Nemesia ring necrosis virus: a new tymovirus with a genomic RNA having a histidylatable tobamovirus-like 3’ end

R. Koenig,1 S. Barends,2 A. P. Gultyaev,3 D.-E. Lesemann,1 H. J. Vetten,1 S. Loss1 and C. W. A. Pleij2

1Biologische Bundesanstalt für Land- und Forstwirtschaft, Institut für Pflanzenvirologie, Mikrobiologie und biologische Sicherheit, Messeweg 11, D-38104 Braunschweig, Germany
2Leiden Institute of Chemistry, Leiden University, PO Box 9502, 2300 RA Leiden, The Netherlands
3Leiden Institute of Biology, Leiden University, Kaiserstraat 63, 2311 GP Leiden, The Netherlands

The complete nucleotide sequence of the genomic RNA of the new virus Nemesia ring necrosis virus (NeRNV), which is widespread in various ornamental plant species belonging to the Scrophulariaceae and Verbenaceae, i.e. Nemesia, Diascia, Alonsoa, Sutera and Verbena (Koenig & Lesemann, 2000; Seigner, 2003; Skelton et al., 2004; D.-E. Lesemann and R. Koenig, unpublished). High concentrations of ‘full particles’ and ‘empty shells’ have been found using electron microscopy in negatively stained extracts from symptomatic infected plants and also from symptomless leaves of infected plants. In immuno-electron microscopic decoration tests, these particles react with antisera to several other tymoviruses, but molecular studies have indicated that the virus is only rather distantly related to them (Koenig et al. studies have indicated that the virus is only rather distantly related to them (Koenig et al.). Obviously, it represents a new virus species, for which the name Nemesia ring necrosis virus (NeRNV) is proposed. In the present paper, the entire sequence of the genomic RNA of a Nemesia isolate of NeRNV is described. The RNA shows the typical gene arrangement and 5’-untranslated region (UTR) folding of tymoviral RNA but, surprisingly, it is predicted that its 3’ end folds in a tobamovirus RNA-like manner, i.e. into an upstream pseudoknot domain (UPD) followed by a tRNA-like structure (TLS). Like tobamoviral RNAs, but unlike the valylatable tymoviral RNAs, it can be charged with histidine even more efficiently than tobacco mosaic virus RNA.

INTRODUCTION

In recent years, a virus with a tymovirus-like morphology has been found to be widespread in commercially grown cultivars of species in various genera in the Scrophulariaceae and Verbenaceae, i.e. Nemesia, Diascia, Alonsoa, Sutera and Verbena (Koenig & Lesemann, 2000; Seigner, 2003; Skelton et al., 2004; D.-E. Lesemann and R. Koenig, unpublished). NeRNV particles were purified by homogenizing 250 g infected N. benthamiana leaves in 200 ml 0·1 M sodium phosphate buffer, pH 7, containing 0·02 M Na2SO4. The mixture was strained through cheesecloth and 0·2 volumes of a 1:1 (v/v) mixture of chloroform and butanol were added to the leaf extract. After vigorous stirring for 15 min at room temperature, the aqueous phase was separated by low-speed centrifugation. The virus was then sedimented by high-speed centrifugation and resuspended in 100 ml 0·01 M Tris/HCl, pH 7·5. After overnight incubation at 4 °C and another cycle of low- and high-speed centrifugation, the virus was again resuspended in the same buffer and purified further by sucrose rate-zonal centrifugation at 25000 r.p.m. for 3 h at 10 °C. A broad opalescent zone containing mainly ‘full particles’ rather than ‘empty shells’ was collected from the centre of the gradient. After high-speed centrifugation, the virus was resuspended in 0·01 M Tris/HCl, pH 7·5.

METHODS

RNA and virus. The RNA of NeRNV was obtained from crude extracts of mechanically inoculated Nicotiana benthamiana or from purified virus preparations with a Qiagen RNasy Plant Mini kit. NeRNV particles were purified by homogenizing 250 g infected N. benthamiana leaves in 200 ml 0·1 M sodium phosphate buffer, pH 7, containing 0·02 M Na2SO4. The mixture was strained through cheesecloth and 0·2 volumes of a 1:1 (v/v) mixture of chloroform and butanol were added to the leaf extract. After vigorous stirring for 15 min at room temperature, the aqueous phase was separated by low-speed centrifugation. The virus was then sedimented by high-speed centrifugation and resuspended in 100 ml 0·01 M Tris/HCl, pH 7·5. After overnight incubation at 4 °C and another cycle of low- and high-speed centrifugation, the virus was again resuspended in the same buffer and purified further by sucrose rate-zonal centrifugation at 25000 r.p.m. for 3 h at 10 °C. A broad opalescent zone containing mainly ‘full particles’ rather than ‘empty shells’ was collected from the centre of the gradient. After high-speed centrifugation, the virus was resuspended in 0·01 M Tris/HCl, pH 7·5.

The GenBank/EMBL/DDBJ accession number for the complete RNA sequence of a Nemesia isolate of Nemesia ring necrosis virus is AY751778; partial RNA sequences of Diascia and Verbena isolates of the virus have accession numbers AY751781 and AY885256, respectively.
Native turnip yellow mosaic virus (TYMV) RNA was isolated from infected Chinese cabbage according to Dunn & Hitchborn (1965) and native brome mosaic virus (BMV) RNA was purchased from Promega. Tobacco mosaic virus (TMV) particles (U1 strain) were purified from infected Nicotiana tabacum leaves that were frozen in liquid nitrogen prior to grinding in 10 mM EDTA/10 mM NaH2PO4 (adjusted to pH 7.5 with NaOH). Cell debris was removed by centrifugation at 6000 g, 4 °C. The supernatant was filtered and virus particles were precipitated by adding 4% (w/v) polyethylene glycol 20000 and 4% (w/v) NaCl followed by gentle shaking for 2 h at 4 °C. The pellet obtained after 30 min centrifugation at 25 000 g at 4 °C was dissolved in the grinding buffer and the precipitation and centrifugation steps were repeated twice. The final pellet was dissolved in H2O to a concentration of ~30 mg ml−1. RNA was extracted from the particles by three phenol extractions.

Sequence analysis. The first genome portions of NeRNV RNA were amplified by PCR using primers derived from sequences that, in alignments, were found to be highly conserved in tymoviral RNAs. The starter sequences thus obtained were bridged up using PCRs with specific primers or were extended by a random primed cDNA approach (Koenig et al., 2004). The sequence of the 5′ end of NeRNV RNA was determined using the 5′ RACE system of rapid amplification of cDNA ends (Invitrogen) and that of the 3′ end using an RNA ligase-mediated RACE method essentially as described by Coutts & Livieratos (2003). For cloning into the pGEM-T vector (Promega), PCR products were purified using the Jetsorb Gel Extraction kit (Genomed). Sequencing was done by a commercial company (MWG-Biotech). Sequences were analysed using UWCGC software version 8 (Devereux et al., 1984). The program STAR was used for prediction of RNA secondary structure, including pseudoknots (Guliyev et al., 1995).

Aminoacylation. Yeast histidyl-tRNA synthetase (HisRS) was partially purified on chromatography on a hydroxyapatite column (Bio-Rad). Fractions with enriched histidyltRNA activity were pooled and dialysed against 50 mM KH2PO4/K2HPO4 (pH 7.5) with 2 volumes protein sample buffer (Bio-Rad), prior to separation by SDS-PAGE and analysis by phosphor imaging (Bio-Rad).

Translation. In vitro translation was essentially carried out as described previously (Barends et al., 2003). Briefly, wheat germ extract (Promega) was complemented with 100 mM KCl, 100 μM each amino acid except methionine, 1 U RNaseOUT μl−1 (Invitrogen), 100 nM RNA and 5% (v/v) yeast HisRS extract. Reactions were followed at 30 °C and, at the indicated times, 20 μl samples were precipitated in 1 ml 5% (w/v) ice-cold trichloroacetic acid and filtered over GF/C filters (Whatman). Radioactivity was measured by liquid scintillation counting.

RESULTS AND DISCUSSION

Genome organization

The genomic RNA of a Nemesis isolate of NeRNV comprises 6285 nt. Like the genomic RNAs of other tymoviruses that have been fully sequenced so far, it has three major open reading frames (ORFs) (Fig. 1). Comparison of the deduced amino acid sequences of the putative gene products of these ORFs with the corresponding ones of other tymoviruses suggests that ORF I encodes a protein that may act as a movement protein (Bozarth et al., 1992) and also as a suppressor of RNA silencing (Chen et al., 2004). ORF I almost entirely overlaps with the 5′ end of ORF II, which starts, as in the RNAs of all previously analysed tymoviruses, 7 nt further downstream (Fig. 1 and Fig. 2). The translation product of ORF II contains the motifs of methyltransferases (aa 65–219), papain-like proteases (aa 732–818), helicases (aa 915–1143) and RNA-dependent RNA polymerases (aa 1443–1670), which are typically found in the tymoviral polyproteins (Koonin & Dolja, 1993; Bransom & Dreher, 1994). The translation product of ORF III shows similarities with the coat proteins of other tymoviruses. Its closest relationship (68% amino acid sequence identity) was found to the coat protein of Ononis yellow mosaic virus (OYMV). A detailed account of the relationships of the NeRNV proteins with those of other tymoviruses has been given by Koenig et al. (2005).

The in vitro translation patterns of TYMV RNA and NeRNV RNA confirmed that the latter has typical properties of a tymoviral RNA (Fig. 3). The ORF II product of NeRNV RNA, with a predicted molecular mass of 200 kDa, migrates slightly faster than the 206 kDa ORF II product of TYMV RNA. The TYMV ORF II product has previously been shown to be proteolytically cleaved into products of 140 and 66 kDa (Kadare et al., 1995; Bransom et al., 1996). In the experiments shown in Fig. 3, a distinct band corresponding to ~140 kDa is produced by TYMV RNA translation and a slightly faster migrating one (as might be expected from the shorter full-length ORF II product) by NeRNV RNA translation. Bands for the 66 kDa cleavage product and the ORF I-encoded movement proteins are less pronounced due to the presence of translational arrested protein products from ORF II. The very similar translation patterns obtained with the RNAs of NeRNV and TYMV greatly differ from those obtained with the other two aminocytalplant virus RNA, i.e. those of BMV and TMV (Fig. 3).
The 5′ UTR

The 153 nt 5′-UTR of the NeRNV RNA can be folded into a series of characteristic hairpin structures (HP 1 to 4) in an otherwise U- and C-rich single-stranded context (Fig. 2). These hairpins are characterized by internal loops containing C–C or U–C mismatches. Similar hairpins have been predicted for all other fully sequenced tymoviral RNAs (Hellendoorn et al., 1996b; Koenig et al., 2005; C. W. A. Pleij, unpublished observations). However, U–C mismatches, which occur in HP 2 and 3 of NeRNV RNA, have previously been found only in HP 2 of the 5′-UTR of OYMV RNA (Hellendoorn et al., 1996b) and in the RNAs of the related Anagyris vein clearing virus, Plantago mottle virus and Scrophularia mottle virus (Koenig et al., 2005). For TYMV, it has been shown that the C–C (and also C–A) pairs in the internal loops of the hairpins of the 5′-UTR form protonated base pairs at low pH and play an important role in the initiation of RNA encapsidation (Hellendoorn et al., 1997; Bink et al., 2002).

The 3′ end

In view of the many above-described similarities of the NeRNV RNA with those of previously analysed tymoviruses, it was surprising to find that the predicted secondary structure of the 3′ end of NeRNV RNA greatly differs from those of other tymoviral RNAs. For the majority of tymoviral RNAs, 3′-terminal valine-specific tRNA-like structures (TLS) are predicted that are usually preceded upstream by various stem–loops or one or more pseudoknots (Hellendoorn et al., 1996a; Koenig et al., 2005). The 3′ end of NeRNV RNA, however, strongly resembles that of tobamoviral RNAs (Rietveld et al., 1984; van Belkum et al., 1985) in that it can be folded into a so-called UPD and a TLS with an anticodon for histidine (Fig. 4). This unusual 3′ end is obviously not a specific feature of the Nemesia isolate of NeRNV. Short stretches of 441 and 675 nt, respectively, of Verbena and Diascia isolates of NeRNV were also amplified. The RNA sequence of the Diascia isolate contained the entire coat protein gene and the entire UPD. The sequence of the Verbena isolate contained the 3′-terminal part of the coat protein gene, the entire UPD and, in addition, a 5′ portion of the TLS including most of the stem–loop with the anticodon for histidine. Sequences of the Diascia and Verbena isolates of NeRNV showed 99.4 and 99.1% identity with the corresponding RNA parts of the Nemesia isolate suggesting that the three isolates are essentially identical.

The 3′ UPD

Tobamovirus-like UPDs consisting of at least two very characteristic pseudoknot structures (PK1 and PK2 in Fig. 5) have been described not only for tobamoviruses, but also for many hordeiviruses, furoviruses and pomo-viruses and even for one tymovirus, i.e. *Wild cucumber mosaic virus* (WCMV) (van Belkum et al., 1985; Pleij et al.,...
Alignment of the PK1- and PK2-containing regions of the UPDs of representatives of various virus genera reveals that pseudoknot PK1 of NeRNV RNA and the corresponding pseudoknot structures of other viruses have a loop 2 (mostly UAAAUC) that is highly conserved among many viruses in different genera (Fig. 5). They all have a bulged A residue at a specific position in the 3’ strand of stem 2 (marked by an arrow in Fig. 5), which may ensure linkage and coaxial stacking of the two stem regions of this pseudoknot. In addition, the PK2 structures have a typical 3 bp stem S1, a single nucleotide loop L1, a 6 bp stem S2 and a loop L2 of 3 nt with a conserved 3’-proximal A residue. Interestingly, the UPD in NeRNV RNA shares a higher degree of identity with those of several tobamoviruses (e.g. 81 % with that of the crucifer tobamovirus described by Shimamoto et al., 1998) and pomoviruses than with that of WCMV (Fig. 5). This suggests that the UPD regions in the RNAs of NeRNV and WCMV may have different origins.

The hairpin structure between the UPD and the TLS of NeRNV RNA is not found in tobamovirus RNAs (Fig. 4). However, some pomoviral and furoviral RNAs do show a similar structural organization (Koenig et al., 1998, 1999, 2000).
The TLS of NeRNV RNA (Fig. 4) has the typical 11 bp acceptor domain, including the pseudoknot structure (PKa) and the strongly conserved seven-membered T-like loop (Mans et al., 1991). Furthermore, five out of the six base pairs that form pseudoknot PKb are identical in both TMV and NeRNV RNA and the D-like loop in NeRNV RNA also contains the conserved sequence UGGA. The two G residues in this sequence are supposed to interact with the T-like loop in a way analogous to elongator tRNAs (Mans et al., 1991; Felden et al., 1996). NeRNV RNA, like TMV RNA, was effectively charged with histidine (Fig. 6), thus proving the functionality of the anticodon arm in its TLS. Neither NeRNV nor TMV RNA were charged with valine (not shown).

Interestingly, there are a few dissimilarities between the TLS of the NeRNV and TMV RNAs that involve identity elements for recognition by yeast HisRS. Instead of the GUU anticodon in TMV RNA, NeRNV RNA, like in many other tobamovirus RNAs, harbours the more active GUG anticodon. With yeast tRNA\textsubscript{His}, such an exchange results in a fourfold increase in charging efficiency (Nameki et al., 1995). Another difference lies in the equivalent of the major identity element, located in the acceptor arm. In all elongator tRNA\textsubscript{His} species, there is an extra nucleotide at the 5' end, G\textsubscript{–}1, which pairs in a non-canonical way with A\textsubscript{73}. The possible equivalent pairing in NeRNV RNA, A\textsubscript{1–}G\textsubscript{18} (numbering is from the 3' end under the assumption that a terminal A is added by nucleotidyl transferase in the cytoplasm of the host) is exactly the same as for yeast tRNA\textsubscript{His}, whereas TMV RNA has a C\textsubscript{4–}A\textsubscript{18} pairing at the corresponding position, which would lead to a twofold reduction in aminoacylation efficiency in yeast tRNA\textsubscript{His} (Rudinger et al., 1994; Nameki et al., 1995). Indeed, in line with all these observations, it is not surprising that NeRNV RNA exhibits significantly higher charging efficiency than TMV RNA (Fig. 6).

Concluding remarks

In summary, NeRNV RNA, which is tymovirus-like in most of its parts, has a 3' end with almost the same organization as tobamovirus RNAs, including a TLS that can be specifically aminoacylated with histidine. The tymovirus WCMV was shown previously to also have a UPD domain, but this domain is followed by the valylatable TLS typical for tymoviral RNAs (Hellendoorn et al., 1996a). In fact, NeRNV is more or less the counterpart of Sunn-hemp mosaic virus (SHMV or, previously, CcTMV) (Meshi et al., 1981; Rietveld et al., 1982), the only tobamovirus known to have a valine-specific TLS downstream of a UPD domain. Interestingly, both SHMV and NeRNV have a hairpin structure...
between the UPD domain and the TLS (van Belkum et al., 1985). In both cases, this may represent a remnant of the recombination event, although it should be kept in mind that SHMV RNA could have received its 3'-UTR from a pomo- or furoviral RNA as an alternative to a tymoviral RNA. Another possibility is that this hairpin is needed to ensure proper mutual orientation of the UPD and TLS. The same phenomenon is observed in the 3'-UTR of tyrosylatable hordeivirus RNAs, which also have a UPD domain (Pleij et al., 1987). Interestingly, a chimeric TYMV RNA harbouring the TLS of TMV RNA was not infectious to plants (Skuzeski et al., 1996) and it was only after introducing TYMV sequences into the amino acid acceptor arm and anticodon loop (including the valine anticodon) and passingage in plants, which produced further mutations, that the resulting viruses, TYMC-XX and TYMC-YY, became highly infectious to plants (Goodwin et al., 1997; Filichkin et al., 2000).

Fig. 7 summarizes the various compositions of the RNA 3' ends of tobamoviruses and tymoviruses and compares them with those of pomo- and furoviruses. It illustrates the high flexibility in the make-up of 3' ends among these virus genera, which is presumably due to recombination events in doubly infected plant hosts. This suggestion is supported by the occurrence of frequent duplications of UPDs and parts of the TLS in several tobamoviruses (Gultyaev et al., 1994; Bodaghi et al., 2000; Ruiz del Pino et al., 2003). In view of the observations summarized in Figs 5 and 7, it might not be surprising if some day a pomo- or furovirus would be detected with an RNA that has a histidylatable TLS on its 3' end rather than the common valylatable TLS.

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