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Red clover mottle virus from Ukraine is an isolate of RCMV strain S

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Abstract

The relationship between red clover mottle virus (RCMV) isolated in the Ukraine (designated RCMV-Uk) and well-characterised strains from Sweden has been investigated. Nucleic acid hybridisation indicate that both RNAs from RCMV-Uk are highly homologous to their counterparts from RCMV strain S, a conclusion supported by protein sequence analysis of the two viral capsid proteins. Nucleic acid sequence analysis of a portion of RCMV-Uk RNA 2 confirmed the high degree of similarity between RCMV-Uk and RCMV strain S. This information suggests that RCMV-Uk should be considered an isolate of RCMV strain S.

Red clover mottle virus (RCMV) is a member of the comovirus group of plant viruses. Its genome consists of two molecules of positive-strand RNA, RNA 1 and RNA 2, which are separately encapsidated in isometric particles. The virus occurs in Europe and has been independently isolated in several countries including England (Sinha, 1960), The Netherlands (Bos and Maat, 1965), Slovakia (Musil, 1966), Germany (Schumann and Umland, 1970; 1971), Sweden (Gerhardson and Lindsten, 1973) and Ukraine (Lapchic et al., 1975). The Swedish isolates have been further resolved into three strains, RCMV-S, -N and -O (Oxelfelt, 1976). Though all the isolates give similar reactions on *Trifolium pratense* (systemic mosaic), *Vicia faba* (systemic necrosis) and *Phaseolus vulgaris* (necrotic local lesions), they cause a range of different symptoms on *Pisum sativum* and *Chenopodium quinoa*. The Ukrainian isolate (designated RCMV-Uk), and RCMV-S cause a systemic mosaic on both hosts. RCMV-O causes top necrosis in *P. sativum* and no symptoms on *C. quinoa*. Strain N appears to be somewhat intermediate causing slowly developing chlorotic lesions on the inoculated leaves of *C. quinoa* but no systemic spread and gives rosette-type symptoms on *P. sativum*. The Dutch isolate causes top necrosis on *P. sativum* but also causes a systemic infection on *C. quinoa* whereas the German isolate produces a mosaic on *P. sativum* but no symptoms on *C. quinoa*.

Of all the isolates of RCMV, only those from Sweden have been examined in any detail at the molecular level. The nucleotide sequences of both RNAs of strain S have been determined (Shanks et al., 1986; Shanks and Lomonossoff, 1992) and partial sequences from both RNAs of strain O are available (Oxelfelt et al., 1992; Clark et al., 1993). Though the virus particles of all three strains are serologically identical, the RNAs from strains S and O can be differentiated by hybridisation using strain-specific cDNA probes (Oxelfelt et al., 1992). In order to increase our understanding of the epidemiology of RCMV and how the nucleotide sequence of the RNAs might relate to symptomatology, we have examined the relationship between RCMV-Uk and the Swedish strains S and O.

RCMV-Uk, RCMV strain S and RCMV strain O were propagated and purified from *P. sativum*. RNA was extracted from purified particles and 1μg samples were denatured, electrophoresed on formaldehyde-containing agarose gels (Lehrach et al., 1977) and the RNA transferred to nitrocellulose membranes (Hy-

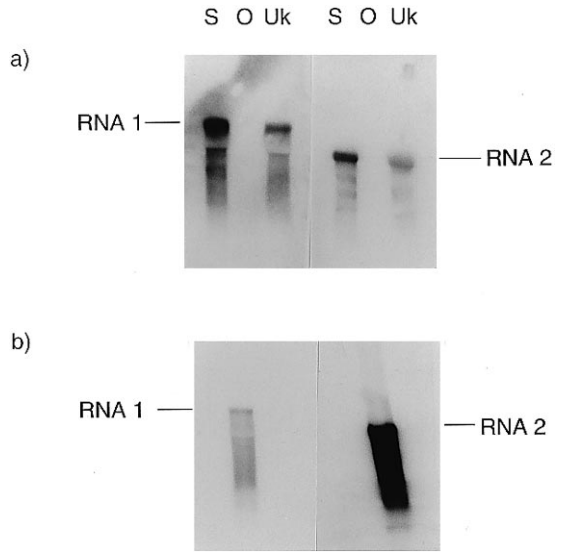

Figure 1. Northern blot analysis of RNA extracted from purified RCMV particles. RNA prepared from each strain (S, O and Uk) was hybridised with (a) probes specific for RNA-1 (Left-hand panel) and RNA 2 (Right-hand panel) of RCMV strain S and (b) RNA-1 (Left-hand panel) and RNA 2 (Right-hand panel) of RCMV strain O. The positions of RNA 1 and RNA 2 are indicated by the side of each blots.

bond N, Amersham, U.K.). Probes specific to RNA 1 and 2 of RCMV strains S and O (derived from clones M13-Sma/Pst-2, M13-T26, pRCOB-D28 and pRCOM-D1 (Oxelfelt et al., 1992)) were prepared by oligo-labelling (Feinberg and Vogelstein, 1983) and used to probe the blots. After hybridisation the blots were subjected to a high stringency wash at 65 ° C in 0.1 × SSC (1 × SSC is 0.15M sodium chloride, 0.015M sodium citrate) containing 0.1%(w/v) SDS. The probes specific to RNAs 1 and 2 of strain S, hybridised to the corresponding RNA in the samples from both strain S and strain Uk (Figure 1a). No hybridisation to either RNA from strain O could be detected. When replica blots were probed with strain O-specific probes, hybridisation could only detected with the RNAs in the strain O sample (Figure 1b). These results indicate that both RNAs from RCMV-Uk have a high degree (>80%) of sequence homology to their counterparts from RCMV-S and can be distinguished from strain O.

To further characterise RCMV-Uk, a sample of virus particles was applied directly to a ProBlott membrane (Applied Biosystems) and subjected to automated Edman degradation using an Applied Biosystems 470A gas phase protein sequencer. It was anticipated that this procedure would give a unique sequence since, when it is applied to CPMV, sequence from only the N-terminus of the small (S) coat protein is obtained (van Wezenbeek et al., 1983). No sequence was obtained from the large (L) protein because of acetylation of its amino terminus (Bruening, 1978). Somewhat surprisingly, two distinct amino acid sequences were obtained. Although a mixture of proteins was being sequenced it was possible to unambiguously deduce the N termini of the two proteins as being TDTD and GGVV. These tetrapeptide sequences correspond exactly to the predicted N-termini of the L and S proteins of RCMV-S (Shanks et al., 1986). The corresponding sequences from strain O coat proteins are EDID and GGVI (Clark et al., 1993). These results are consistent with the conclusion that RCMV-Uk is more closely related to RCMV-S than it is to RCMV-O. The fact that amino acid sequence was obtained from the L protein indicates that, unlike the situation with CPMV, its N-terminus is not blocked.

To obtain detailed nucleotide sequence information about RCMV-Uk, double-stranded cDNA specific to the 3′ terminal 2.0kb of RNA 2 was synthesised by the RACE procedure (Frohman et al., 1988). The primer for first-strand synthesis reverse transcription reaction was GACTCGAGTCGACATCGA$(T)_{17}$. PCR amplification was carried out using a primer, GGGATCCAT(T,C,A)GA(T,C)CC(T,C,G,A)AA(G,A)GA, which consists of the sequence of a *BamH1* site linked to a consensus sequence from strains S and O immediately upstream of the region of RNA 2 which encodes the viral coat proteins (Shanks et al., 1986; Clark et al., 1993) and a primer, GACTCGAGTCGACATCG, identical to the 5′ end of the first strand cDNA. The PCR reactions were carried out as previously described (Achon et al., 1994). Agarose gel electrophoresis revealed the expected product of approximately 2kb. The PCR products were digested with *BamH1* and *Sal1* and ligated into similarly digested, phosphatase-treated replicative form DNA of M13mp19. Following transformation of *Escherichia coli* strain JM101, a recombinant, designated M13-RCUM-N2, containing the 2.0kb *BamH1/Sal1* fragment was identified by restriction digestion. The sequence of a 265 nucleotide-long segment of the clone encompassing the region encoding the C-terminus of the RCMV 48/58K protein and the N-terminus of the L protein was determined by the 'dideoxy' technique using the universal primer (Figure 2). The cleavage site for the L protein could be identified by comparison with protein sequence information described above.

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  G  N  V  F  A  N  P  Q /T  D  T  D  L  F  K  L  S  L  D  D  T  S
TGGCAATGTATTTGCAAATCCCCAGACTGACACTGATTTGTTCAAACTAAGTTTGGATGATACGTCAA
C  T                                                 T                 G

S  P  K  G  S  L  L  D  T  R  F  A  Q  K  K  V  L  I  P  K  A  M  A
GTCCCAAAGGATCACTACTAGACACAAGATTTGCCCAAAAGAAAGTCTTGATTCCAAAAGCAATGGCA
                 T                                   T

 G  G  A  D  L  L  S  S  N  L  Y  D  V  L  S  G  S  S  F  R  A  S  L
GGGGGGGCTGACTTACTGAGTTCAAATCTTTATGATGTGCATTCAGGAAGTTCCTTTAGAGCCTCTCT
        A                                    G

  A  L  A  R  T  H  V  V  E  G  K  I  R  C  I  C  T  I  N  L
AGCATTAGCAAGAACTCATGTAGTTGAAGGAAAGATCAGATGTATTTGCACCATAAATTTAC
                        G                 G                 C
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Figure 2. Nucleotide sequence of the region of RNA 2 of RCMV-Uk encoding the C-terminus of the 48/58K protein and N-terminus of the L protein. Nucleotide differences in RCMV-S are indicated below the main sequence. The deduced amino acid sequence of the encoded translation product is shown above. / indicates the cleavage site between the 48K protein and the L protein.

Comparison of the sequence with the corresponding region from RCMV-S revealed only 10 nucleotide differences. Of these, 9 were transitions and none of the 10 changes caused an alteration in the amino acid sequence of the encoded protein. Although only a small portion of RNA 2 was sampled, the results suggest that RNAs from RCMV-Uk and RCMV-S are approximately 96% identical. By contrast when the sequence of RCMV-Uk was compared with that of RCMV-O, 70 nucleotide changes were found, implying an identity of only 73% between these two isolates.

The results described in this paper show that RCMV-Uk is closely related to RCMV-S and should probably be considered an isolate of it. The results also indicate that the ability to systemically infect *C. quinoa* is associated with strains similar to RCMV-S. This is consistent with the results obtained with pseudo-recombinants between RCMV-S and -O which showed that the ability to grow in *C. quinoa* is determined by the presence of RNA 1 from RCMV-S (Oxelfelt et al., 1992). This suggests that the ability to spread systemically in *C. quinoa* could be diagnostic of an isolate being closely related to RCMV-S. It will be of considerable interest to extend our findings to include other isolates of RCMV which have been obtained from different parts of Europe. As isolates of RCMV that produce top necrosis on *P. sativum* have been obtained in Ukraine it is possible that strain O co-exists with strain S there, as it does in Sweden. Futher characterisation is required to confirm this.

It is possible that some of the isolates reported in the literature may effectively be 'pseudo-recombinants' between strains. A pseudo-recombinant containing the RNA 1 of strain O and the RNA 2 of strain S produces a systemic mosaic on *P. sativum* but does not produce any symptoms on *C. quinoa* (Oxelfelt et al., 1992; Clark et al., 1993), a situation similar to that reported for the German isolate of RCMV. However, though pseudo-recombinants can be formed artificially between different strains of RCMV (Oxelfelt and Abdelmoeti, 1978) it appears that the replication complex encoded by the RNA 1 of a given strain replicates its homologous RNA 2 preferentially (Oxelfelt et al., 1992). This feature could be responsible for preventing wholesale reassortment among gene segments of RCMV, allowing distinct virus strains to co-exist.

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References

Achon MA, Medina V, Shanks M, Markham PG and Lomonossoff GP (1994) Characterisation of a maize-infecting potyvirus from Spain. European Journal of Plant Pathology 100: 157–165

Bos LD and Maat DZ (1965) A distinctive strain of red clover mottle virus in the Netherlands. Netherlands Journal of Plant Pathology 71: 8–13

Bruening G (1978) Biochemistry of plant viruses. In: Stumpf PK, Conn EE, and Marcus A (eds) The Biochemistry of Plants. A Comprehensive Treatise. Vol. 6 (pp 571–631) Academic Press, New York

Clark AJ, Oxelfelt P, Lomonossoff GP and Shanks M (1993) Investigation of the symptom and host-range determinants of red clover mottle comovirus. International Congress of Virology, Glasgow, Abstact P64–9

Feinberg AP and Vogelstein B (1983). A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Analytical Biochemistry 132: 6–13

Frohman MA, Dush MK and Martin GR (1988). Rapid production of full-length cDNAs from rare transcripts: Amplification using a single gene-specific oligonucleotide primer. Proceedings of the National Academy of Sciences USA 85: 8998–9002

Gerhardson B and Lindsten K (1973) Red clover mottle virus and red clover necrotic mosaic virus in Sweden. Phytopathologische Zeitschrift 76: 67–79

Lapchic LG, Kuznetzova LL, Melniczenko VS, Vocelko SK and Sjedin AA (1975) Red clover mottle virus in Ukraine. Phytopathologische Zeitschrift 82: 339–346

Lehrach H, Diamond D, Wozney JM and Boedtker H. (1977) RNA molecular weight determination by gel electrophoresis under denaturing conditions, a critical examination. Biochemistry 16: 4743–4751

Musil M (1966) O vyskytu viru strakatosti jetele lucniho na Slovensku. Biologia (Bratislava) 21: 663–670

Oxelfelt P (1976) Biological and physico-chemical characteristics of three strains of red clover mottle virus. Virology 74: 73–80

Oxelfelt P and Abdelmoeti M (1978) Genetic complementation between natural strains of red clover mottle virus. Intervirology 10: 78–86

Oxelfelt P, Shanks M, Widmark AK and Lomonossoff GP (1992) Identification and characterisation of pseudo-recombinants of red clover mottle comovirus. Journal of General Virology 73: 2121–2124

Schumann K and Umland T (1970) Zum vorkommen des scheckungs-virus des rotklees (red clover mottle virus) in der Deutschen Demokratischen Republik. Phytopathologische Zeitschrift 67: 73–77

Schumann K and Umland T (1971) Zur charackterisierung des scheckungs-virus des rotklees (red clover mottle virus) in der Deutschen Demokratischen Republik. Archiv für Pflanzenschutz 7: 163–169

Shanks M, Stanley J and Lomonossoff GP (1986) The primary structure of red clover mottle virus middle component RNA. Virology 155: 687–706

Shanks M and Lomonossoff GP (1992) The nucleotide sequence of red clover mottle virus bottom component RNA. Journal of General Virology 73: 2473–2477

Sinha R C (1960) Red clover mottle virus. Annals of Applied Biology 48: 742–748

van Wezenbeek P, Verver J, Harmsen J, Vos P and van Kammen A. (1983) Primary structure and gene organisation of the middle component RNA of cowpea mosaic virus. EMBO Journal 2: 941–946