Different Tomato Bushy Stunt Virus Strains That Cause Disease Outbreaks in Solanaceous Crops in Spain

Marisol Luis-Arteaga, Emilio Rodríguez-Cerezo, Aurora Fraile, Elisa Sáez, and Fernando García-Arenal

First author: Servicio de Investigación Agraria, Apdo.727, 50080 Zaragoza, Spain; second author: Centro Nacional de Biotecnología (CSIC), Cantoblanco, 28049 Madrid; third and fifth authors: Departamentode Biotecnología, E.T.S. Ingenieros Agrónomos, 28040 Madrid; fourth author: Servicio de Protección de los Vegetales, El Ejido, Almería, Spain.

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ABSTRACT

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Tomato bushy stunt virus (TBSV) was detected for the first time in Spain in 1994 in two main horticultural areas of the southeast. TBSV was the cause of economically important diseases in greenhouse-grown tomato and eggplant in the El Ejido area and in greenhouse- and open field-grown tomato in the Mazarrón area. Field isolates from both regions and several reference strains of TBSV were characterized by serology and by determining the nucleotide sequence of a 0.9-kb segment at the 3′ end of the viral genomic RNA. Isolates from El Ejido were closely related to the BS3 strain of TBSV, a strain that is responsible for disease outbreaks in solanaceous plants in neighboring countries. In contrast, isolates from Mazarrón were very closely related to the cherry strain of TBSV, a strain that typically infects rosaceous trees worldwide and that has never been reported to infect tomato naturally. The results reported here contribute to a better understanding of the taxonomy of tombusviruses.

Tomato bushy stunt virus (TBSV) is the type member of the genus *Tombusvirus*. Tombusviruses are characterized by positive-strand RNA genomes of about 4.7 kb packaged within small (30 nm) isometric particles (24). The genus comprises at least 13 viruses with narrow natural host ranges restricted to dicotyledonous plants and with limited geographic distribution. TBSV, however, is an exception because it is widespread and causes economically important diseases in several crops (19,20). TBSV was first described in tomato (*Lycopersicon esculentum* Mill.) in England (25), but until the mid-1960s, the virus was not considered economically important in this crop because of its low incidence. However, in the last 30 years, TBSV epidemics have been reported in field- and greenhouse-grown tomato crops in South America (21), California (9), Morocco (6), Portugal (2), and Tunisia (3). Symptoms induced by TBSV in tomato plants include stunting and bushy growth, deformation and chlorotic spots on young leaves, and purpling and necrosis of older leaves. Yields are reduced, and fruits are smaller and show chlorotic rings and blotches that lower the economic value of the crop. In Tunisia, TBSV also has caused epidemics in other horticultural solanaceous crops, such as eggplant (*Solanum melongena* L.) and pepper (*Capsicum annuum*) (3).

The TBSV isolates that cause disease outbreaks in tomato crops in Morocco and Tunisia (3,6) are indistinguishable serologically from the BS3 strain of TBSV (TBSV-BS3) (26)—the latter was derived from the type tomato strain (TBSV-type) (25). In addition to these tomato strains, many other strains of TBSV have been isolated worldwide from diverse crops, but the relationships among them have not been established satisfactorily (20). TBSV is related serologically to several tombusviruses to varying degrees among species and strains (16,20). At the nucleic acid level, the complete nucleotide sequence of the genomic RNA (11) is known for the cherry (*Prunus avium*) strain of TBSV (TBSV-Ch [1]), but no information is available for other TBSV strains that could aid in classification or detection.

Although TBSV is endemic in neighboring countries (2,3,6), it has not been reported in Spain. In 1994, symptoms of new diseases were recorded in tomato and eggplant in two regions of the southeastern Spanish Mediterranean littoral, where there is intensive field and greenhouse production of horticultural crops. In this paper we provide evidence supporting the conclusion that both the tomato and eggplant diseases are caused by TBSV. We have characterized Spanish TBSV isolates and reference tomato TBSV strains at the biological, serological, and partial nucleotide sequence levels. Our results show that different TBSV strains are found in solanaceous crops in Spain depending on the geographic origin of the isolates. The results reported here may contribute to understanding the taxonomic relationships among tombusviruses.

MATERIALS AND METHODS

TBSV isolates and strains. Samples were obtained during either April 1994 or February 1995 from symptomatic tomato plants (including isolates A14, A16, and A23) or from symptomatic eggplants (including isolates B2 and B8) grown in greenhouses in the region of El Ejido (Almería Province in southeastern Spain). During November 1994, isolates (including JA6 and JA9) were obtained from open field-grown tomato plants in the region of Mazarrón (Murcia Province in southeastern Spain, 200 km east of El Ejido). TBSV-type and TBSV-BS3 were obtained as dried tissue from J. Burgyán (Gödöllo, Hungary) and V. Lisa (CNRS, Tor-

Corresponding author: F. García-Arenal
E-mail address: agrovirus@samba.cnb.uam.es

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ino), respectively. TBSV-Ch genomic RNA was obtained from M. Borja (Madrid) in the form of in vitro transcripts of the full-length cDNA clone, pTBSV100 (11).

Virus transmission and maintenance. Extracts from diseased tomato and eggplant leaves prepared in 30 mM Na_2HPO_4 and 0.2% Na diethyldithiocarbamate were used to mechanically inoculate a number of indicator plant species (Table 1). Field isolates A14, A16, A23, JA6, JA9, B2, and B8 were multiplied in *Nicotiana clevelandii* Gray. The TBSV reference strains also were multiplied in *N. clevelandii* inoculated with extracts of dried tissue or with viral RNA transcripts in the case of TBSV-Ch.

Virion and RNA purification. Systemically infected *N. clevelandii* leaves were collected 1 week after inoculation, and TBSV virions were purified as described (8) without the polyethylene glycol concentration step and further CsCl purification. TBSV genomic RNA was extracted from sodium dodecyl sulfate (SDS)-disrupted purified virions with phenol-chloroform (8).

Serological tests. Serological analyses were done with a polyclonal antiserum to TBSV-BS3 provided by V. Lisa. Agar double-diffusion tests were carried out in plates of 0.6% agarose and 1% sodium azide prepared in phosphate-buffered saline, pH 7.4 (PBS). Preparations of purified virions (100 µg diluted in PBS) from *N. clevelandii* plants were used in all cases as antigen. Antigens were added to the wells 5 h prior to the placement of the antiserum. For Western blot analysis, samples of 20 µg of purified virions were disrupted in sample buffer (17) and analyzed by electrophoresis in duplicate 12.5% polyacrylamide gels containing SDS. One gel was stained with Coomassie brilliant blue, and the duplicate gel was used to transfer the proteins overnight to nitrocellulose membranes at 30 V (29). The membranes were incubated with TBSV antiserum (1/1,000 dilution), and the bound antibody was detected in Western blot with goat anti-rabbit immunoglobulin G-alkaline phosphatase conjugate (Sigma Chemical Company, St. Louis) and the chromogenic substrates nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma).

TABLE 1. Symptoms induced in indicator species inoculated with extracts of symptomatic tomato or eggplant samples from El Ejido, Spain

Family Species	Reaction[a] Local	 Systemic
Amarantaceae		
Gomphrena globosa	nll	o
Apocinaceae		
Vinca rosea	o	o
Compositae		
Lactuca sativa cv. Kwick	o	o
Cucurbitaceae		
Cucumis sativus cv. Marketer	nll	o
Cucurbita pepo cv. F1 Diamante	nll	o
Chenopodiaceae		
Chenopodium amaranticolor	nll	o
C. quinoa	nll	o
Labiatae		
Ocimun basilicum	nll	o
Leguminosae		
Vigna unguiculata cv. Walp	nll	o
Solanaceae		
Capsicum annuum		
cv. Doux des Landes	o	Mo
C. annuum cv. Yolo Wonder	nll	Mo
Datura stramonium	nll	NS, Mo, StN
Lycopersicon esculentum		
cv. Montserrat	o	Mo, AN
cv. San Pedro	o	o, NS
Nicotiana glutinosa	nll	o
N. megalosiphon	nll	o
N. rustica	nll	o
N. sylvestris	o	o
N. tabacum		
cv. Paraguay	o	o
cv. Samsun	nll	o
cv. Xanthi-nc	nll	o
N. clevelandii	nll	Mo, AN
Petunia hybrida	o	o
Physalis floridana	nll	o
Solanum melongena		
cv. Cerna Krazavitska	o	Mo

[a] o = no symptoms; nll = necrotic local lesions; Mo = mosaic; NS = necrotic spots; AN = apical necrosis; and StN = stem necrosis.

RNA sequencing and sequence analyses. Nucleotide sequence data were obtained from virion-purified viral genomic RNA by direct sequencing with reverse transcriptase and dideoxynucleotides as described by Fichot and Girard (5). Based on reported tombusvirus RNA sequences (10,11,23,27), three degenerate oligonucleotides were designed as sequencing primers. Oligonucleotide 5′-CATCCCGGG CTGCATT(G/T)CTGC-3′ contained a consensus sequence (underlined) complementary to the 3′ terminal 16 residues of tombusviral RNA sequence. Oligonucleotides 5′-CA(A/C)(C/T)AAGAGTAACCTGTA-3′ and 5′-CCGTCCAAGATCCAA-3′ consisted of consensus sequences complementary to regions 300 and 615 nt upstream from the 3′ end.

RNA sequences and deduced amino acid sequences were aligned by the CLUSTAL V program (12), and were used to establish phylogenetic relationships according to Wagner parsimony (15) with the aid of the PHYLIP 3.5 package of J. Felsestein (University of Seattle). Genetic distances among the sequences were expressed as percent identities.

RESULTS

Symptomatology and host range. In 1994, severe symptoms of stunting, apical chlorosis, veinal necrosis, and conspicuous purpling of leaves were observed on tomato plants grown in greenhouses in El Ejido. In some tomato greenhouses, 40 to 50% of the plants developed total necrosis. Fruit from diseased tomato plants showed chlorotic and necrotic spots and deformation, rendering them unmarketable (Fig. 1A). Symptoms of stunting and yellowing of leaves and severe fruit deformation were recorded concomitantly in greenhouse-grown eggplants in the same area (Fig. 1B). The tomato disease symptoms were observed in open fields as well as in greenhouse-grown tomatoes in the Mazarrón area during the fall of 1994.

As a first step toward the identification of the casual agent of these diseases, extracts from symptomatic tomatoes and eggplants collected from the El Ejido greenhouse area during spring 1994 were inoculated onto 25 indicator plants. Both tomato and eggplant isolates induced similar symptoms on this set of indicator plants (Table 1). Tomato and eggplant mechanically inoculated with these extracts reproduced the symptoms observed in the field. Uranyl-acetate–stained leaf extracts examined by electron microscopy showed the presence of isometric virion particles, approximately 30 nm in diameter, with the knobbed surface typical of tombusviruses (data not shown). For the plant species tested (Table 1), the host range of the Almería tomato and eggplant tombusvirus was very similar to that of the isolates of TBSV-BS3 associated with disease outbreaks in solanaceous crops in Tunisia (3). The only difference was that the Almería isolates did not induce the formation of local lesions in petunia. In addition to TBSV, eggplant crops have been infected in the Mediterranean area by another tombusvirus, eggplant mottled crinkle virus (EMCV) (18), but unlike the virus found in eggplants in Almería (Table 1), EMCV does not infect tomato or pepper.

Symptoms similar to those observed at El Ejido also were observed on tomato crops at Mazarrón. TBSV was identified serologically (described below) in these plants, but a host-range study of the Mazarrón isolates was not done. Nevertheless, when the Mazarrón tomato isolates of TBSV were used to inoculate *N.*

clevelandii plants for multiplication, we consistently observed reduced severity and a delay in the development of systemic necrosis (9 to 10 days postinoculation) compared to the El Ejido isolates (6 to 7 days postinoculation). When reference TBSV strains were multiplied in *N. clevelandii*, TBSV-BS3 and TBSV-type behaved the same as the El Ejido tomato and eggplant isolates, whereas TBSV-Ch behaved the same as the Mazarrón tomato isolates. Defective interfering (DI) RNAs were not found in any of the cases by any of the methods used (Northern blot and polymerase chain reaction analyses; data not shown), thus the differences in symptoms in *N. clevelandii* cannot be attributed to the accumulation of DI RNAs, which is known to attenuate the severity of symptoms induced by tombusviruses in this host plant (14).

Serological characterization. The Spanish TBSV isolates from tomato were characterized serologically by immunodiffusion experiments with an antiserum to TBSV-BS3. In one experiment, El Ejido tomato isolate A23 and strains TBSV-type, TBSV-BS3, and TBSV-Ch were tested against TBSV-BS3 antiserum. A specific reaction to the antiserum occurred with all four viruses. The precipitin line of A23 fused completely only with TBSV-BS3 and spurred with TBSV-type and TBSV-Ch (data not shown). Experiments with additional El Ejido isolates from tomato (A14 and A16) yielded similar results. In contrast, when tomato isolate JA6 from Mazarrón was tested together with the three TBSV reference strains, its precipitin line fused completely only with that of TBSV-Ch (data not shown). Therefore, the serology experiments confirm that the tombusvirus found in Spain is TBSV and suggest the presence of two serologically distinct groups of TBSV isolates in Spain.

To obtain more information about possible differences between TBSV isolates from southeastern Spain and their coat proteins (CPs), SDS-polyacrylamide gel electrophoresis (PAGE) and Western blot analyses of virion CP were performed. In Coomassie-stained gels, the CP monomers of isolates TBSV-BS3, A14, A16, A23, and B8 had a similar apparent molecular mass of about 41 kDa (Fig. 2A, lanes 5 through 9), slightly larger than the CP monomers of isolate B2 (Fig. 2A, lane 10). The CP of a second group of isolates that included TBSV-Ch, TBSV-type, JA6, and JA9 was degraded into two fragments with apparent molecular masses of 25 and 22 kDa (Fig. 2A, lanes 1 through 4). The observed differences in the CPs of TBSV-Ch and TBSV-BS3 are in agreement with a previous report by Hillman et al. (13). Western blot analyses did not show appreciable differences in the binding of TBSV antiserum to the CP of any of the isolates tested (Fig. 2B). Only the smaller fragment derived from the CP of isolates TBSV-type, TBSV-Ch, JA6, and JA9 reacted with the antiserum. Thus, both immunodiffusion analyses and SDS-PAGE of CPs showed that TBSV isolates can be divided into two main groups: TBSV-BS3, A14, A16, A23, B2, and B8; and TBSV-Ch, TBSV-type, JA6, and JA9.

Nucleotide sequence analyses. The relationships among TBSV isolates, including the Spanish tomato isolates A16, A23, JA6, and JA9, the Spanish eggplant isolates B2 and B8, and the three reference strains of TBSV, were studied further by direct nucleotide sequencing of viral RNA. A region of 887 nt at the 3′ end of the genomic RNA, corresponding to positions 3856 to 4733 of the published TBSV-Ch RNA sequence (11), was sequenced. This sequence includes the complete coding region for the nested open reading

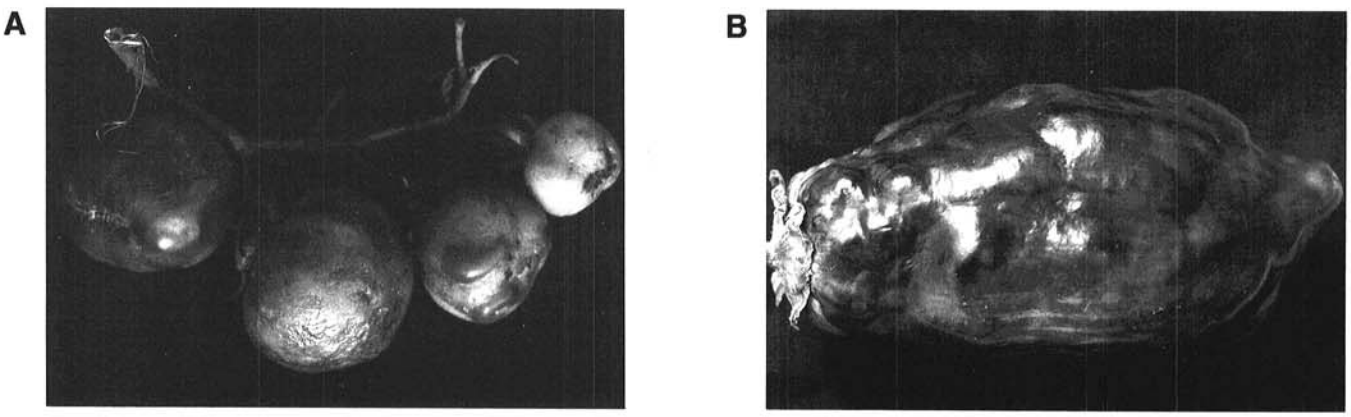


Fig. 1. Symptoms observed in tomato bushy stunt virus-infected field-grown **A,** tomato or **B,** eggplant fruits.

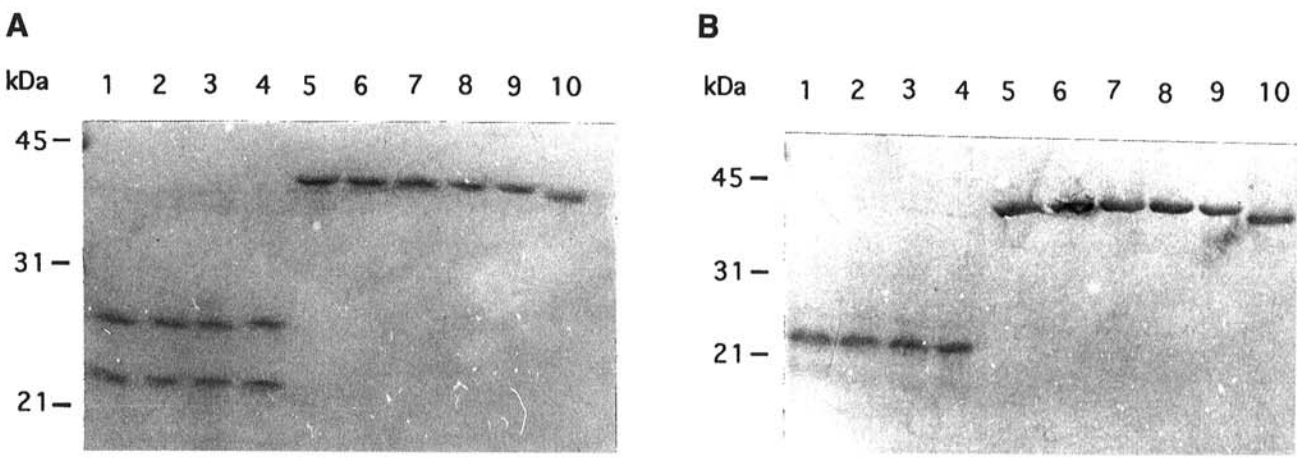
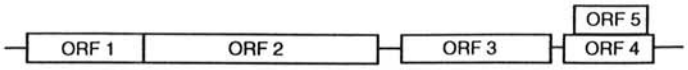


Fig. 2. A, Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the coat proteins of tomato bushy stunt virus (TBSV) isolates TBSV-Ch (lane 1), TBSV-type (lane 2), JA6 (lane 3), JA9 (lane 4), TBSV-BS3 (lane 5), A14 (lane 6), A16 (lane 7), A23 (lane 8), B8 (lane 9), and B2 (lane 10). The positions of molecular weight standards from Bio-Rad Laboratories are shown to the left. **B,** Western blot of a duplicate gel of that shown in **A** reacted with TBSV antiserum. Positions of prestained molecular weight standards from Bio-Rad are shown to the left.

frames (ORFs) 4 and 5 and the 3′-terminal untranslated region (UTR) of the tombusvirus genomic RNA, excluding the 3′-terminal 41 nt (24).

The region for which the nucleotide sequence was determined was chosen primarily because of the availability of a primer complementary to the 3′ end of the genomic RNA. This primer was obtained for other reasons, so the selection of the sequenced region may be considered as random and, thus, is most appropriate for genetic and taxonomic analysis of these TBSV isolates. The sequences obtained in this work, together with the sequence of

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                3860              3880              3900              3920
TBSV-Ch         ATGGATACTGAATACGAACAAGTCAATAAACCATGGAACGAGCTATACAAGGAAGCGACGCTAGGGAACAAGCTAACAGT
TBSV-Type       ................................................................................
JA6             ................................................................................
TBSV-BS3        ...........................................................T.............T.TG..
A116            ................................................................................T.TG..
A123            ................G...............................................................T.TG..
JA9             ................................................................................
B2              ................................................................................T.TG..
B8              ..............................................C.........A.........C....T.TG..

                3940              3960              3980              4000
TBSV-Ch         GAACGTTGGGATGGAGGATCAGGAGGTACCACTTCTCCCTTCAAACTTCCTGACGAAAGTCCGAGTTGGACTGAGTGGCG
TBSV-Type       ........................A..T.............C.....................................
JA6             ................................................................................
TBSV-BS3        .........A..............A..T.....................................................
A116            .........A..................T.....................................................
A123            .........A........C........T..............C.....................................
JA9             ................................................................................
B2              ................................................................................
B8              .........A........C.C......T..............C.....................................

                4020              4040              4060              4080
TBSV-Ch         GCTACATAACAATGAGACGAATTCGAATCAAGATAATCCCCTTGGTTTCAAGGAAAGCTGGGGTTTCGGGAAAGTTGTAT
TBSV-Type       ................................................................................
JA6             ................................................................................
TBSV-BS3        ......................T.........................................................
A116            ......................T.........................................................
A123            ................................................................................
JA9             ................................................................................
B2              ......................T.........................................................
B8              .......T................................................................................

                4100              4120              4140              4160
TBSV-Ch         TTAAGAGATATCTCAGATACGACAGGACGGAAGCTTCACTGCACAGAGTCCTTGGATCTTGGACGGGAGATTCGGTTAAC
TBSV-Type       ........................A.........A.....T.....................................
JA6             ................................................................................
TBSV-BS3        ........................A.....A...A.............................................
A116            ........................A.........A.....T.....................................
A123            ........................A.........A.............................................
JA9             ................................................................................A.....
B2              ..................................A.....T.......................................
B8              ........................G........A.....................................................

                4180              4200              4220              4240
TBSV-Ch         TATGCAGCATCTCGATTTTTCGGTTTCGACCAGATCGGATGTACCTATAGTATTCGGTTTCGAGGAGTTAGTATCACCGT
TBSV-Type       ........................G..A.....................................................G.....A.
JA6             ................................................................................
TBSV-BS3        ..................C.....................G.....................................G.....A.
A116            ..................C.....................................................G.....A.
A123            ..................C.....................................................G.....A.
JA9             ................................................................................
B2              ........................G..A.....................................................G.....A.
B8              ................................................................................G.....A.
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(*continued on next page*)

Fig. 3. Nucleotide sequences for 887 nt at the 3′ end of the genomic RNA of field isolates and reference strains of tomato bushy stunt virus (TBSV). The gene map of the 4.7-kb genomic RNA of the tombusvirus is shown at the top. Sequence coordinates correspond to positions 3856 to 4733 of the published TBSV-Ch sequence (11) to which the rest of the sequences were aligned. Initiation and stop codons for open reading frame 4 (underlined) and 5 (double underlined) are indicated; dashes indicate deletions. The positions of the primers used for sequencing are underlined.

the corresponding region of TBSV-Ch RNA (11), were aligned as shown in Figure 3. Overall nucleotide sequence identities (Table 2) ranged from 87.5 to 99% for TBSV sequences. Similar values (88 to 92%) were obtained when artichoke mottle crinkle tombusvirus (AMCV) was included in the comparison. In contrast, lower values (67 to 77%) were obtained when cucumber necrosis (CuNV) or cymbidium ringspot (CyRSV) tombusviruses were included in the comparison (Table 2). These results indicate that the Spanish field isolates and the three reference TBSV strains belong to a single tombusvirus species and suggest that AMCV may be a strain of TBSV.

Fig. 3. (*continued from preceding page*)

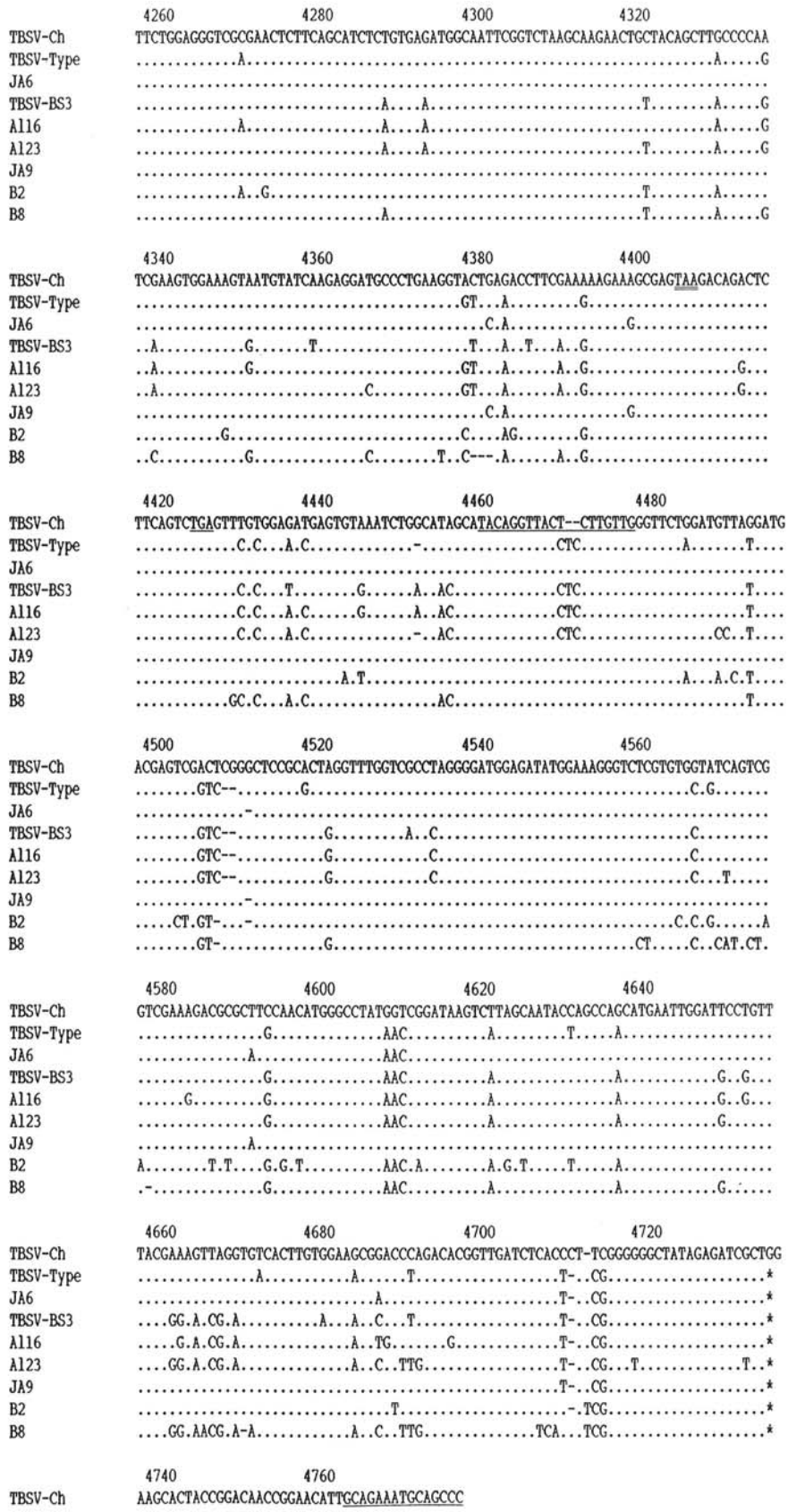

TABLE 2. Percent nucleotide sequence identities (below the diagonal: the entire 887 nt sequence of Figure 3; above the diagonal: the 3′ untranslated regions)[a]

	CyRSV	AMCV	TBSV-Ch	TBSV-type	TBSV-BS3	TBSV-A16	TBSV-A23	TBSV-JA6	TBSV-JA9	TBSV-B2	TBSV-B8	CuNV
CyRSV		61.8	66.6	61.8	62.6	62.3	62.5	64.1	64.1	62.1	63.4	54.6
AMCV	76.4		80.3	81.2	76.7	76.7	76.1	80.6	79.9	82.2	76.7	48.8
TBSV-Ch	77.6	90.3		83.2	81.0	80.6	78.0	93.9	95.2	83.2	79.0	58.1
TBSV-type	76.4	91.2	92.1		89.6	90.3	90.6	86.7	86.1	83.5	78.3	53.6
TBSV-BS3	75.5	88.0	90.0	93.6		96.5	93.5	85.1	84.1	79.3	82.5	44.0
TBSV-A16	75.7	89.2	90.2	94.6	97.4		93.2	84.8	83.1	79.6	82.2	51.9
TBSV-A23	75.8	88.5	89.0	94.3	95.9	96.5		81.9	79.9	76.4	82.8	50.9
TBSV-JA6	76.6	90.3	97.5	93.0	91.3	91.6	90.3		98.7	83.2	79.9	53.3
TBSV-JA9	76.6	90.0	97.7	92.8	90.7	91.0	89.7	99.2		82.5	79.0	53.6
TBSV-B2	76.6	91.7	91.8	92.4	89.7	90.6	88.8	91.7	91.3		74.8	54.0
TBSV-B8	75.1	87.5	88.4	88.7	90.6	90.7	91.5	88.8	88.5	87.5		52.6
CuNV	74.7	71.0	76.5	71.4	69.5	72.0	69.7	71.7	71.7	75.4	67.5	

[a] CyRSV = cymbidium ringspot virus; AMCV = artichoke mottle crinkle virus; TBSV = tomato bushy stunt virus; and CuNV = cucumber necrosis virus.

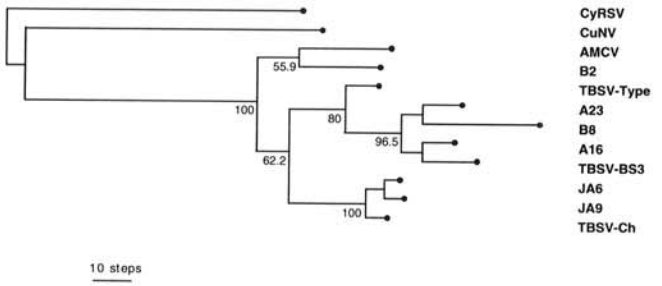

Fig. 4. Consensus most parsimonious phylogenetic tree of tomato bushy stunt virus (TBSV) field isolates and strains obtained with the 887 nt sequence of Figure 3 as input. Values at nodes indicate significance in a bootstrap analysis with 100 replicates. Published nucleotide sequences obtained from references are those of artichoke mottle crinkle virus (AMCV) (27), cucumber necrosis virus (CuNV) (23), cymbidium ringspot virus (CyRSV) (10), and TBSV-Ch (11).

The nucleotide sequence of the coding regions for ORFs 4 and 5 was more conserved than the sequence of the 3′ UTRs (Table 2). The identity values for the 3′ UTRs discriminated better between TBSV isolates at the strain level and can be used to delineate two groups of high homology (over 93% nucleotide sequence identity) within them: the first formed by TBSV-BS3 and the El Ejido isolates A16 and A23 and the second formed by TBSV-Ch and the Mazarrón isolates JA6 and JA9. This grouping was confirmed and improved when the phylogenetic relationships between the TBSV isolates were inferred by Wagner parsimony with the whole 887-nt sequence as input data. The consensus most parsimonious phylogenetic tree obtained by this method is shown in Figure 4. The Spanish TBSV field isolates in the tree formed two clear phylogenetic clusters: the El Ejido tomato isolates A16 and A23 and the El Ejido eggplant isolate B8 grouped with TBSV-BS3 with high significance (the cluster was found in 96.5% of the trees in a bootstrap analysis with 100 replications) and the Mazarrón tomato isolates JA6 and JA9 grouped with TBSV-Ch (the cluster appeared in 100% of the trees in the bootstrap). The rest of the isolates were not closely related to these; although TBSV-type clustered with TBSV-BS3, A16, A23, and B8, the significance was 80%. Isolate B2 from eggplant clustered with AMCV with low significance; the significance of the cluster (AMCV, B2) rose to 93% when only the 3′ UTR was considered (data not shown). The relevance for virus taxonomy of 3′ UTRs has been described (7). It is interesting that all the TBSV isolates and strains plus AMCV formed a single highly significant cluster clearly different from the other two tombusviruses (CuNV and CyRSV) included in the analysis.

The sequence variation among TBSV isolates was studied at the protein level for the region corresponding to ORFs 4 and 5. The product of ORF 4 of the tombusvirus genome is a 22-kDa protein and that of ORF 5, which is completely nested within ORF 4 with a −1 frameshift, is a 19-kDa protein (24). Because both ORFs share most of their coding region, the number of changes at the RNA level was very similar (Fig. 3). However, in the case of the 22-kDa protein, only 20% of the changes resulted in a change of amino acid, whereas in the case of the 19-kDa protein 73% of the mutations resulted in amino acid changes. The deduced amino acid sequences of the 19-kDa protein were used as input to construct an evolutionary tree by Wagner parsimony (data not shown), and again, the TBSV isolates were clearly divided into two main evolutionary clusters formed by TBSV-BS3 plus the El Ejido isolates or by TBSV-Ch plus the Mazarrón isolates. The sequence of the 22-kDa protein did not place the isolates into clusters (data not shown), in agreement with a higher similarity of this protein among the tombusviruses as compared with the 19-kDa protein (Table 3).

DISCUSSION

We have shown that a new economically important disease of tomato and eggplant in two main horticultural areas of the Mediterranean coast of Spain is incited by TBSV. Although biological, serological, and molecular characterization studies were made with only some TBSV field isolates, Northern blot analyses of more than 50 field isolates (data not shown) support the conclusion that TBSV is the cause of the new disease. This is the first report of TBSV in Spain. In the El Ejido region, TBSV affects greenhouse-grown tomato and eggplant crops, whereas in the Mazarrón region TBSV infects both open field- and greenhouse-grown tomato crops. The characterization of several tomato TBSV isolates from Spain strongly suggests they belong to different TBSV strains in correlation with the geographic origin of the isolates: those collected from Mazarrón are related to TBSV-Ch, and most of those collected from El Ejido are related to TBSV-BS3. This conclusion is supported by electrophoretic and serological analyses of the CPs and by nucleotide sequence analysis of 887 nt at the 3′ end of the genomic RNA of field isolates and reference TBSV strains. Furthermore, the two main groups of TBSV isolates also could be distinguished at the biological level by the severity of symptoms induced in *N. clevelandii*.

The presence of TBSV-BS3-like isolates in the El Ejido greenhouses has a parallel in the TBSV-BS3 outbreaks recorded in the past 3 decades in North and South America (9,21) and, significantly, in neighboring Mediterranean countries (3,6). Because no invertebrate or fungal vector of TBSV is known (20), TBSV-BS3 may have been introduced into the area via contaminated soils, plant material, or even seeds (3,28) due to the heavy trading of plant and soil material, as well as of temporary agricultural labor, between Morocco and Spain. TBSV-BS3 may be a considerable threat to the intensive horticulture of southeastern Spain because it causes serious diseases in tomato, eggplant, and pepper (3). So far, pepper infection by TBSV-BS3 has not been reported in El Ejido.

In contrast, the detection of TBSV-Ch–like isolates infecting tomato crops in the Mazarrón area is striking and constitutes the

TABLE 3. Percent amino acid sequence identities (below the diagonal: 22-kDa protein; above the diagonal: 19-kDa protein)[a]

	CyRSV	AMCV	TBSV-Ch	TBSV-type	TBSV-BS3	TBSV-A16	TBSV-A23	TBSV-JA6	TBSV-JA9	TBSV-B2	TBSV-B8	CuNV
CyRSV		75.6	82.0	72.7	70.9	72.7	73.3	72.7	72.7	75.0	69.6	83.1
AMCV	83.6		90.1	91.3	88.4	90.1	90.1	89.0	89.0	92.4	84.2	73.3
TBSV-Ch	83.6	97.9		92.4	87.8	89.5	89.0	98.8	98.8	91.9	84.2	72.7
TBSV-type	83.6	97.9	98.9		91.3	92.4	93.0	91.3	91.3	93.0	86.6	71.5
TBSV-BS3	81.0	94.7	94.7	95.8		97.9	94.8	86.6	86.6	89.5	88.3	67.4
TBSV-A16	82.0	96.8	96.8	97.9	96.8		97.1	88.4	88.4	91.3	90.1	69.2
TBSV-A23	82.0	97.3	97.3	98.4	96.3	99.4		87.8	87.8	89.5	91.8	69.8
TBSV-JA6	84.1	98.4	99.5	99.4	95.2	97.3	97.9		98.9	90.7	83.0	72.7
TBSV-JA9	84.1	89.4	98.9	99.4	94.7	96.0	97.3	99.4		90.7	84.2	71.5
TBSV-B2	81.0	98.4	98.4	98.9	96.3	98.4	97.9	98.9	98.4		84.8	73.3
TBSV-B8	84.1	95.2	95.2	95.7	92.0	94.1	94.7	95.7	95.2	95.7		67.3
CuNV	88.4	87.8	88.9	99.4	85.1	87.3	87.3	89.4	89.4	88.9	85.6	

[a] CyRSV = cymbidium ringspot virus; AMCV = artichoke mottle crinkle virus; TBSV = tomato bushy stunt virus; and CuNV = cucumber necrosis virus.

first report of TBSV-Ch causing a disease in tomato. The TBSV-Ch preparation used in our serological and sequence analyses was derived from a full-length cDNA clone (11) of the original isolate obtained from cherry (*P. avium*) in Canada (1), a host in which TBSV-Ch causes a disease known as detrimental canker. TBSV-Ch is able to infect tomato via mechanical inoculation, although when very young plants are inoculated symptoms can be milder than those induced by the type tomato strain (1). In the field, we have not observed differences between the symptoms induced in tomato by either TBSV-BS3– or TBSV-Ch–like isolates. The latter strain and the canker disease have been reported frequently on cherry trees in central and eastern Europe (20) but not in Spain. The presence of TBSV-Ch in Mazarrón may represent an introduction from an area where TBSV-Ch is endemic and indicates that in spite of geographic proximity and intense interchange of plant, soil, and labor between the areas of El Ejido and Mazarrón disease outbreaks in these two areas must have different origins. Isolate B2 from El Ejido may be the result of a third introduction event.

The taxonomy of the genus *Tombusvirus* has been based on serology and has resulted in unsatisfactory classification of isolates (20). TBSV and a cluster of tombusviruses, such as petunia asteroid mosaic virus (PeAMV) and AMCV, are serologically related, and moreover, there is serological variation within strains of the individual species (13,16). For instance, TBSV-Ch is serologically closer to PeAMV than to TBSV-type, and therefore, TBSV-Ch and PeAMV have been considered synonymous (20). The similarity (Tables 2 and 3) and the phylogenetic analysis of sequences (Fig. 4) indicate that all the isolates of TBSV (including TBSV-Ch) whose sequence is reported here belong to a single virus species. These analyses also suggest that AMCV could be considered a strain of TBSV.

Within the TBSV species, two main clusters of field isolates represented by the strains TBSV-Ch and TBSV-BS3 were distinguished by phylogenetic analysis of sequence data (Fig. 4). TBSV-type, the tomato isolate originally described in the 1930s and from which the BS3 strain was derived (26), the eggplant field isolate B2, and AMCV were placed as separate TBSV strains. This agrees with the results of the biological assays (in which TBSV-type behaved as TBSV-BS3) and with CP analyses (in which TBSV-type behaved as TBSV-Ch and B2 was similar but not identical to the TBSV-BS3–like isolates). The information reported here may aid in TBSV strain classification and also can serve in designing nucleic acid probes for strain-specific diagnosis of TBSV.

The comparison of the deduced amino acid sequences of the putative 22- and 19-kDa proteins (encoded by nested ORFs 4 and 5) of several TBSV isolates shows a clear difference in the evolution of two proteins that share essentially the same coding region and, therefore, present a similar variation at the nucleotide level. The 19-kDa protein is less conserved than the 22-kDa protein (Table 3). The 22-kDa protein is needed for the movement of tombusviruses in infected plants, probably acting as a cell-to-cell movement protein (24) and, therefore, plays a key role in the infection process. This finding is consistent with the high degree of conservation found for the 22-kDa protein among isolates of TBSV. In contrast, the 19-kDa protein does not seem to be strictly necessary for tombusvirus infection of plants: mutants of CuNV and CyRSV in which the initiation codon of ORF5 was suppressed were able to infect plants, but in both cases a marked attenuation of symptoms was observed (4,22). The dispensability of the 19-kDa protein may be associated with a relaxation of purifying selection of random point mutations and with a higher variability of its amino acid sequence compared to the 22-kDa protein. The TBSV isolates analyzed in this work can be divided into two clusters according to the amino acid sequence of the 19-kDa protein. Interestingly, the two clusters can be distinguished based on the severity of the symptoms induced in *N. clevelandii*, a result that does not contradict a role for ORF 5 of tombusvirus in symptom induction.

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