European Journal of Plant Pathology **110:** 429–433, 2004.
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Isolation of two strains of a new Tombusvirus (*Havel river virus*, HaRV) from surface waters in Germany

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Accepted 10 December 2003

Key words: comparisons of Tombusvirus coat protein sequences, immunoelectron microscopy, Tombusvirus relationships, viruses in surface waters

Abstract

Two virus isolates from water samples – one from a small stream in South Western Germany and another one from the Havel river in North Eastern Germany c. 500 km away, proved to be strains, named S and H, respectively, of a new Tombusvirus for which the name *Havel river virus* (HaRV) had been suggested previously in a brief account. Immunoelectron microscopical decoration tests and sequence comparisons of the coat proteins indicated that the two HaRV strains are only distantly related to known Tombusviruses. The closest relationships were found to *Cucumber necrosis virus*. Nothing is known about their natural hosts. Because the S strain of HaRV was isolated in a woody area from a small stream close to its origin, they may be pathogens of trees or wild plants in such habitats.

Introduction

Various Tombusviruses have previously been isolated from surface waters. For some of them, e.g. *Tomato bushy stunt virus* (TBSV) (Tomlinson and Faithfull, 1984; Koenig and Lesemann, 1985), *Carnation Italian ringspot virus* (CIRV) (Büttner et al., 1987; Pfeilstetter, 1992), *Petunia asteroid mosaic virus* (PetAMV) (Pfeilstetter, 1992), *Cymbidium ringspot virus* (CymRSV) (Pfeilstetter, 1992) and *Grapevine Algerian latent virus* (GALV) (Li et al., 1992) natural hosts are known, whereas for others, e.g. the *Neckar river virus* (NRV) (Koenig and Lesemann, 1985), the *Lato river virus* (LRV) (Gallitelli et al., 1989) and the *Sikte waterborne virus* (SWBV) (Li et al., 1992) they have not yet been identified. In the present paper, we have studied two of such virus isolates in more detail. One was obtained in 1990 in Eastern Germany from the Havel river shortly before it flows into the Elbe river (Proll et al., 1991), the other one in 1991 about 500 km away in a woody area in South Western Germany (Upper Frankonia) from a small stream (St. Moritz stream) close to its origin (Pfeilstetter, 1992). Their morphological properties and a weak reactivity with an antiserum to *Cucumber necrosis virus* (CuNV) suggested that they might be Tombusviruses. This was confirmed by means of sequence analyses which indicated that the two virus isolates represent strains of a new Tombusvirus for which the name *Havel river virus* (HaRV) had previously been suggested in a brief account (Proll et al., 1991). The Havel river isolate will be designated as strain H and the St. Moritz stream isolate as strain S of HaRV.

Materials and methods

Viruses were sedimented from c. 500 ml water samples by high-speed centrifugation for 2 h at 100 000 × g. The pellets obtained were resuspended in 0.03 M phosphate buffer pH 7.0 and the suspensions were rubbed on leaves of *Chenopodium quinoa*. Local lesions which

developed were ground up in the same phosphate buffer and the homogenates were rubbed on leaves of *Nicotiana clevelandii* which became infected systemically. The viruses were maintained on this host for further studies. The H strain was purified and an antiserum was produced as described previously for other Tombusviruses (Koenig and Lesemann, 1985).

Virus particles were visualized by negative staining with 1% aqueous uranyl acetate. For assessing their serological reactivity, adsorbed particles were decorated with antisera diluted 1 : 50 (Milne, 1984). Decoration titers were determined with two-fold serial dilutions of antisera starting at a dilution of 1 : 2.

Total RNA was extracted from infected *N. benthamiana* leaves by means of the Qiagen RNeasy Plant Mini Kit (Cat. No. 74904). cDNA was obtained by means of reverse transcription using the primer Cir2 (5′-ggtttattgacttgttcgtattcag-3′) which is complementary to nucleotides 3847–3870 of CIRV RNA (accession no. X85215) downstream of the coat protein gene (Figure 1). PCRs were done using the primer pair Cir2/Cir1. The latter primer (5′-gactccgccgtagcttgacc-3′) corresponds to nucleotides 2616–2635 of CIRV RNA upstream of the CIRV coat protein gene (Figure 1). The regions on the CIRV RNA from which Cir2 and Cir1 were derived are highly conserved also in the RNAs of other Tombusviruses. Amplification of open reading frames (ORFs) 3 and 4 downstream of the coat protein gene of the S strain of HaRV was achieved by using the primer Cu2 for cDNA synthesis. This primer is complementary to the sequence of the 22 3′-terminal

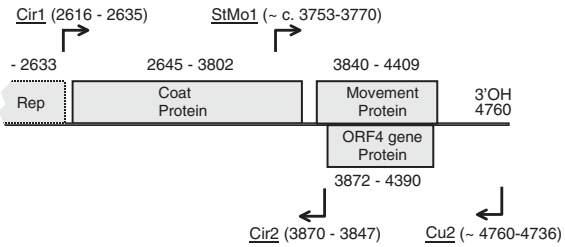

Figure 1. Organization of the 3′-part of a Tombusvirus genome (CIRV) showing the coding regions for the C-terminal part of the replication-associated proteins (Rep), the coat protein, the movement protein and the ORF4 gene protein as well as the positions and orientations of the primers Cir1, Cir2, Cu1 and StMo1. Cir1 and Cir2 are derived from the CIRV sequence, Cu1 from the CuNV sequence and StMo1 from the HaRV-S sequence. The sequences of these pimers except for that of StMo1 are highly conserved also in other Tombusviruses.

nucleotides of CuNV RNA (accession no. M25270) which is highly conserved also in the genomic RNAs of other Tombusviruses. Polymerase chain reactions (PCRs) were done using the primers Cu2 and StMo1 (5′-aaatattcgtcgccaagg-3′). The latter corresponds to nucleotides 1112–1129 of the newly determined coat protein gene-containing sequence of the S strain of HaRV. PCR products were purified using the Jetsorb Gel Extraction Kit (Genomed), cloned into the pGEM-T vector (Promega) and sequenced by a commercial company (MWG-Biotech, D85560 Ebersberg/Germany). Sequences were analysed by means of the LINEUP/PILEUP programs of the UWGCG software version 8 (Devereux et al., 1984). The program DNAMAN (Lynnon Bio/Soft) was used for determining the percentages of identity between the deduced amino acid sequences for the coat proteins, the movement proteins and the ORF4 gene products and to generate 'homology trees' based on the UPGMA method of Sneath and Sokal (1973). The accession numbers for the 2088 nucleotide sequence of the S strain and the 1200 nucleotide sequence of the H strain of HaRV are AY370535 and AY370534, respectively.

Results and discussion

The resuspended sediments obtained after high-speed centrifugation of the water samples from the Havel river and from the small St. Moritz stream (see Introduction and Materials and methods sections) produced one local lesion each on the leaves of *Ch. quinoa*. From this host, the viruses were further transmitted mechanically to *N. clevelandii* which became infected systemically. Its leaves contained large amounts of isometric particles with a diameter of 28–30 nm showing a distinctly 'knobby' surface fine structure.

Sap from *N. clevelandii* infected with the Havel river isolate served as a source of inoculum for mechanical transmission studies to a total of 25 different test plants. Local lesions which were often small and necrotic were produced on *Beta vulgaris* cv. Ponemo, *Capsicum annuum*, *Ch. capitatum*, *Ch. foetidum*, *Ch. foliosum*, *Ch. murale*, *Ch. quinoa*, *Celosia argentea*, *Cucumis sativus* cv. Eva, *Datura stramonium*, *Gomphrena globosa*, *Lycopersicon esculentum* cv. Harzfeuer, *N. benthamiana*, *N. clevelandii*, *N. glutinosa*, *N. megalosiphon*, *N. occidentalis*, *N. tabacum* cv. Samsun, *Petunia hybrida*, *Phaseolus vulgaris*,

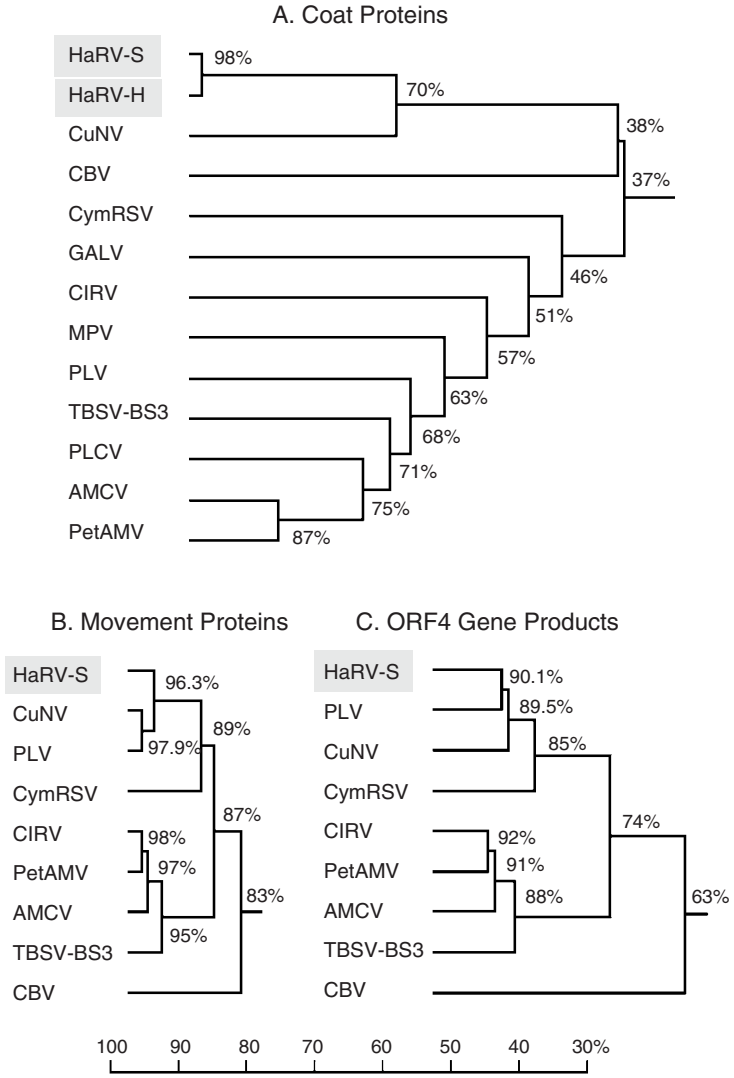

Figure 2. 'Homology' trees showing the percentages of amino acid sequence identities for the coat proteins (A), the movement proteins (B) and the ORF4 gene products (C) of the *Havel river virus* (HaRV – strains H and S) and other Tombusviruses, i.e. AMCV – *Artichoke mottled crinkle virus* (X62493), CBV – Cucumber Bulgarian virus (NC_004725), CIRV – *Carnation Italian Ringspot virus* (X85215), CuNV – *Cucumber necrosis virus* (M25270), CymRSV – *Cymbidium ringspot virus* (X155112), GALV – *Grapevine Algerian latent virus* (AF540885), MPV – Moroccan pepper virus (AF540885), PetAMV – *Petunia asteroid mosaic virus* (M21958 – this isolate has been designated as Cherry strain of *Tomato bushy stunt virus* – TBSV, but Hollings and Stone, 1975, found that it was serologically different from the type strain of TBSV, but undistinguishable from PetAMV), PLCV – *Pelargonium leaf curl virus* (AF290026), PLV – pear latent virus (AY100482) and TBSV BS3 (AJ249740 – statice isolate) (accession numbers in parentheses). The values given at the nodes represent average percentages of identity between the viruses shown on each side of the branches and NOT boot strap values. The scale at the bottom represents percentages of identity.

Vicia faba cv. Tinova. Systemic infections where observed in *N. clevelandii*, *N. benthamiana*, *N. megalosiphon*, *C. annuum* and occasionally in *Ch. quinoa*, *N. tabacum* 'Samsun' and *P. vulgaris*.

The virus isolate from the Havel river was purified from *N. clevelandii* and used for the production of an antiserum which in the immunoelectron microscopical decoration test reacted equally well with both water

virus isolates up to a dilution of 1 : 10 000. The reactivity of the two isolates was also checked in this test with antisera to 36 other isometric viruses, mainly belonging to the Tombusvirus and Carmovirus genera which have particles with a surface fine structure similar to that of the two water virus isolates. The only antiserum which decorated the particles of these two isolates was one to CuNV. This decoration, however, was only rather weak and was observed only up to a dilution of c. 1 : 50, whereas with its homologous virus the CuNV antiserum reacted up to a dilution of 1 : 10 000. This indicates that the two closely related water virus isolates are only rather distantly related serologically to CuNV. Antisera to other Tombusviruses, i.e. *Artichoke mottled crinkle virus* (AMCV), CIRV, CymRSV, *Eggplant mottled crinkle virus*, GALV, LRV, *Moroccan pepper virus*, NRV, PetAMV, *Pelargonium leaf curl virus* (PLCV), SWBV, the BS3 strain of TBSV and to viruses in other genera, especially in the Carmoviruses, failed to decorate the particles.

Most of the gene products of Tombusviruses show a high degree of sequence identity and are therefore not well suited for the differentiation of isolates (Hearne et al., 1990; Rochon et al., 1991; Rubino et al., 1995). The highest degree of sequence diversity is found in the coat proteins, especially in the internally located R domains and the protruding P domains (Tavazza et al., 1989; Hearne et al., 1990). The coat protein genes of the two water virus isolates were readily amplified using the primers Cir1 and Cir2 which are derived from areas in the CIRV genome which are highly conserved also in the RNAs of other Tombusviruses (see Materials and methods section). High percentages of sequence identity were found between the coat protein genes (94.7%) and even more so between the deduced coat protein amino acid sequences (98.2%; Figure 2A: HaRV-H and HaRV-S) of the two water virus isolates. Most of the 61 nucleotide exchanges in their coat protein genes were silent; thus, only seven exchanges were found in the deduced amino acid sequences. Four of these exchanges occurred in the internally located R domain and only one in the protruding P domain which presumably is mainly responsible for the serological properties of Tombusviruses.

The coat protein amino acid sequences of these two closely related water virus isolates proved to be rather different from those of the other Tombusviruses for which sequence data are available (Figure 2A), especially in the R and P domains (results not shown). The closest coat protein relationship of the two HaRV strains was found with CuNV. However, with only ∼70% amino acid sequence identity this relationship is more distant than the relationships between the coat proteins of PetAMV, AMCV, PLCV and the statice isolate of the BS3 strain of TBSV (Figure 2A) which are regarded to represent distinct virus species (Lommel et al., 2000).

From the molecular comparisons presented in Figure 2A and the serological observations described above it is concluded that the two water isolates should be regarded as strains of a distinct new Tombusvirus. In a preliminary report, the name HaRV had been proposed for the Havel river isolate by Proll et al. (1991). We propose to designate this isolate as H strain and the St. Moritz stream isolate as S strain of HaRV, respectively.

For the S strain of HaRV, we have amplified also the region downstream of the coat protein gene which contains ORF3 and ORF4 present in the genomes of all Tombusviruses (Figure 1) (Lommel et al., 2000). ORF3 codes for a 21–22 kDa movement protein. ORF4 which is totally included in ORF3 codes for a 19–20 kDa protein which acts as a suppressor of the RNA-mediated defence mechanisms in plants (Voinnet et al., 1999). Because of the high percentages of amino acid sequence identities, especially in the movement proteins (Figure 2B), small changes in the amino acid sequences may have a strong influence on the clustering of the viruses. This may, at least in part, explain why the grouping of the viruses in Figure 2B (movement proteins) and Figure 2C (ORF4 proteins) differs from that shown in Figure 2A (coat proteins). Nevertheless, also the percentages of sequence identities between the movement proteins and the ORF4 proteins suggest that the relationships of HaRV to CuNV are closer than to most other Tombusviruses.

So far we know nothing about the natural hosts of the two HaRV strains. Because the S strain was isolated in a woody area from a small stream close to its origin, they may be pathogens of trees or wild plants in such habitats.

Acknowledgements

We are greatly indebted to Steffi Loss, Sabine Bonse and Christina Maaß for expert technical assistance and to the Deutsche Forschungsgemeinschaft for financial support.

References

Büttner C, Jacobi V and Koenig R (1987) Isolation of *Carnation Italian ringspot virus* from a creek in a forested area South West of Bonn. Journal of Phytopathology 118: 131–134.

Devereux J, Haeberli P and Smithies O (1984) A comprehensive set of sequence analyses for the VAX. Nucleic Acids Research 12: 387–395.

Gallitelli D, Di Franco A, Vovlas C and Martelli GP (1989) Coat protein-derived small particles in a tombusvirus from river Lato. Journal of Phytopathology 126: 213–218.

Hearne PQ, Knorr DA, Hillman BI and Morris TJ (1990) The complete genome structure and synthesis of infectious RNA from clones of tomato bushy stunt virus. Virology 177: 141–151.

Koenig R and Lesemann D-E (1985) Plant viruses in German rivers and lakes I. Tombusviruses, a potexvirus and carnation mottle virus. Phytopathologische Zeitschrift 112: 105–116.

Li Y, Lesemann D-E, Koenig R, Rüdel M and Pfeilstetter E (1992) Isometric plant viruses in ditches and streams in agricultural areas: Recovery of previously found viruses and identification of hitherto unrecorded carmo- and tombusviruses including grapevine Algerian latent virus. Journal of Phytopathology 134: 121–132.

Lommel SA, Martelli GP and Russo M (2000) Tombusviridae. Virus Taxonomy. Seventh Report of the International Comittee on Taxonomy of Viruses, pp. 791–825.

Milne RG (1984) Electron microscopy for the identification of plant viruses in *in vitro* preparations. In: Maramorosch K and Koprowski H (eds) Methods in Virology VII. Academic Press, New York, pp. 87–120.

Pfeilstetter E (1992) Untersuchungen zur Virösen Zweignekrose an Süßkirschen Oberfrankens: Vorkommen, Ausbreitung und Möglichkeiten des Nachweises der Erreger petunia astroid mosaic virus (PAMV) und carnation Italian ringspot virus (CIRV). Ph.D. Thesis, Technical University Munich, 134 pp.

Proll E, Kegler H and Stanarius A (1991) Isolierung und erste Charakterisierung eines isometrischen Virus aus der Havel. Jahresbericht Institut für Phytopathologie Aschersleben 17–18.

Rochon DM, Johnston JC and Riviere CJ (1991) Molecular analysis of the cucumber necrosis virus genome. Canadian Journal of Plant Pathology 13: 142–154.

Rubino L, Burgyan J and Russo M (1995) Molecular cloning and complete nucleotide sequence of carnation Italian ringspot tombusvirus genomic and defective interfering RNAs. Archives of Virology 140: 2027–2039.

Sneath PH and Sokal RR (1973) *Numerical Taxonomy: The Principles and Practice of Numerical Classification*. Freeman, San Fransisco, 573 pp.

Tavazza M, Lucioli A, Ancora G and Benvenuto E (1989) cDNA cloning of artichoke mottled crinkle virus RNA and localization and sequenzing of the coat protein gene. Plant Molecular Biology 13: 685–692.

Tomlinson JA and Faithfull EM (1984) Studies on the occurrence of tomato bushy stunt virus in English rivers. Annals of Applied Biology 104: 485–495.

Voinnet O, Pinto YM and Baulcombe DC (1999) Supression of gene silencing: A general strategy used by diverse DNA and RNA viruses in plants. Proceedings of the National Academy of Sciences, USA 96: 14147–14152.