SUMMARY

A tombusvirus was isolated from plants of statice (Goniolimon tataricum) showing malformation, mosaic and necrosis of the leaves and poor flowering. Electron microscopy of infected tissues revealed the presence of vesiculated structures derived from altered peroxisomes. In Northern blots four major RNA species were detected, corresponding to viral genomic, subgenomic and defective interfering (DI) RNAs. Both genomic and DI RNAs were cloned and sequenced, and shown to have structure and organization similar to those of viruses of the genus Tombusvirus. In agar gel diffusion tests, spurs were formed when the statice isolate was compared with the cherry strain but not with the BS3 strain of Tomato bushy stunt virus.

Key words: statice, TBSV, Goniolimon tataricum.

In 1989 a virus disease of statice [Goniolimon tataricum (L.) Boiss.] was recorded from several localities of Palatinate (Germany). Affected plants showed malformation, mosaic and necrosis of the leaves and severely reduced flowering (Krczal and Beutel, 1994). An isometric virus was transmitted by sap inoculation to a number of herbaceous hosts, and tentatively identified as a strain of Tomato bushy stunt virus (TBSV), synonym of Petunia asteroid mosaic virus (PAMV) (Koenig and Kuntze, 1982), whereas a continuous line was observed in the reaction with TBSV-BS3, regardless of whether antisera TBSV-Sta or to TBSV-BS3 were used (Fig. 1).

For electron microscopy, tissues were sampled from the upper leaves of systemically infected plants one week after inoculation, and processed as described by Martelli and Russo (1984), i.e., fixation in 4% glutaraldehyde in 0.05 M phosphate buffer (pH 7.4), post-fixation in 1% osmium tetroxide for 2 h at 4°C, and staining overnight in 0.5% aqueous uranyl acetate at 4°C, dehydration in a graded ethanol series, and embedding in Spurr’s medium. Thin sections were stained with lead citrate and viewed with a Philips 201 C electron microscope. Infected cells contained cytopathic structures typical of most tombusviruses (Russo et al., 1987), i.e., multivesicular bodies consisting of aggregates of many vesicles resulting from proliferation of the limiting membrane of peroxisomes (Fig. 2).

Total RNA from infected tissue was extracted as detailed in Dalmay et al. (1993). For Northern blot analysis, RNA samples were denatured with glyoxal, electrophoresed in 1.2% agarose gel, transferred to nylon membranes and probed with digoxigenin-labelled clones. Hybridization signals were detected by chemiluminescence using a kit from Boehringer (Mannheim). Three major viral RNA species were observed (Fig. 3), which were interpreted as the genomic and subgenomic RNAs, respectively, as reported for tombusviruses (Russo et al., 1994). In addition total RNA extracts contained an RNA species of approx. 500 nt.

For cDNA cloning and sequencing, viral genomic RNA was extracted from virus particles by adding 1 vol. of buffer (100 mM glycine-NaOH, pH 9.0, containing 100 mM NaCl, 10 mM EDTA, 2% sodium dodecyl sulfate, and 1% sodium lauroyl sarcosine) and 2 vol. of phenol. The aqueous phase was further extracted...
with equal volumes of phenol and chloroform, then chloroform alone, and precipitated with 2.5 vol. of ethanol in the presence of 0.3 M sodium acetate, pH 5.5. The RNA was resuspended in sterile water. Approximately 2 µg RNA were denatured by heating at 65°C. After cooling, the 3’ end was polyadenylated with poly(A) polymerase (BRL) according to the manufacturer’s protocol. The polyadenylated RNA was extracted with phenol-chloroform, precipitated with ethanol, resuspended in 10 µl of water and used as template for oligo(dT)-primed cDNA synthesis using the cDNA Synthesis Module (Amersham) according to the manufacturer’s protocol.

The sequence of the 5’ region was determined according to Hirzman et al. (1993). Briefly, first-strand cDNA was primed with an oligonucleotide complementary to nucleotides 5’GGCTAGCGCGCATATAGG3’ in genomic RNA, digested with ribonuclease H, purified through a QIAquick column (QIAGEN), and tailed with dGTP using terminal deoxynucleotidyl transferase (BRL). The dG-tailed cDNA was amplified by polymerase chain reaction (PCR) using a (C)14 oligonucleotide primer and the oligonucleotide complementary to nucleotides 5’TGAACCACTCCATATTGG3’. To clone the rest of the molecule, cDNA first-strand synthesis was primed with random hexanucleotide primers and double-stranded DNA of various sizes was obtained by PCR using appropriate overlapping couples of primers. The resulting double-stranded DNA was made blunt-ended with Klenow enzyme ligated to Smal-digested, dephosphorylated pUC18 (Pharmacia) or directly to pGEM-2T (Promega) and cloned in *Escherichia coli* strain DH5α. DNA was extracted from a selection of recombinant clones and sequenced on both strands by the dideoxy chain termination method (Sanger et al., 1977) with T7 DNA polymerase (Sequenase, US Biochemicals).

Sequence data were analyzed using the DNA Strider.
software (Marck, 1988) and the GAP procedure of the Genetics Computer Group (GCG) software package of the University of Wisconsin (Version 8). Analysis of cDNA clones obtained after extending the cDNA products with terminal deoxynucleotidyl transferase showed that the 5’ two terminal nucleotides were A and G, respectively, thus excluding the presence of a cap structure at the 5’ end. The sequence of the 3’ terminal region, obtained from cDNA clones made to in vitro polyadenylated RNA using an oligo(dT) primer, ended with -CCC which is a feature common to the genus Tombusvirus and related genera (Russo et al., 1994).

The complete genome was 4770 nt long and contained five open reading frames (ORFs) (EMBL accession number AJ249740). The first ORF started from an AUG at nt 169-171 and ended with an amber stop codon UAG at nt 1057-1059. Readthrough would extend the frame up to a termination codon at nt 2623-2625 (ORF 2). ORF 3 was from nt 2649 terminating with a UGA at nt 3813-3815. ORFs 4 initiated at nt 3851 ending with a UGA at nt 4418-4420. ORF 5 was nested in ORF 4 in a different frame beginning with an AUG at nt 3883-3885 and terminating with UAG at nt 4399-4401. The predicted Mr of polypeptides encoded by the five ORFs were respectively: 33,476 (33 K), 92,362 (92 K), 40,928 (41 K), 21,558 (22 K), and 19,352 (19 K). The 3’ untranslated region was 369 nt long.

Table 1. Pairwise amino acid sequence comparison of proteins encoded by the genome of the statice isolate with corresponding gene products of other tombusviruses. RT: readthrough domain of viral polymerase; CP: capsid protein; –: not determined.

<table>
<thead>
<tr>
<th>Virus</th>
<th>RT</th>
<th>CP</th>
<th>22K</th>
<th>19K</th>
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<tr>
<td>AMCV</td>
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<td>TBSV-BS3</td>
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<td>95</td>
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<td>96</td>
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Table 1 shows pairwise comparisons between the proteins encoded by TBSV-Sta and those of other sequenced tombusviruses taken from databanks except for the CP gene of TBSV-strain BS3 which was cloned and sequenced by us. This was done because three sets of residues (246-258, 269-276, and 365-379) of this strain had been obtained by electron density assignment (Hopper et al., 1984), rather than by direct sequencing. TBSV-BS3 was obtained from Dr. R. Koenig (Braunschweig, Germany) and purified as above.

To clone the CP gene, a random-primed cDNA to genomic RNA was used as template for PCR using as primers two oligonucleotides in the highly conserved regions flanking the CP gene of all sequenced tombusviruses. The PCR product was cloned in pUC18 and sequenced.

The deduced amino acid sequence of TBSV-BS3 CP (EMBL accession number AJ271328) was in fact different from the published sequence in the three sets of residues mentioned above. Table 1 shows that the highest degree of homology occurred in the pairwise comparison of the readthrough domain of ORF 2. This region is highly conserved since it contains the motifs of RNA polymerases of many positive-stranded RNA viruses (Koonin, 1991; Russo et al., 1994). The same applied to the pre-readthrough domain (ORF 1), except for the comparison with CIRV due to the different targeting signal contained in this protein (Rubino and Russo, 1998). The protein encoded by ORF 4 (22K) was also very similar to the corresponding products of other tombusviruses, whereas the product of ORF 5 (19K) was more similar to that of the TBSV cluster. Finally, a striking similarity was found in the composition of TBSV-Sta CP (ORF 3) with the CP of TBSV-BS3, which is in line with serological data.

As shown in Fig. 3, TBSV-Sta-infected N. benthamiana tissue contained a virus-related RNA species of approximately 500 nt in addition to genomic and subgenomic RNAs. This RNA was cloned using total RNA extracts as template. First-strand synthesis was primed with oligonucleotide 5’GGGCTGCATTCTG-CAATG3’, which is complementary to the last 19 nucleotides of TBSV-Sta genome, and cDNA was amplified by 35 PCR cycles using as second primer 5’GAAATCCCCAGGATTTC3’, which is homologous to nt 2-18 in the virus genome, and Taq DNA polymerase (Boehringer, Mannheim). The resulting ds DNA was made blunt-ended with Klenow enzyme, cloned in pUC18 and sequenced as described above.

Sequence analysis showed that this RNA species was indeed a DI RNA of 521 nt, made up of four blocks (I-IV) entirely derived from genomic RNA: (i) block I was comprised of the first 171 nt of the genomic RNA including the 5’ leader sequence and the start codon of ORF 1; (ii) the 168 nt long block II was identical to the
central part of the polymerase gene (ORF 2) from nt 1356 to nt 1524; (iii) block III was 54 nt long and contained 23 nucleotides of the carboxyl terminus of ORF 4 and 31 nt of the 3' non-coding region; (iv) block IV was 128 nt long and comprised the 3'terminal nt of the viral genome. Further passages in N. benthamiana did not modify the composition of this molecule, indicating that it had reached the minimal functioning size in the evolution from larger molecules (Russo et al., 1994).

A full-length clone of TBSV-Sta genome was prepared, from which infectious transcripts could be synthesized, with a view to deriving specific sequences for inducing transgenic resistance in statice. The 5' region was cloned by priming first-strand cDNA synthesis with the oligonucleotide 5'AAACCTGGAGTACATTAGC3', complementary to nt 2357-2375 of the TBSV-Sta genome sequence. Hybrid RNA-DNA molecules were melted at 100 °C for 1 min, and the cDNA was amplified by 35 PCR cycles. The second-strand primer was 5'TAATACGACTCACTATAGG AAA TTC CCC AGG3', which contains 23 nucleotides of the carboxyl terminus of ORF 4 and 31 nt of the 3' non-coding region; (iii) central part of the polymerase gene (ORF 2) from nt 154 to nt 1524; (iii) block III was 54 nt long and comprised the 3'terminal nt of the viral genome. Further passages in N. benthamiana did not modify the composition of this molecule, indicating that it had reached the minimal functioning size in the evolution from larger molecules (Russo et al., 1994).


