

SHORT COMMUNICATION

MOLECULAR CHARACTERIZATION OF A TOMBUSVIRUS ASSOCIATED WITH A DISEASE OF STATICE [*GONLIOLIMON TATARICUM* (L.) BOISS.]

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SUMMARY

A tombusvirus was isolated from plants of statice (*Gonolimon 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, *Gonolimon tataricum*.

In 1989 a virus disease of statice [*Gonolimon 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), (genus *Tombusvirus*, family *Tombusviridae* (Martelli and Russo, 1994).

In this paper serological properties, cytopathological characteristics and the complete nucleotide sequence of the genomic and defective interfering (DI) RNAs of this virus (named TBSV-Sta) are reported and comparatively analysed with those of other tombusviruses.

The virus was propagated in *Nicotiana benthamiana* and purified essentially as in Rubino *et al.* (1995). Purified virus preparations (0.5-2 mg) were mixed with an

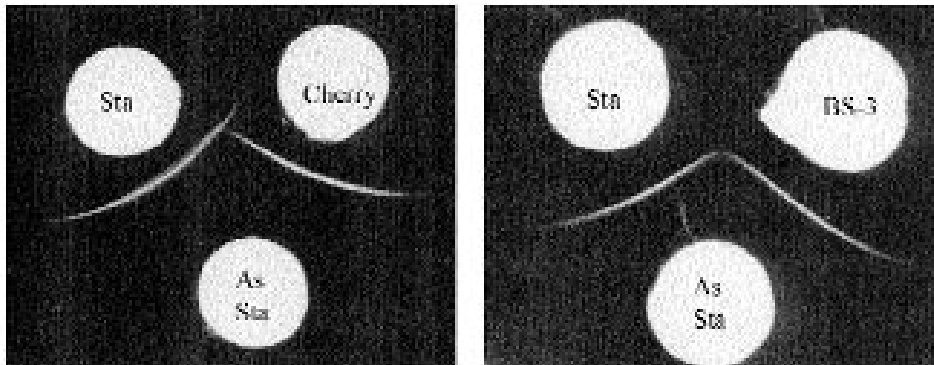
equal volume of Freund's incomplete adjuvant and injected intramuscularly in a rabbit at weekly intervals. Bleeding was done two weeks after the third injection. In agar double diffusion assays a clear spur developed when the statice isolate was tested with TBSV-cherry isolate, synonym of *Petunia asteroid 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

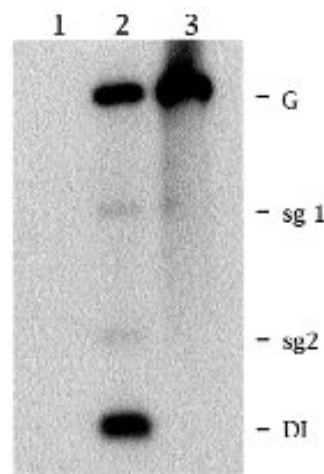
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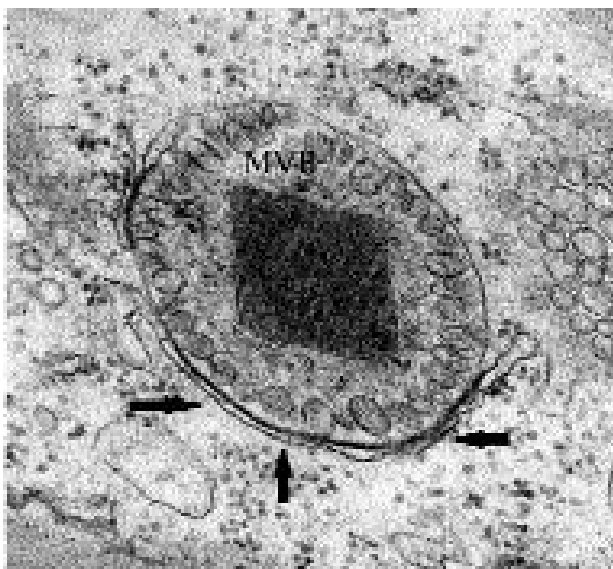
**Fig. 1.** Serological comparison of the static isolate (Sta) with the Cherry and BS-3 isolates of TBSV in agar gel double diffusion tests. Lower wells contain antiserum (As) to TBSV-Sta; upper wells contain antigens as indicated. Identical results were obtained when lower wells contained antisera to TBSV-Cherry or BS-3, respectively.

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



**Fig. 3.** Northern blot analysis of RNA extracted from TBSV-Sta-infected tissues (lane 2) or virus particles (lane 3) showing genomic (G), subgenomic (sg 1 and sg 2) and defective interfering (DI) RNAs. Lane 1 contains extract from uninfected control plants. sg 2 and DI RNAs were not encapsidated at a detectable level. Hybridization was with a cloned probe representing the 3'-terminal 1000 nucleotides of TBSV-Sta RNA.



**Fig. 2.** A multivesicular body (MVB) derived from an altered peroxisome in a *N. benthamiana* cell infected with TBSV-Sta. Arrows point to endoplasmic reticulum in close contact with the modified peroxisomal membrane. Bar = 200 nm.

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)<sub>14</sub> oligonucleotide primer and the oligonucleotide complementary to nucleotides 5'TGAACCACTCCCATTTGG3'. 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 *Sma*I-digested, dephosphorylated pUC18 (Pharmacia) or directly to pGEM-2T (Promega) and cloned in *Escherichia coli* strain DH5a. 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  $M_s$  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.

Virus	Amino acid identity (%)				
	33K	RT	CP	22K	19K
AMCV	93	97	71	95	87
CIRV	62	96	57	94	87
CNV	87	94	38	87	69
CymRSV	87	95	50	81	71
TBSV-BS3	-	-	93	95	94
TBSV-cherry	94	97	73	96	90
TBSV-pepper	93	95	73	96	90

Table 1 shows pairwise comparisons between the proteins encoded by TBSV-Sta and those of other se-

quenced 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 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'GGGCTGCATTTCTGCAATG3', 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'GAAATTCCCCAGGATTC3', 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 static. The 5' region was cloned by priming first-strand cDNA synthesis with the oligonucleotide 5'AACCTGGAGTA-GAATCGC3' 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 the first 14 nt (underlined) of CIRV genome fused to 17 nt of the bacteriophage T7 RNA polymerase promoter consensus sequence, and five bases contributing to the formation of a *Cla*I restriction site. The PCR product of the expected size (2400 bp) was eluted in 1% low melting point agarose, made blunt-ended with Klenow enzyme, ligated to *Sma*I-digested, dephosphorylated pUC18 and cloned in *E. coli* strain DH5a.

The 3' region was cloned by priming first-strand cDNA synthesis with an oligonucleotide 5'GGGCTG-CATTTCTGCAATG3', complementary to the last 19 nucleotides of the genomic RNA sequence. The cDNA was amplified by PCR using this oligonucleotide and the oligonucleotide 5'GCGGCATATGGAATC-CAAG3' homologous to nt 1610-1627 in the CIRV genome. The PCR product (3160 bp) was cloned into *Sma*I-digested and dephosphorylated pUC18 so that a *Sma*I site was regenerated precisely at the 3' terminus of the TBSV-Sta sequence. The 5' and 3' regions were then fused at the common *Sna*BI restriction site, which is present once within the viral sequence. A number of clones were submitted to restriction analysis and two of them (pDM1 and pDM2) were selected. RNAs were obtained from 2 µg plasmid DNAs, linearized with *Sma*I, using T7 RNA polymerase and a T7 transcription kit according to the manufacturer's (BioLabs) instructions. RNA was diluted with an equal volume of inoculation buffer containing 1% celite and 1% bentonite (Heaton *et al.*, 1989) to a final concentration of ca 75 µg µl<sup>-1</sup>, and successfully inoculated to *N. benthamiana* plants with a sterile glass spatula.

In conclusion, it was ascertained that the virus asso-

ciated with the diseased static plants is an isolate of the BS3 strain of TBSV, which has a worldwide distribution and is the cause epidemics in several horticultural crops (Martelli *et al.*, 1988; Luis-Arteaga *et al.*, 1996). TBSV-Sta was recently isolated from dandelion plants (*Taraxacum officinale*) growing in an infected static field (unpublished result). This observation adds concern to static growers, since it shows that wild plants may be important reservoir of the virus in addition to persistence in the soil (Krczal and Beutel, 1994).

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