Complete nucleotide sequence of tobacco necrosis virus strain DH and genes required for RNA replication and virus movement

Attila Molnár, Zoltán Havelda, Tamás Dalmay, Henrietta Szutorisz and József Burgyán

Agricultural Biotechnology Center, Plant Science Institute, PO Box 411, 2101 Gödöllő, Hungary

The complete genome sequence of tobacco necrosis virus strain D (Hungarian isolate, TNV-DH) was determined. The genome (3762 nt) has an organization identical to that reported for TNV-D. Highly infectious synthetic transcripts from a full-length TNV-DH cDNA clone were prepared, the first infectious necrovirus transcript reported. This clone was used for reverse genetic studies to map the viral genes required for replication and movement. Protoplast inoculation with Δ22 and Δ82 mutants revealed that both the 22 kDa and 82 kDa gene products are required for RNA replication. Although the products of three small central genes (p7a, p7b) were not essential for RNA replication in protoplasts, mutations in these ORFs prevented infection of plants. In contrast, viral RNA accumulation and cell-to-cell movement were observed in the inoculated, but not the systemically infected, leaves of Nicotiana benthamiana challenged with RNA lacking the intact coat protein (CP) gene. These results strongly suggest that p7a, p7b and CP are involved in TNV-DH cell-to-cell and long-distance movement, respectively.

Tobacco necrosis virus (TNV) is a small icosahedral virus which belongs to the genus Necrovirus (Russo et al., 1994). Particles of TNV contain a single-stranded positive-sense RNA genome approximately 3-8 kb long. The complete nucleotide sequence of TNV strain D (TNV-D) reported by Couvts et al. (1991) and revised recently (Offei & Couvts, 1996) indicates the presence of five open reading frames (ORFs). The genomic RNA of TNV-D probably directly translates both ORF1 (p22) and ORF2 (p82) from the same initiation codon, and p82 is likely to be synthesized by translational readthrough of the amber stop codon. The readthrough part of p82 contains the GDD motif typical of viral RNA polymerases (Argos, 1988).

The two small ORFs located centrally in TNV-D RNA encode two different 7 kDa proteins (p7a and p7b) and the coat protein (CP) is encoded at the 3′ end. The two 7 kDa proteins show limited sequence similarity with the corresponding gene products of related TNV strain A (TNV-A) (p8 and p6; Meulewaeter et al., 1990) and the carmovirus turnip crinkle virus (TCV) (p8 and p9; Carrington et al., 1989). These small proteins of TNV-A and TNV-D are thought to be involved in cell-to-cell movement (Coults et al., 1991; Meulewaeter et al., 1992; Offei et al., 1995; Drouzas et al., 1996) of the two viruses, similar to that demonstrated for TCV (Hacker et al., 1992).

In the present study, we provide evidence for the roles of proteins encoded by the TNV genome using a full-length infectious cDNA clone of a new TNV-D isolate (TNV-DH) found in our greenhouse associated with Cymbidium ringspot tombusvirus (CymRSV). TNV-DH was found in Nicotiana clevelandii plants infected with CymRSV. After purification of the virus through several local lesion passages in N. clevelandii, it was propagated in Nicotiana benthamiana and purified from systemically infected leaves by the procedure described for tombusviruses (Gallitelli et al., 1985). The RNA was extracted from virus particles (Dalmay et al., 1993 a) and the 3′ end was polyadenylated with poly(A) polymerase (Amersham). The polyadenylated RNA was used as template for oligo(dT)-primed cDNA synthesis using the cDNA System Plus (Amersham) according to the manufacturer’s protocol. Alternatively, first-strand cDNA synthesis was primed with random hexanucleotide primers using the same kit. Double-stranded DNA was ligated to Smal-digested dephosphorylated pUC18 and cloned in Escherichia coli strain DH5α. TNV-DH-specific recombinant clones were sequenced by the dideoxy chain-termination method (Sanger et al., 1977) with T7 DNA polymerase (Sequenase, Amersham-USBiochemicals) on both strands of several independent cDNA clones. The sequence of the 5′ region of TNV-DH RNA was determined by dideoxy-
Fig. 1. (a) Comparison of the genome organizations of TNV-D, -DH and -A and TCV. The ORFs for each genome are shown as boxes and the approximate sizes of the predicted proteins are indicated. ORFs encoding proteins with similar putative functions are shown with the same type of shading. rt indicates the amber stop codon of ORF1. (b) Cluster dendrograms generated by the PILEUP, DISTANCES and GROWTREE programs showing the relationship between the predicted small internal ORF products (see (a)) of TNV-DH (p7₁, p7ₐ and p7ₚ) and other necroviruses [TNV-D (p7₁, p7ₐ and p7ₚ); Offei & Coutts, 1996], TNV-NE (p6 and p11; Zhang et al., 1993), TNV-A (p6, p7 and p8; Meulewaeter et al., 1990), OLV-1 (p6 and p8; Grieco et al., 1996) and LSWV (p6 and p11; Lot et al., 1996)] and TCV carmovirus (p8 and p9; Carrington et al., 1989).
length in vitro transcripts for biological activity tests. This clone was constructed using newly prepared cDNA clones of the 5' half of the TNV-DH and a clone (A67) containing approximately the last 2500 nt and the added poly(A) tail. The 5' half was cloned by priming first-strand synthesis with the oligonucleotide 5' TGGGAGAAA-TACGGAGGCCTAT 3' complementary to nt 2143–2162 of the genomic RNA sequence. The cDNA was amplified by PCR using the above oligonucleotide and 5' ATCGATAATACGACTCAGTG 3', which contained the first 24 nt of TNV-DH genomic RNA (bold) fused to a 17 nt bacteriophage T7 RNA polymerase promoter consensus sequence and five bases contributing to the formation of a Clal restriction site (underlined italic). The major PCR product of approximately 2200 bp was cloned as described above. The resulting clones A152 and A67 contained the 5' and 3' halves of the viral genome, and were fused using the EcoRV restriction site at position 2157. The resulting full-length cDNA clone (A172) contained the complete sequence of TNV-DH and a poly(A) tail at the 3' end.

The genomic transcript of TNV-DH was designed to start with two G residues for good T7 promoter activity (Dunn & Studier, 1983). Since the A172 clone contained an A20 tail at the 3' terminus of the viral sequence, linearization was downstream using SalI, which resulted in the addition of 10 non-viral nucleotides 3' of the poly(A) tail. The RNA transcripts had a specific infectivity similar to natural viral RNA in local lesion assays on Chenopodium quinoa. Moreover, there was 100% infection in N. benthamiana, eliciting necrotic local lesions on inoculated leaves and systemic stunting, withering and severe chlorotic mottling. These symptoms are very similar to those described for OLV-1 (Martelli et al., 1996), but are different from necrotic infection (necrotic local lesions) elicited by TNV-D in N. benthamiana (Martelli et al., 1996). Northern blot analysis of RNA extracted from TNV-DH-infected N. benthamiana revealed the presence of genomic and subgenomic (sg1 and sg2) RNAs are marked.

Fig. 2. Northern blot analysis of nucleic acids extracted from inoculated leaves of N. benthamiana plants (a) or protoplasts (b) inoculated with RNA transcribed from wild-type (A172) and mutant TNV-DH clones. The positions of wild-type genomic (G) and subgenomic (sg1 and sg2) RNAs are marked.

Table 1. Percentage amino acid sequence similarity between proteins encoded by TNV-DH and the corresponding ORFs of necroviruses and TCV carmovirus

<table>
<thead>
<tr>
<th>Virus</th>
<th>p22</th>
<th>p82</th>
<th>p71</th>
<th>p7a</th>
<th>p7b</th>
<th>CP</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNV-D</td>
<td>94 (85)</td>
<td>95 (89)</td>
<td>82 (73)</td>
<td>95 (91)</td>
<td>94 (89)</td>
<td>97 (95)</td>
</tr>
<tr>
<td>LWSV</td>
<td>62 (43)</td>
<td>76 (64)</td>
<td>—</td>
<td>63 (40)a</td>
<td>71 (56)b</td>
<td>48 (29)</td>
</tr>
<tr>
<td>TNV-NE</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>46 (19)c</td>
<td>48 (21)d</td>
<td>59 (45)</td>
</tr>
<tr>
<td>TNV-A</td>
<td>50 (24)</td>
<td>61 (40)</td>
<td>40 (20)f</td>
<td>44 (19)f</td>
<td>48 (21)f</td>
<td>62 (47)</td>
</tr>
<tr>
<td>OLV-1</td>
<td>48 (24)</td>
<td>59 (39)</td>
<td>—</td>
<td>50 (25)h</td>
<td>48 (21)h</td>
<td>56 (41)</td>
</tr>
<tr>
<td>TCV</td>
<td>42 (19)</td>
<td>56 (35)</td>
<td>—</td>
<td>47 (19)i</td>
<td>51 (33)i</td>
<td>43 (22)</td>
</tr>
</tbody>
</table>

Numbers in parentheses show the percentage amino acid sequence identity. Superscript letters indicate proteins which have names other than the corresponding proteins of TNV-DH: a, LWSV p11; b, LWSV p6; c, TNV-NE p11; d, TNV-NE p6; e, TNV-A p7; f, TNV-A p6; g, TNV-A p6; h, OLV-1 p6; i, OLV-1 p6; j, TCV p8; k, TCV p9. For references see legend to Fig. 1(b).
was mutated to ATA (position 2213) using the oligonucleotide 5’ CTCTGGGCGATTTAACTCACC 3’; ∆7a, in which the initiation codon was mutated to GTG (position 2251) using the oligonucleotide 5’ TCGAAGCTAGGGAAAAATTC 3’; ∆7b, in which the initiation codon was mutated to CTG (position 2448) using the oligonucleotide 5’ TTCATTTC-TGCTGAATACAT 3’; Finally, all three oligonucleotides were used simultaneously to prepare the triple mutant ∆7_ab.

In vitro RNA transcripts of A172 mutants were prepared from SalI-linearized template DNA, and were used to inoculate N. benthamiana plants as described previously (Dalmay et al., 1993b). Samples of inoculated leaves were taken 2–3 days post-infection regardless of whether lesions had appeared, and 7–10 days later from upper uninoculated leaves. Inoculated plants which did not develop symptoms were kept for up to 6 weeks, and leaf samples were collected and examined periodically. Protoplasts were isolated from N. benthamiana plants and transfected with in vitro-synthesized transcripts from A172 or its mutants using the polyethylene glycol (PEG) method (Dalmay et al., 1993b). Total RNA was extracted from 50 mg leaf tissue or 0.5 × 10⁶–1 × 10⁶ protoplasts according to White & Kaper (1989), as modified by Dalmay et al. (1993b). The presence of virus-related RNA was assessed by Northern blot analysis with a ³²P-labelled probe prepared by nick-translation or random priming (Sambrook et al., 1989) of a clone representing the 3’-terminal 600 nt of the TNV-D genome.

Plants inoculated with in vitro transcripts of clone A82 lacking a large central portion of the readthrough region of the 82 kDa ORF remained symptomless for several weeks after inoculation in four independent experiments. Furthermore, Northern blot analysis of total RNA extracted from inoculated plants (Fig. 2a) or protoplasts (Fig. 2b), failed to show the presence of viral RNA. However, expression of p82 alone may not be sufficient for genomic RNA replication since a mutant in which the 22 kDa amber stop codon was replaced with a tyrosine codon (Δ22) also failed to replicate in both plants and protoplasts (Fig. 2a, b). These results strongly suggest that both the 22 and 82 kDa readthrough product of ORFs 1 and 2 are essential for replication of TNV-D RNA.

Plants inoculated with in vitro-transcribed RNA from the initiation codon mutants of the three 7 kDa genes mutated singly (∆7γ, 7a and ∆7b) or simultaneously (∆7γab), failed to show symptoms for several weeks and total RNA extracts from inoculated leaves did not contain any detectable virus-specific RNA. In contrast, Northern blot analysis of RNA samples from protoplasts transfected with the same inoculum showed that each of the initiation codon mutants was able to replicate (Fig. 2b). The presence of subgenomic RNAs in infected protoplasts indicated that the mutations did not interfere with viral RNA replication. It is likely, therefore, that these three small proteins are involved in cell-to-cell translocation in plants and that the virus cannot spread beyond the initial infection site when any one of them is not produced.

The ACP mutant had most of the coat protein deleted (i.e. the sequence between the Ata1 and EcoRV sites, nt 2853–3395). If the truncated CP gene was expressed, a defective coat protein of approximately 7 kDa would be synthesized. N. benthamiana plants infected with in vitro transcripts of ACP showed large dark necrotic lesions on the inoculated leaves which were similar, if not identical, to those produced in plants inoculated with A172 RNA. The necrotic lesions produced by the ACP mutant grew continuously until the inoculated leaf became completely necrotized. However, the upper non-inoculated leaves remained symptomless. Northern blots of RNA samples extracted from the inoculated leaves showed the presence of three virus-specific RNAs, which had the sizes expected for mutant genomic and subgenomic RNAs (Fig. 2a).

The quality of total RNA extracts from ACP- as well as A172-infected plants was usually poor due to the very rapid (2 days post-infection) appearance of necrotic lesions on inoculated leaves. The accumulation of the ACP mutant in transfected protoplasts was similar to wild-type virus (Fig. 2b), indicating that replication of the viral genome was not affected. An unusual accumulation of a small viral RNA (approximately 600 nt) was frequently observed in ACP-infected plants and protoplasts. At first, it was believed that this was a defective interfering RNA; however, this RNA hybridized strongly with the 3’-specific probe (Fig. 2a, b), but not with a nick-translated probe of a clone containing the 5’ half of the viral genome (results not shown). Furthermore, repeated attempts to amplify this RNA with RT–PCR using two oligonucleotides containing the first 20 nt and a sequence complementary to the last 17 nt of the viral RNA, respectively, were unsuccessful. Based on these results this RNA was identified as the smaller subgenomic RNA of the ACP mutant.

Sequence data revealed that p82 contained the GDD motif common to RNA-dependent RNA polymerases (Argos, 1988), suggesting that p82 is the viral replicate. However, it could not be predicted from sequence information alone whether or not other TNV-D proteins might be required for replication. Elimination of the amber termination codon of the p22 kDa gene (Δ22) prevented RNA replication. When expression of the 82 kDa protein, but not the 22 kDa protein, was prevented (Δ82), replication of RNA was also blocked. This result strongly suggests that expression of both p22 and p82 is required for RNA replication, in agreement with data obtained with TCV and TNV-A (Hacker et al., 1992; White et al., 1995; Andriessen et al., 1995).

By analogy with the p8 and p9 proteins of TCV (Hacker et al., 1992), it was suggested that the proteins expressed from the small centrally located ORFs of necroviruses (TNV-A and -D) may be involved in cell-to-cell movement (Couitts et al., 1991; Meulewaeter et al., 1992; Oftei et al., 1995; Drouzas et al., 1996). This proposal has been verified by experiments in this paper in which each of the three p7 proteins of TNV-D were shown to be required for efficient spread of the virus in inoculated plants. Nevertheless, the amino acid sequences of
these small proteins do not contain the conserved motifs of movement proteins (Mushegian & Koonin, 1993).

The ability of the ΔCP mutant to infect leaves indicates that CP is not essential for cell-to-cell spread. However, the ΔCP mutant was not able to infect N. benthamiana plants systemically. This behaviour indicates that TNV-DH requires its coat protein (or perhaps intact particles) for efficient long-distance movement, in a similar way to the related TCV and its coat protein (or perhaps intact particles) for efficient long-distance movement, in a similar way to the related TCV and its coat protein (or perhaps intact particles) for efficient long-distance movement, in a similar way to the related TCV and its coat protein (or perhaps intact particles) for efficient long-distance movement.

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References


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