This paper reports the properties of a tombusvirus isolated by mechanical inoculation from severely stunt-ed and mottled cucumber plants, infected also by Watermelon mosaic virus (WMV), grown in glasshouses in the Plovdiv area (Bulgaria). After separation from WMV, this virus was characterized biologically (host range reaction), physicochemically, molecularly, and ultrastructurally. The virus invaded systemically with difficulty different cucumber cultivars, producing latent infections. It was not serologically related to 24 different viruses in the genera Tombusvirus, Carmovirus, Necrovirus and Sobemovirus, but it had physico-chemical and ultrastructural properties typical of tombusviruses. The genome, which was totally sequenced, is a single-stranded positive-sense RNA of 4576 nucleotides. The genome structure was identical to that of tombusviruses but the sequence homology of the protein cistrons was not higher than 41% when compared to that of all sequenced members of the genus Tombusvirus. Distinct differences were also found in the N-terminal region of the ORF 1 sequence. The cucumber virus was therefore identified as a new putative tombusvirus species provisionally named Cucumber Bulgarian latent virus (CBLV).

Key words: cucumber, tombusvirus, sequencing, cytopathology, serology, taxonomy.

INTRODUCTION

A disease characterized by stunting and severe mot-tling of the leaves was observed in glasshouse-grown cucumbers of cvs. Gergana and Mirei in the Plovdiv area of Bulgaria. Diseased plants contained two viruses one of which had filamentous particles and was identified serologically as Watermelon mosaic virus (WMV). The other virus, which could not be immediately identified, had isometric particles with a rounded contour and a diameter of ca. 30 nm. Both viruses were mechanically transmitted from naturally infected cucumber plants to Nicotiana benthamiana. Pure cultures of the isometric virus were established by inoculating herbaceous hosts with sap from doubly infected N. benthamiana that had been heated at 75°C for 10 min to inactivate WMV.

As reported in the present paper, the isometric virus was characterized and shown to be a hitherto undescribed member of the genus Tombusvirus. The virus is provisionally named Cucumber Bulgarian latent virus (CBLV).

MATERIALS AND METHODS

Host range. Sap from N. benthamiana infected only with CBLV, as ascertained by serology and electron microscopy, was extracted in 0.1 M phosphate buffer pH 7.0 and inoculated to 21 herbaceous hosts of six different families. Inoculated plants were grown in a glasshouse at 25°C and observed for symptom expression.

Virus and viral RNA purification. Systemically infected N. benthamiana leaves were collected 7-10 days after inoculation and homogenized. The slurry was mixed with 10% chloroform and shaken for 15 min. After low-speed centrifugation (20,000 g for 10 min), virus particles were precipitated with 10% polyethylene glycol (m.w. 8,000) and 0.05 M NaCl, followed by one cycle of differential centrifugation (10 min at 20,000 g; 1.5 h at 36,000 g). Pellets were suspended in 0.05 M phosphate buffer, pH 7.0, and centrifuged in a preformed 10-40% caesium sulphate gradient at 52,000 rpm for 1 h in a Beckman Sw60 rotor. The single band formed after centrifugation was collected, diluted 1:3 with 0.05 M NaCl, followed by one cycle of differential centrifugation (10 min at 20,000 g; 1.5 h at 36,000 g). Pellets were suspended in 0.1 M phosphate buffer, layered on 10-40% sucrose density gradient columns, and centrifuged at 141,000 g for 3 h in a Beckman SW41 rotor. The viral band was collected and concentrated by high-speed centrifugation (360,000 g for 1.5 h). Pellets were suspended in 0.05 M phosphate buffer, pH 7.0, and centrifuged in a preformed 10-40% caesium sulphate gradient at 52000 rpm for 1 h in a Beckman Sw60 rotor. The single band formed after centrifugation was collected, diluted 1:3 with 0.05 M phosphate buffer, pH 7.0, and concentrated by high speed centrifugation (60,000 rpm in a Beckman TLA100.3 rotor) in a small volume of 0.05 M phosphate buffer.

Viral proteins were analysed in 12.5% denaturing gels (Laemmli, 1970) stained with Coomassie blue.
RNA was extracted from virus particles essentially as described by Rubino et al. (1995). Briefly, one volume of RNA extraction buffer (0.1 M glycine-NaOH, pH 9.0, containing 100 mM NaCl, 10 mM EDTA, 2% sodium dodecyl sulphate, and 1% sodium lauryl sulphate) and 2 volumes of phenol were added to virus preparations. The aqueous phase was further extracted with equal volumes of phenol and chloroform, washed with chloroform, and precipitated with 2.5 volumes of ethanol in the presence of 1/10 volume of 3 M sodium acetate. The RNA was finally suspended in sterile water.

**Virus transmission to cucumber from *N. benthamiana***. Cucumber plants ( cvs Gergana, Marketer and Mirei) were grown in Agriperlite to the first true leaf stage and then inoculated by three different procedures.

1. The cotyledons were mechanically inoculated, after transplanting into sterilized soil.
2. The roots were mechanically inoculated by rubbing with virus extract containing carborundum and immediately transplanted into sterilized soil.
3. The roots were dipped in a tube containing virus extract with carborundum and gently shaken with a Wirli mixer for 3 min, then transplanted as above.

Plants were kept in a glasshouse for 2 months. Virus presence was evaluated by back inoculation to *N. benthamiana*.

**Serology**. A rabbit antiserum was raised by injecting 900 µg of purified virus (emulsified with Freund’s incomplete adjuvant) intramuscularly three times (on days 0, 8 and 21). Bleeding was 6 weeks after the first injection. Serological tests were done by agar gel double diffusion.

**Northern blot analysis**. Total RNA from infected *N. benthamiana* was extracted by homogenizing leaf tissue (ca. 100 mg) with ice-cold mortar and pestle and suspending the extract in 600 µl of RNA extraction buffer and an equal volume of phenol. The aqueous phase was further extracted with phenol-chloroform, then chloroform, and precipitated with ethanol. The RNA was suspended in 25 µl sterile water. For Northern blot analysis, RNA samples were denatured with formamide and formaldehyde, electrophoresed in 1.2% agarose gels, transferred to nylon membranes and probed with radioactive transcripts obtained from a clone comprising the 3' terminal 220 nt of the viral genomic RNA.

**Cloning and sequencing**. Approximately 2 µg RNA were denatured by heating at 65°C. After cooling, the 3' end was polyadenylated with yeast poly(A) polymerase (USB, Cleveland, USA) according to the manufacturer’s protocol. The polyadenylated RNA was extracted with phenol-chloroform, precipitated with ethanol, suspended in sterile water and used as template for oligo(dT)-primed cDNA synthesis using the cDNA Synthesis Module (Amersham, Little Chalpant, UK) according to the manufacturer’s protocol. Double-stranded DNA was ligated to Smal-digested dephosphorylated pUC18 plasmid and cloned in *Escherichia coli* strain DH5α. Several clones were sequenced (MWG Biotech, Ebersberg, Germany), and sequence information from these clones was used to construct primers, then used for RT-PCR amplification of viral RNA. PCR products were cloned as above.

To clone and sequence the 5' region the method by Hirzmann et al. (1993) was adopted. Briefly, a random-primed first strand cDNA was digested with ribonuclease H, purified through a QIAquick column (QIAGEN Valencia, CA, USA), and tailed with dGTP using terminal deoxynucleotidyl transferase (BioLabs Beverly, MA, USA). The dG-tailed cDNA was amplified by PCR using a (dC)14 oligonucleotide primer and a second primer complementary to an internal region of the viral genome. To obtain additional information on the 3' region, the procedure of Shirakao and Wilson (1993) was used, which allows the precise determination of the terminal 3' nucleotide.

**Electron microscopy**. Purified virus preparations were negatively stained with 2% aqueous uranyl acetate and observed with a Philips CM10 electron microscope. For thin sectioning, leaf fragments of systemically infected *N. benthamiana* were fixed in 4% glutaraldehyde in phosphate buffer pH 7.0, postfixed in 1% osmium tetroxide in the same buffer, bulk stained with uranyl acetate, dehydrated in a graded ethanol series and embedded in Spurr’s resin (Martelli and Russo, 1984). Thin sections were stained with lead citrate and viewed and photographed with a Philips Morgagni electron microscope.

**RESULTS AND DISCUSSION**

**Host range and symptomatology**. As shown in Table 1, CBLV infected several test plants most of which reacted with localized infections only. *Ocimum basilicum* showed the dark brown necrotic lesions with a lighter centre that are typically induced by tombusviruses and are of diagnostic value (Martelli, 1981). Only *N. benthamiana* and *N. clevelandii* were consistently infected systemically. Symptoms were chlorotic or necrotic local lesions on inoculated leaves 3 to 5 days after inoculation, followed in 2-3 days by mosaic and distortion of apical leaves, and after an additional week by necrosis and death of the plant. In cucumber the virus easily infected cotyledons inducing chlorotic local lesions. Latent systemic infection was found only in a few plants, 45 days after inoculation. Root inoculation with both methods resulted in systemic invasion of some plants and 45 days after inoculation the virus was recovered by back inoculation. Infection was always symptomless regardless of the cultivar. This was taken as an indication that field symptoms were likely elicited by WMV,
although a synergistic effect of the two viruses cannot be excluded, and that its presence assisted CBLV in invading systemically naturally infected cucumbers.

**Virus purification and physico-chemical properties.** Routinely, 30 mg virus could be extracted from 100 g of infected tissue. The UV absorption spectrum was typical of nucleoproteins with E$_{max}$ = 260 nm and E$_{min}$ = 242 nm. The E$_{280}$/E$_{260}$ ratio of ca. 0.6 indicated the presence of approximately 15% nucleic acid. In the electron microscope, negatively stained CBLV particles appeared isometric, with a diameter of ca. 30 nm (Fig. 1).

**Serology.** The antiserum had an homologous titre of 1:1024 and did not react with healthy plant material. No reaction was observed between CBLV and antisera to the following viruses: Cucumber leafspot virus (CLSV), Pothos latent virus (PoLV), Cucumber soil-borne virus (CSBV), Turnip crinkle virus (TCV), Carnation mottle virus (CARMV), Pelargonium flower break virus (PFBV), Bean mild mosaic virus (BMMV), Tobacco necrosis virus (TNV), Sowbane mosaic virus (SoMV), Cucumber necrosis virus (CNV), Petunia asterid mosaic virus (PAMV), Pelargonium leaf curl virus (PLCV), Tomato bushy stunt virus (TBSV), Carnation Italian ringspot virus (CIRV), Artichoke mottled crinkle virus (AMCV), Grapevine Algerian latent virus (GALV), Moroccan pepper virus (MPV), Cymbidium ringspot virus (CymRSV), Narcissus tip necrosis virus (NTNV), Pelargonium ringspot virus (PRSV), Pelargonium line pattern virus (PLPV), and Escarole mosaic virus (EMV).

**Cloning and nucleotide sequence analysis.** Several overlapping clones were obtained with each of the

![Fig. 1. Purified particles of CBLV negatively stained with uranyl acetate. Bar = 150 nm.](image-url)
cloning strategies used. Labeling the 5' and the 3' termini allowed their unequivocal determination. The 5' two terminal nucleotides were A and G, i.e. identical to those of other tombusviral genomes (Rubino et al., 1995; Galetzka et al., 2000; Szittya et al., 2000), whereas the 3' terminal sequence -GCC is a feature common to all members of the family Tombusviridae (Russo et al., 1994). All clones were sequenced on both strands and sequence data were assembled using the DNA Strider software (Marck, 1988). CBLV genomic RNA was of 4,576 nt in size and contained five open reading frames (ORFs) (GenBank accession number AY163842).

ORF 1 began at the first AUG at nt 135 continuing to an amber stop codon at nt 941 to yield a protein with predicted Mr of 30,273 (p30). Readthrough of the amber terminator of ORF 1 to the stop codon at nt 2507 would result in a polypeptide with Mr of 89,200 (p89). An 18 nt intergenic region separated ORF 2 from ORF 3 that began at nt 2526 and terminated at nt 3668 encoding a protein with Mr of 41,090 (p41). A 40-nt intergenic sequence separated ORF 3 from ORF 4, which began at nt 3709 and terminated at nt 4,260, encoding a polypeptide of Mr of 21,056 (p21). ORF 5, completely nested within ORF 4, started at nt 3,741 and terminated at nt 4,247, encoding a protein of Mr of 19,042 (p19). The 5' and 3' noncoding regions were of 134 and 329 nt, respectively. As shown in Fig. 3, the order of the genes corresponded to that of members of the genus Tombusvirus (Russo et al., 1994).

To ascertain the relation of CBV with members of this genus and to identify the possible functions of the encoded proteins, the amino acid sequence of each protein was compared with that of the corresponding products of tombusviruses using the GAP program in the software package of the University of Wisconsin Genetics Computer Group (GCG; Deveraux et al., 1984). Percent homology (Table 2) was rather high for the readthrough (rt) domain of the ORF2-encoded p89 (85-87%) and the ORF4-encoded p21 (80-84%), and lower for the proteins encoded by ORF 1, 3, and 5. Based on published information, p89, p41, p21 and p19 were identified as the replicase, coat, movement and symptom modulation/virus spread/gene-silencing proteins, respectively (Russo et al., 1994; Scholthof et al., 1995; Voinnet et al., 1999; Szytta et al., 2002).

As to ORF1-encoded protein p30, recent studies have shown that this tombusviral product has a role in the induction of cytopathological structures (multivesicular bodies) derived from peroxisomes (CymRSV) or mitochondria (CIRV) (Russo et al., 1987), in which virus replication takes place (Burgyan et al., 1996; Rubino and Russo, 1998). The pairwise comparison of CBLV p30 with ORF1-encoded proteins of CymRSV (p33) and CIRV (p36) showed that the three proteins were conserved in the C-terminal region, but diverged in the N-terminal portion (Fig. 4).

Table 2. Pairwise amino acid sequence comparison of proteins encoded by the genome of CBLV with corresponding gene products of other tombusviruses.

<table>
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<tr>
<th>Virus</th>
<th>Amino acid identity (%)</th>
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<td></td>
<td>p30</td>
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<tr>
<td>AMCV</td>
<td>69</td>
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<tr>
<td>CIRV</td>
<td>53</td>
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<tr>
<td>CNV</td>
<td>68</td>
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<tr>
<td>CymRSV</td>
<td>67</td>
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<td>Pear latent virus, PeLV</td>
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<tr>
<td>TBSV-Sta</td>
<td>68</td>
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<td>TBSV-Ch</td>
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AMCV (X62493); CIRV (X85215); CNV (M29270); CymRSV (X15511); Pear latent virus, PeLV (AY100482); TBSV (TBSV-Sta, AJ249740; TBSV-Ch, M21958). rt = readthrough domain of ORF 2.

Fig. 2. Northern blot analysis of CBLV RNA extracted from N. benthamiana-infected leaf tissue (lanes 1 and 2) and from virus particles (lane 3) hybridized with a radioactive riboprobe representing the 3’-terminal 220 nt of the CBLV genome. Arrow points to the genomic RNA band.

Fig. 3. Schematic representation of the genomic organization of CBLV.
The N-terminus of CBLV p30 was then further analysed to identify hydrophobic regions and transmembrane domains (TMDs) using the programme of Rost et al. (1995). Two TMDs were found, spanning from aa 86 to 103 and from aa 108 to 125, suggesting that also this protein, similarly to CymRSV p33 and CIRV p36, can integrate into cell membranes and play a role in virus replication.

A comparison of the CBLV genome with that of unclassified tombusvirids using the BLAST programme (Altschul et al., 1990) showed that CBLV p30 had the same size as the protein encoded by ORF1 of Maize necrotic streak virus (MNSV), which resembles members of the family Tombusviridae (Louie et al., 2000). However, percent homology (59%) was no higher than that with true tombusviruses (Table 2). The hydrophathy plot, however, was similar to that of CBLV p30.

Electron microscopy. The most striking ultrastructural modification of *N. benthamiana* mesophyll cells was the presence of intranuclear membranous inclusions consisting of groups of electron-lucent apressed tubules, cisternae or vesicles from 50 to 100 nm in diameter (Fig. 5 A). Although these inclusions recalled the stacks of the Golgi apparatus when transversely sectioned, they were not cytoplasmic dictyosomes embedded in the nuclear matrix, but seemed to originate from proliferation of the inner nuclear membrane (Fig. 5 B). The cytoplasm of many cells contained cytopathic structures closely resembling the peroxisome-derived multi-vesicular bodies (Fig. 5 C), elicited by a number of members of the genus *Tombusvirus* (Russo et al., 1987). Other cell organelles appeared unaffected. Virus particles were scattered in the cytoplasm (Fig. 5 C).

Thus, the cytopathology of CBLV infections differs from that generally reported for tombusviruses, since proliferation of the inner nuclear membrane is a very uncommon feature, reported only once in cells of necrotic local lesions of leaves of *Chenopodium quinoa* infected with AMCV (Martelli and Russo, 1973).

In conclusion, CBLV was shown to have the general properties (biological, morphological, ultrastructural, and molecular) of the genus *Tombusvirus*. However, it was serologically unrelated to a number of tombusviruses and had molecular and ultrastructural traits differing enough from those of characterized members of this genus to be regarded as a species in its own right.

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REFERENCES


Fig. 5. Cytopathology of CBLV-infected *N. benthamiana* cells. **A.** Intranuclear (N) membranous inclusions. **B.** A large vesicle originating from the invagination of the inner lamella (IL) of the nuclear envelope. **C.** A cytoplasmic multivesicular body (MB) surrounded by scattered virus particles (V). NE = nuclear envelope; OL = outer lamella of the nuclear envelope. All bars = 200 nm.


