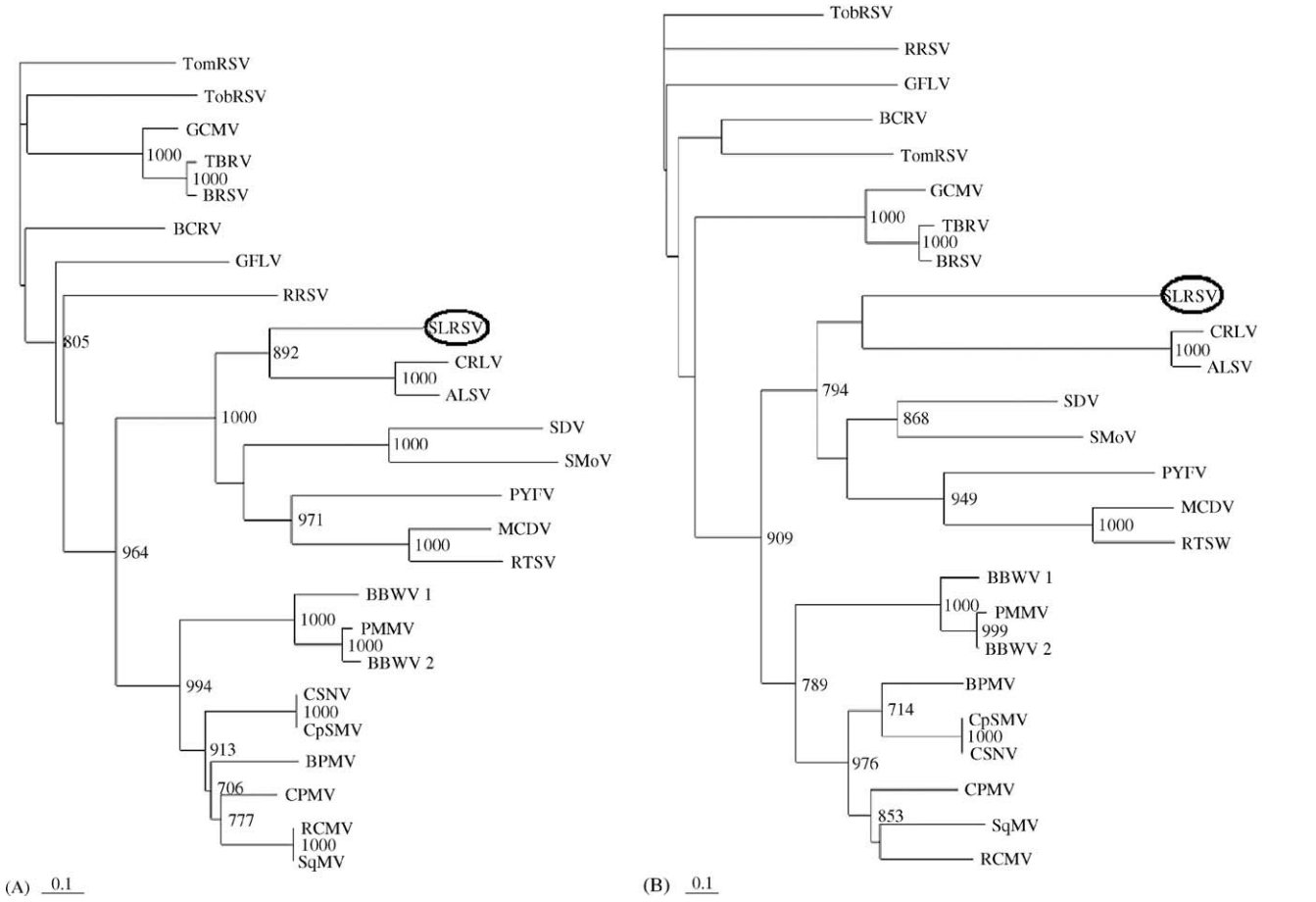

Fig. 3. Unrooted phylograms of the (A) helicase and (B) polymerase motifs of *Strawberry latent ringspot virus* and related viruses. The sequences used for the construction of the phylogram were obtained from the GenBank accessions of the complete genome of the viruses. *Abbreviations*: ALSV, *Apple latent spherical virus*; BPMV, *Bean pod mottle virus*; BRSV, *Beet ringspot virus*; BCRV, *Blackcurrant reversion virus*; BBWV 1, *Broad bean wilt virus 1*; BBWV 2, *Broad bean wilt virus 2*; CRLV, *Cherry rasp leaf virus*; CPMV, *Cowpea mosaic virus*; CpSMV, *Cowpea severe mosaic virus*; CNSV, *Cycas necrotic stunt virus*; GCMV, *Grapevine chrome mosaic virus*; GFLV, *Grapevine fanleaf virus*; MCDV, *Maize chlorotic dwarf virus*; PYFV, *Parsnip yellow fleck virus*; PMMV, *Patchouli mild mosaic virus*; RRSV, *Raspberry ringspot virus*; RCMV, *Red clover mottle virus*; RTSV, *Rice tungro spherical virus*; SDV, *Satsuma dwarf virus*; SqMV, *Squash mosaic virus*; SLRSV, *Strawberry latent ringspot virus*; SMoV, *Strawberry mottle virus*; TobRSV, *Tobacco ringspot virus*; TBRV, *Tomato black ring virus*; TomRSV, *Tomato ringspot virus*. The numerical values of the nodes with bootstrap values of less than 70% are not shown, as they are not considered significant. The bars represents 0.1 amino acid changes per site.

in the United States, it was decided to characterize the virus at the molecular level in order to provide insight into any alternative means of transmission. This is the first report of the molecular characterization of RNA 1 of SLRSV, and this study revealed important differences between the RNA 2 molecules of SLRSV-NZ, SLRSV-H and SLRSV-M. The complete nt sequence of SLRSV reveals further variability among the picorna-like plant viruses.

Gly-Ser proteolytic cleavage sites are not common in the *Comoviridae* or *Cheravirus* genus. Protein sequencing of several proteins from the SLRSV-H and -NZ isolates (Everett et al., 1994; Kreiah et al., 1994), in addition to the presence of hydrophobic residues at position-4 in all the putative sites, a possible determinant of the catalytic reaction (Wellink and van Kammen, 1988) provides strong evidence that the virus uses this cleavage site for processing of the polyproteins. The protease cofactor motif and the transmembrane region in the helicase, shown to anchor the protein and the replication complex onto the membranes in the case of *Tomato ringspot virus* (Wang et al., 2004) indicate conservation of motifs and probably function between SLRSV and other species in the *Comoviridae*. The polymerase is the most conserved region of the genome, having about 50% identity and 63% similarity with the orthologous regions of the *Cheravirus* members (Mayo, 2005).

Phylogenetic analysis of the conserved helicase and polymerase motifs (Fig. 3) indicated that SLRSV is closely related to ALSV (Li et al., 2000) and CRLV (James and Upton, 2002; Thompson et al., 2004), making SLRSV a provisional member of the newly formed *Cheravirus* genus rather than the *Sadwavirus* genus (Mayo, 2005). Based on analysis of the replication-associated proteins, SLRSV, ALSV and CRLV are more closely related than any other viruses available in the GenBank, but they differ in several ways. SLRSV has two CPs instead of the three of ALSV and CRLV (Fig. 2). The similarity between the CPs of the 25 viruses used in the phylogenetic analysis typically having less than 25% aa identity, is not sufficiently significant to determine evolutionary relationships (Fig. 2). The number of the structural peptides may be indicative of a possible

genetic exchange between viruses or alternatively may provide an insight into the evolution of the picorna-like plant viruses. For example, SLRSV or a virus similar to it may have been the progenitor of the *Cheravirus* and *Sadwavirus* genera and the *Comoviridae*. Transmission studies using the mint aphid and the spotted cucumber beetle, failed to demonstration transmission of the virus by either vector.

SLRSV-H RNA 2 has a stop codon at 731–733 nt not found in SLRSV-M. As a consequence SLRSV-M encodes a 120 aa larger polyprotein. Because the N-termini of the polyproteins have the Leu-Pro-Leu motif and therefore could be identified as putative movement proteins, it appears that of SLRSV-M MP is 13 kDa larger than the 27 kDa of its SLRSV-H counterpart. Another difference between the two isolates is an additional 24 aa at the C-terminus of the SLRSV-H RNA 2 polyprotein. SLRSV-M RNA 2 polyprotein terminates at position 3282 in contrast to the termination signal of SLRSV-H found at 3342 nt. Since UTRs of SLRSV-M and SLRSV-NZ are about 90% identical and are of similar sizes (560 and 552, respectively), the predicted extension of the SLRSV-H is found in a region that is occupied by the UTRs. In addition, the last 57 nt of the SLRSV-H polyprotein extension share 90% identity with both 3′ UTRs of SLRSV-M, another indication that the extension is probably part of the 3′ UTR, fused to the protein probably by an analysis or sequencing mistake. Further work is under way to determine the transmission efficiency of the United States' isolates of SLRSV with different species of nematodes.

Acknowledgments

This project was funded by the Mint Industry Research Council and the U.S. Department of Agriculture.

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