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Sequence analysis and location of capsid proteins within RNA 2 of strawberry latent ringspot virus

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The nucleotide sequence of the RNA 2 of a strawberry isolate (H) of strawberry latent ringspot virus (SLRSV) comprised 3824 nucleotides and contained one long open reading frame with a theoretical coding capacity of 890 amino acids equivalent to a protein of 98·8K. The N-terminal amino acid sequences of virion-derived proteins were determined by Edman degradation allowing the capsid coding regions to be located and serine/glycine cleavage sites to be identified within the polyprotein. The amino acid sequence in the capsid coding region of an isolate of SLRSV from flowering cherry in New Zealand was 97% identical to that of SLRSV-H. Except in the 3′ and 5′ terminal non-coding sequences, computer-based alignment and comparison algorithms did not reveal any substantial homologies between RNA 2 of SLRSV-H and the equivalent genomic segments in the nepoviruses arabis mosaic, cherry leaf roll, grapevine fanleaf, raspberry ringspot, grapevine hungarian chrome mosaic, tomato blackring, tomato ringspot, tobacco ringspot, or in the comoviruses cowpea mosaic and red clover mottle. Despite the similarities in overall genome organization, data from RNA 2 remain insufficient for unambiguous positioning of SLRSV in relation to species/genera in the *Comoviridae*.

Strawberry latent ringspot virus (SLRSV), which is transmitted by soil-inhabiting nematodes (*Xiphinema diversicaudatum* and *X. coxi*: Lister, 1964; Murant, 1974), has a bipartite genome composed of two positive ssRNA molecules. RNA 1 (M_r $2{\cdot}9 \times 10^6$) and RNA 2 (M_r $1{\cdot}4 \times 10^6$) are 3′-polyadenylated (Mayo *et al.*, 1979) and have a genome-linked protein (VPg) covalently attached to their 5′ termini (Mayo *et al.*, 1982). The genomic RNAs, each of which encodes a polyprotein that is cleaved post-translationally from a common precursor (Hellen *et al.*, 1991), are encapsidated separately in isometric particles composed of two polypeptides of M_r 44K and 29K (Mayo *et al.*, 1974; Gallitelli *et al.*, 1982). SLRSV has been grouped, as a tentative member, within the nepoviruses which (because of similarities in their genome organization) are themselves classified with the insect-transmitted comoviruses and fabaviruses as genera in the *Comoviridae* (Mayo & Martelli, 1993). Although extra-genomic RNAs have not been reported in comoviruses or fabaviruses, several nepoviruses encapsidate such RNAs which are satellites as defined by Murant & Mayo (1982). The nucleotide sequence of a satellite RNA (RNA 3) associated with the type isolate (SLRSV-H; from strawberry in Hampshire, U.K.; Lister, 1964) was described by Kreiah *et al.* (1993).

The nucleotide sequence data reported in this paper will appear in the EMBL database under the accession number X77466.

The complete sequence has been determined for M RNA = RNA 2 of cowpea mosaic comovirus (CPMV; Van Wezenbeek *et al.*, 1983) and red clover mottle comovirus (RCMV; Shanks *et al.*, 1986) as have those of the corresponding RNAs of nepoviruses including raspberry ringspot (RRV; Blok *et al.*, 1992), hungarian grapevine chrome mosaic (GCMV; Brault *et al.*, 1989), tomato black ring (TBRV; Meyer *et al.*, 1986), grapevine fan leaf (GFLV; Serghini *et al.*, 1990) and tomato ringspot viruses (ToRSV; Rott *et al.*, 1991). The capsid coding sequence in RNA 2 from an isolate of SLRSV from flowering cherries in New Zealand was published recently (SLRSV-NZ; Everett *et al.*, 1994). Partial sequence data are available for the following nepoviruses: arabis mosaic, ArMV (Bertioli *et al.*, 1991); cherry leaf roll, CLRV (Scott *et al.*, 1993); and tobacco ringspot, TRSV (Buckley *et al.*, 1993). In this paper we report the sequence of RNA 2 of SLRSV-H (culture T39; obtained from the Scottish Crop Research Institute) and assess the relationship of this virus to others in the *Comoviridae*.

After propagation in *Chenopodium quinoa*, virion-derived RNA from SLRSV-H (three size classes) was used as a template for cDNA synthesis and for cloning as described by Kreiah *et al.* (1993). Transformed bacteria containing sequences complementary to SLRSV-H RNA 2 were first identified as described by Grunstein & Hogness (1975) and then cloned cDNA was characterized

by northern transfer hybridizations in which virion-derived RNAs from a lilac isolate of ArMV or a birch isolate of CLRV or SLRSV-H were first denatured at 65 °C for 10 min then fractionated in a 1% (w/v) non-denatured agarose gel at 50 V for 5 h. The separated RNAs were transferred into Hybond-N membrane by capillary elution essentially as described by Sambrook *et al.* (1989) and probed with [α-^{32}P]dATP-labelled cDNA specific for RNA 2 of SLRSV-H. In such tests, cDNA specific for RNA 2 of SLRSV-H did not hybridize to genomic RNAs of ArMV or CLRV or to RNA 3 of SLRSV-H. Nevertheless, the 5′ region of SLRSV-H was cloned using PCR and an upstream primer complementary in sequence to residues 1 to 10 of RNA 3 of SLRSV-H (i.e. UUGAAAAGAA; Kreiah *et al.*, 1993). The reaction was catalysed with 2 units of Taq DNA polymerase (Boehringer Mannheim) and was subjected to 35 cycles of 94 °C for 1 min, 55 °C for 1·5 min and 72 °C for 1 min with a final cycle at 72 °C for 10 min. The ssDNA template for this PCR amplification had been synthesized from virion RNA (8 µg) and avian myeloblastosis reverse transcriptase (Life Sciences Inc.; 20 units) and primed with an oligomer complementary to residues 1276 to 1307 in SLRSV-H RNA 2. These reactions produced one cDNA species which was cloned into the *Eco*RI-digested pT7T3-18U and, as with the other RNA 2 clones, inserted in both orientations into the phagemid pT7T3-18U from which nested deletion libraries were made by controlled digestion with exonuclease III. The helper phage M13K07 (Pharmacia) was used to produce ssDNA from the resulting subclones.

A variety of approaches was used to determine the base sequence. When dsDNA was to be sequenced, it was prepared as described by Chen & Seeberg (1985) and analysed using a dideoxynucleotide chain termination method (Sanger *et al.*, 1977) and the 'universal' or the M13 reverse sequencing primers with the modified DNA polymerase Sequenase (U.S. Biochemical Corp.). Clones were also sequenced using a series of oligonucleotides in addition to the universal and M13 reverse primers. Primers used for this purpose had the following sequences.

5′ TGTAAGACATGGCGGGAG 3′ (complementary to residues 983–1000)
5′ GGGTTCCTCCTCATCAGT 3′ (complementary to residues 703–720)
5′ CCTCCATCTCGCGTGGAG 3′ (complementary to residues 372–389)
5′ GCGACGACTGAATGCGGC 3′ (sense to residues 401–418)
5′ AACTTGGAGTCACGTCCC 3′ (sense to residues 747–764)
5′ GGAGGTGGTTCTCCCGCC 3′ (sense to residues 973–990)

Almost all of the nucleotide sequence of SLRSV-H RNA 2 shown in Fig. 1 was obtained by sequencing cDNA in both orientations. However, the base sequence at the extreme 5′ terminus (110 residues) was determined directly as described by Bauer (1990) and Kreiah *et al.* (1993) from virion-derived RNA 2 purified with RNaid (Bio 101) from agarose gels after electrophoretic separation from RNA 1 and with a primer (5′ ACTGTATCAAAATACAGA 3′) that bound at residues 50 to 66. The sequence of SLRV-H was analysed using programs available on the Oxford University Computing Service OXPATH including: GAP, ALIGN, COMPARE, PILEUP, CLUSTAL V, WORDSEARCH, FASTA, TFASTA and BLAST.

The RNA 2 molecule comprised 3824 nucleotides excluding the poly(A) and had a nucleotide composition of 31·5% U, 24% C, 21·9% A and 22·6% G which was similar to those of the equivalent genomic RNA species of nepoviruses and comoviruses. The 5′ non-coding region of RNA 2 (669 bases) was rich in U residues (32·4%) and included the consensus sequence GAAAA which Fuchs *et al.* (1989) recognized as characteristic for nepoviruses.

The 3′ non-coding region of RNA 2 was very similar in size to that of TobRSV (Silva *et al.*, 1993) but larger than those of ArMV, CPMV, GCMV, GFLV, RRV and TBRV (Bertioli *et al.*, 1991; Van Wezenbeek *et al.*, 1983; Brault *et al.*, 1989; Serghini *et al.*, 1990; Blok *et al.*, 1992; Meyer *et al.*, 1986) and smaller than those of TomRSV and CLRV (Rott *et al.*, 1988; Scott *et al.*, 1992). The U content (37·4%) of the 3′ non-coding region of SLRSV-H RNA 2 was more similar to RRV and TomRSV (Rott *et al.*, 1991) than to ArMV, CPMV, GCMV, GFLV or TBRV. However, the U content of the 3′-terminal 186 nucleotides of SLRSV-H RNA 2 (43%) resembles CPMV. Rott *et al.* (1991) noted that the sequence 5′ AAAAAGC 3′ was present in the 3′ non-coding region of some nepoviruses. This sequence occurred once in the RNA 2 of SLRSV-H (between nucleotides 3815 to 3821) and also in the RNA 2 of RRV (at positions 3574 to 3579; Blok *et al.*, 1992).

To locate the capsid coding sequences, virion proteins were analysed by automated Edman degradation using an Applied Biosystems 470A protein sequencer (Hewick *et al.*, 1981). The fastest sedimenting (bottom) component of SLRSV-H was obtained by rate-density gradient centrifugation in sucrose and, after disruption with 2-mercaptoethanol/SDS, and analysis in a 10% SDS–polyacrylamide gel (Laemmli, 1970) the dissociated capsid proteins were transferred onto an Immobilon-P membrane (Millipore) as described by the manufacturer. The unambiguous sequence of N-terminal amino acids determined for SLRSV-H has been underlined and enboldened in Fig. 1. The corresponding N-terminal

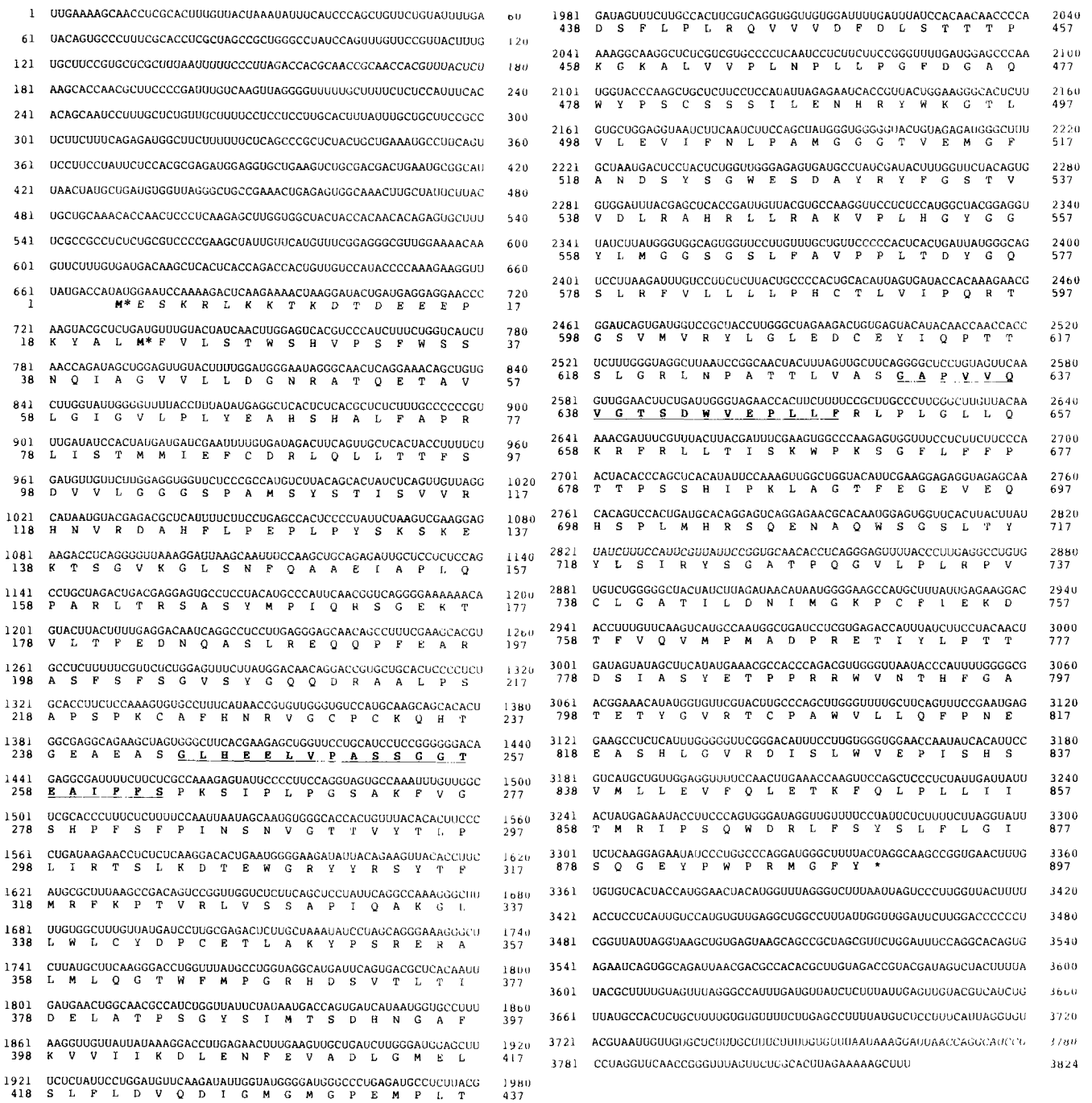

Fig. 1. Nucleotide sequence of the RNA 2 of the H isolate of SLRSV. The deduced amino acid sequence is shown below the nucleotide sequence using a single letter for each amino acid. Amino acids confirmed by Edman degradation to be present in the N-terminal sequences of the capsid components have been underlined and emboldened. The first two in-phase methionines are marked with an asterisk.

amino acids determined for SLRSV-NZ (Everett *et al.*, 1994) were not identical: the differences are shown here in lower case, emboldened (GLHE**d**LVPA**a**SGGTE and GAPVVQVGTSDW**m**EP). The amino acid N-terminal sequence of the larger capsid subunit in the New Zealand isolate of SLRSV was not reported. However, in SLRSV-H, both capsid subunits arise by proteolysis at serine/glycine dipeptides and, in SLRSV-NZ, the small capsid subunit is also cleaved from the large at a serine/glycine dipeptide. Other serine/glycine sites were present in the deduced amino acid sequence but only those at the above positions had alanine residues at their N-terminal site; such residues occur in the capsid coding sequence of tobacco etch potyvirus and may be a prerequisite for

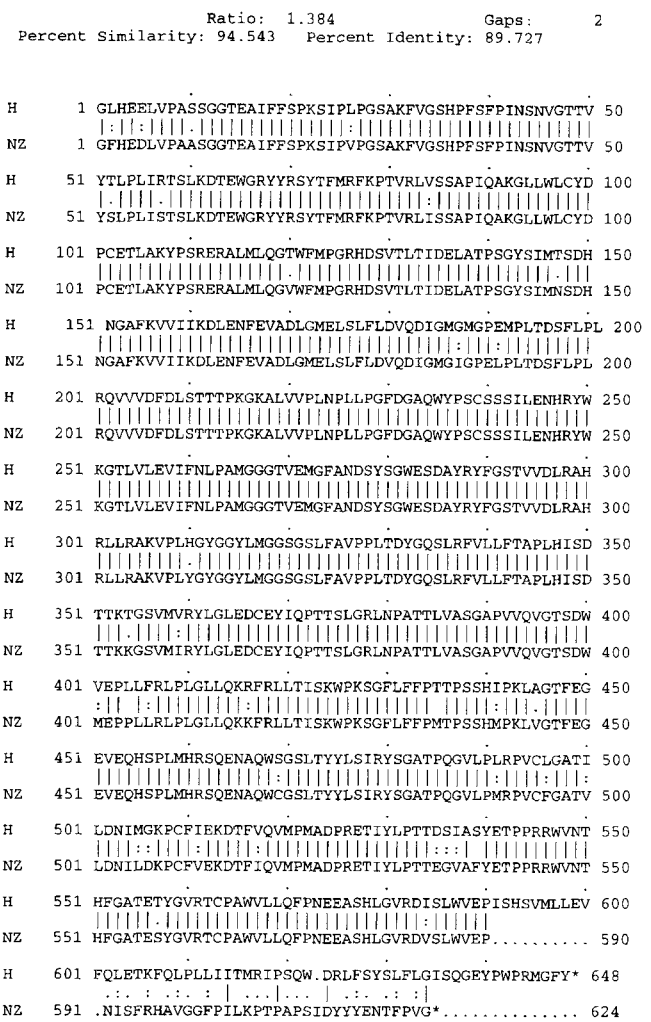

Fig. 2. Amino acid sequence identities in the capsid coding regions of SLRSV-H and SLRSV-NZ. There was 90% identity; identical amino acids are shown by a vertical line and similar amino acids with either one or two dots. Gaps were created to optimize the alignments and the comparisons were made using the programs BESTFIT and GAP (Devereux *et al.*, 1984).

protease cleavage (Dougherty & Carrington, 1988). The cleavage sites in SLRSV are different from those previously recognized in nepo- and comoviruses although, among nepoviruses, a variety of putative cleavage sites have been recognized: e.g. arginine/glycine in both ArMV (Bertioli *et al.*, 1991) and GFLV (Serghini *et al.*, 1990) and cystine/alanine in the RNA 2 of RRV (Blok *et al.*, 1992).

The longest ORF in the SLRSV sequence is capable of encoding a polypeptide of 98·8K (starting at position 670 and terminating with the stop codon UAG at position 3340). This product is similar in size to the larger species generated during *in vitro* translation of virion-derived RNA 2 (99K and 96K; Hellen *et al.*, 1991). The M RNA of CPMV yields a similar pair of *in vitro* translation products (M_r 105K and 95K) with the 95K protein presumed to result from translation initiation at the second in-phase AUG (Holness *et al.*, 1989; Verver *et al.*, 1991). Initiation of the smaller translation product from RNA 2 of SLRSV-H at the second in-phase AUG codon (position 733) would be consistent with the modified scanning hypothesis of translation (Kozak, 1984, 1986 *a*, *b*, 1989). The small capsid subunit (S; predicted M_r 29494) is derived from the C-terminal end of the precursor whereas the large capsid subunit (L; predicted M_r 42662) occupies the N-terminal region (as with comoviruses).

When the sequence of SLRSV-NZ was compared with that of SLRSV-H a few base differences were noticed in the third reading position and correlated with minor differences in the amino acid sequences: notably in the C-terminal region of the smaller capsid subunit (Fig. 2). Although the search programs did not reveal any substantial similarities between the nucleotide sequence of the RNA 2 of SLRSV-H and other viral RNA sequences available in the GenEMBL database, the following three short sequences were shared with those listed below.

(I) The octanucleotide 5′ UUUCUUUU 3′ was shown by Serghini *et al.* (1990) to occur at variable distances from the poly(A) tail in the 3′ non-coding regions of the RNA 2 of GCMV, GFLV-F13 and TBRV-S. This sequence was also reported to be present once in the 5′ non-coding region of the RNA 2 of GFLV-F13 and four times in that of TBRV-S. A search for this sequence in the RNA 2 of RRV, SLRSV-H and TomRSV showed that it was present once in the 3′ non-coding regions of SLRSV-H and TomRSV starting at positions 3743 and 7217 respectively and that it was present once in the 5′ non-coding region of SLRSV-H (starting at position 260) and twice in the same region of TomRSV (starting at positions 64 and 69). Interestingly, this sequence was found twice in the coding region of RRV RNA 2 starting at positions 2458 and 3475 but not in the non-coding regions.

(II) The 3′ non-coding regions in the RNA 2 of SLRSV-H, ArMV and GFLV share an identical sequence of 14 nucleotides (5′ GCUUUUGUGUGUUU 3′) between positions 3673 to 3687 in SLRSV-H and 3654 to 3667 in GFLV and at 119 nucleotides from the poly(A) tail in ArMV.

(III) A stretch of 30 nucleotides located at positions 3290 to 3319 in SLRSV-H and 4356 to 4385 in TBRV-S was almost identical (90%): differences are shown in lower case.

SLRSV-H:

UCUUAGGUAUUUCUcAagGAGAAUAUCCCU

TBRV-S:

UCUUAGGUAUUUCUuAuaGAGAAUAUCCCU

Interestingly, the amino acid triplet 'VQV' was identified in the N-terminal region of the ArMV coat protein, in the S capsid component of SLRSV-H (between residues 636 to 638) and in the same position within the coding sequence of a New Zealand isolate of the virus (Everett *et al.*, 1994). This triplet was not found in six viruses with longidorid vectors other than *X. diversicaudatum* (GCMV, GFLV, RRV, ToRSV and TomRSV) although it was present at a different place (at 204 amino acid residues downstream from the coat protein N-terminus) in the coat protein coding sequence of TBRV. It is tempting to infer that the VQV triplet plays a part in virus transmission by *X. diversicaudatum* analagous to those [DAG (Lee *et al.*, 1993) and NAG (Harrison & Robinson, 1988; Atreya *et al.*, 1991) which have been implicated with the aphid transmission of potyviruses.

The L and the S capsid components contain 7·7% basic and 9% acidic or 8·1% basic and 8·5% acidic amino acids respectively. Within the S component (but in no other region) a MOTIFS search for potential asparagine-glycosylation sites (specific to the consensus sequence N–X–S/T–X, where X is any amino acid) revealed the string NDSY (at positions 519 to 522), perhaps implying that this aspargine is glycosylated (Hubbard & Ivatt, 1981). The alignment and comparison programs did not reveal any significant sequence similarities between the polypeptides encoded by the RNA 2 of SLRSV-H and those encoded by the equivalent genomic segments of the viruses listed earlier. However, SLRSV-H was always grouped, but only distantly, with CPMV in a cluster where CPMV, RCMV and SLRSV-H were equally distant from other viruses in the classification trees. The significance of the similarities between each pair of sequences was calculated using a Monte Carlo method (Lipman & Pearson, 1985). Alignments involving SLRSV-H gave scores indicating uncertain relationships. Thus, taking for example viruses representing two capsid sizes/structures, the similarity (expressed in terms of standard deviation units away from the mean random distribution) between the capsid coding regions of SLRSV-H and CPMV was 2·38 and that between SLRSV-H and ArMV was 0·84 when the corresponding similarity index between ArMV and GFLV was 66·54.

In some nepoviruses, the RNA 2 has the capacity to encode two non-structural proteins but that of SLRSV cannot code for more than one of similar size: a protein (243 amino acids) with a positive net charge of 1 comprising a series of alternating short hydrophilic and hydrophobic regions (Kyte & Doolittle, 1982). The role served by this protein remains unknown but the amino acid triplet 'LPL' which Koonin *et al.* (1991) implicated with virion movement within plants occurred at amino acid positions 63 to 65 in the SLRSV-H 26·6K coding sequence and a movement function for the protein coded in that region by SLRSV-H would accord with the experiences or suggestions of others [viz. CPMV (Wellink *et al.*, 1987; Wellink & Van Kammen, 1989), TBRV (Hibrand *et al.*, 1992; Demangeat *et al.*, 1992) or other nepoviruses (Meyer *et al.*, 1986; Brault *et al.*, 1989; Rott *et al.*, 1991)]. Nevertheless, comparisons between the 26·6K polypeptide of SLRSV-H and the 58/48K polypeptide of CPMV, the 30K protein of tobacco mosaic virus (Atabekov & Dorokhov, 1984; Meshi *et al.*, 1987) or the region upstream of the coat protein coding sequences in ArMV, GCMV, GFLV, RRV, TBRV or TomRSV showed no unexpected affinities. Thus, notwithstanding a degree of similarity in genome organization, our data reaffirm the distinctness of SLRSV-H and the appropriateness of positioning it as a tentative member of the nepovirus genus: the incompletely characterized SLRSV-NZ is slightly divergent.

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