Tomato Bushy Stunt Virus (TBSV) Infecting *Lycopersicon esculentum*

El Sayed E. Hafez[a], Ghada A. Saber[b], and Faiza A. Fattouh[b,*]

[a] Mubarak City for Scientific Research and Technology Application, New Burg Al-Arab, Alexandria, Egypt
[b] Department of Botany and Microbiology, Alexandria University, Alexandria, Egypt. E-mail: faiza_fattouh@yahoo.com

* Author for correspondence and reprint requests

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Tomato bushy stunt virus (TBSV) was detected in tomato crop (*Lycopersicon esculentum*) in Egypt with characteristic mosaic leaf deformation, stunting, and bushy growth symptoms. TBSV infection was confirmed serologically by ELISA and calculated incidence was 25.5%. Basic physicochemical properties of a purified TBSV Egh isolate were identical to known properties of tombusviruses of isometric 30-nm diameter particles, 41-kDa coat protein and the genome of ~ 4,800 nt. This is the first TBSV isolate reported in Egypt. Cloning and partial sequencing of the isolate showed that it is more closely related to TBSV-P and TBSV-Ch than TBSV-Nf and TBSV-S strains of the virus. However, it is distinct from the above strains and could be a new strain of the virus which further confirms the genetic diversity of tombusviruses.

Key words: Tombusvirus, Cloning, Phylogeny

Introduction

Tomato bushy stunt virus (TBSV) is the type member of the genus Tombusvirus within the family Tombusviridae. Over the last 30 years, tombusviruses were reported to cause economically important diseases in greenhouse- and field-grown tomato crops in Italy (Gigante, 1955), Argentina (Pontis *et al*., 1968), Mexico (Martinez *et al*., 1974), Morocco (Fischer and Lockhart, 1977), Portugal (Borges *et al*., 1979), Tunisia (Cherif and Spire, 1983), United States of America (Gerik *et al*., 1990), and Spain (Luis-Arteaga *et al*., 1996). TBSV has also produced disease epidemics in eggplant (*Solanum melongena* L.) in Tunisia (Cherif and Spire, 1983) and Spain (Luis-Arteaga *et al*., 1996) and in pepper (*Capsicum annuum*) in Tunisia (Cherif *et al*., 1983). TBSV has been isolated together with other viruses from diseased lettuce plants in Czechoslovakia and Turkey (Novák *et al*., 1981; Yilmaz, 1981).

TBSV causes symptoms such as stunting, bushy growth, deformation, and necrosis in tomato (Pontis *et al*., 1968; Fischer and Lockhart, 1977; Gerik *et al*., 1990; Luis-Arteaga *et al*., 1996), eggplant (Makkouk *et al*., 1981; Cherif and Spire, 1983; Koenig and Avgelis 1983; Luis-Arteaga *et al*., 1996), and pepper (Cherif and Spire, 1983). Fruits from infected plants develop necrosis and chlorotic blotching, resulting in serious economic damage such as yield loss and deterioration in the quality of commercial solanaceous crops grown in both greenhouses and fields.

TBSV consists of spherical particles, 30 nm in diameter, containing a positive-sense single-stranded RNA genome of approx. 4.8 nt that encodes five major open reading frames (ORFs) (Brunt *et al*., 1996). An ORF1 and an ORF2 are required for viral replication (Scholthof *et al*., 1995a). ORF3 encodes the coat protein while ORF4 encodes the viral movement protein that is necessary for cell-to-cell movement and symptom determination on certain host plants (Russo *et al*., 1994; Scholthof *et al*., 1993, 1995b). Products of ORF5 have a role in the induction of necrotic symptoms and in the long-distance spread of the virus, depending on the host. Tombusvirus particles are very stable and reach high concentrations in infected tissues. In addition, tombusviruses are efficiently spread and can become established in diverse environments. Different tombusviruses have been shown to be spread in nature by a number of means including seed and pollen transmission, transmission through propagation material, and possibly mechanical transmission.

There is little doubt that several tombusviruses are even spread through the soil. Experimentally, tombusviruses are readily transmitted via sap, and infected leaf extracts retain infectivity after freezing for several years. Though there have been no previous reports on the virus in Egypt, a recent survey for the virus has indicated its presence. To our knowledge, this is the first report on TBSV infecting tomatoes in Egypt. In this study characterization and comparison with other known isolates are attempted.

Material and Methods

Survey for TBSV

A survey for TBSV infection of *Lycopersicon esculentum* (tomato) was conducted in 2008 in seven selected governorates in Egypt. During the survey, random samples from different fields were collected. The samples were tested for TBSV by the direct double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA). The assay was conducted essentially as described by Clark and Adams (1977) and Bar-Joseph *et al.* (1979) using TBSV-specific polyclonal antibodies (Agdia, Elkhart, IN, USA). Quantitative measurements of generated *p*-nitrophenol phosphate (PNP) were made by determining the absorbance at 405 nm (A405) in a Micro-ELISA-reader. A positive result was taken as twice the mean of the corresponding negative control after incubation at room temperature for 60 min.

Propagation of the Egyptian isolate of TBSV

Leaves of tomato with typical viral symptoms were used to prepare sap for inoculation with 0.01 M sodium phosphate buffer (pH 7.0). The virus was transferred into the local lesion host *Gomphrena globosa*. Single local lesions were isolated and further propagated in *Lycopersicon esculentum* by mechanical inoculation. Plants used for inoculation were 15 days old. TBSV-infected leaves were ground in liquid nitrogen to a powder using a sterile mortar and pestle. Inoculation buffer (100 ml/g, 0.01 M phosphate buffer, pH 7, 0.5 g Na_2SO_3, and 1% celite) was added to the infected tissue in the ratio 1:10 and mixed well. Leaves were wounded during inoculation by celite to aid viral entry. Mock-inoculated plants were manually inoculated with the inoculation buffer. Following inoculation, the leaves were sprinkled with water and grown at 25–30 °C. 3 d post inoculation, appearance of TBSV-characteristic symptoms on the inoculated leaves was observed.

Virus purification

Infected leaves were collected 7–15 d post inoculation, homogenized with 3 volumes of 0.1 M sodium phosphate buffer (pH 5.5) containing 0.2% 2-mercaptoethanol and 0.1% thioglycolic acid, and filtered through cheesecloth. Filtrates were kept on ice for 2 h and centrifuged at 8,000 rpm for 20 min. 8% PEG (8,000) was added to the supernatant fluid and subjected to two cycles of differential centrifugation. The crude virus was precipitated by ultracentrifugation at 30,000 rpm for 2 h and then suspended in 10 mM sodium phosphate buffer (pH 5.5) for subsequent study (Kim *et al*., 2007).

Electron microscopy

10 μl purified virus particles (approx. 0.1 mg/ml) were stained with 2% uranyl acetate negative stain in bidistilled water, pH 6.0–7.5. 5 μl of the virus preparation were dropped onto a carbon-coated grid. After 1 min, excess liquid was removed by a filter paper. 5 μl of uranyl acetate were added for 1 min to the grid, and then the grid was dried. The grid was examined, and electron micrographs were taken in a transmission electron microscope (TEM, JEOL-CX100), operating at 80 kV.

Estimation of the size of TBSV Egh isolate capsid protein

The size of the TBSV capsid protein was estimated by 12% discontinuous SDS-polyacrylamide gel electrophoresis (Sambrook and Russell, 2001).

Cloning of PCR products and sequence analysis

Viral RNA was extracted using a QIAamp Viral RNA mini column for purification of viral RNA (Qiagen, Valencia, CA, USA). First-strand complementary DNA (cDNA) was synthesized using moloney murine leukemia virus (M-MuLV) reverse transcriptase. 20 μl reaction mixture were added in the presence of a specific TBSV full-length oligonucleotide primer: 5'-AAA TTC TCC AGG ATT TCT CGA CC-3', and 200 units of RT enzyme (M-MuLV) reverse transcriptase. The mixture was incubated

at 40 °C for 60 min, then at 70 °C for 10 min, and held at 4 °C in the PCR thermocycler. Second-strand synthesis and amplification of products were achieved by PCR using the oligonucleotide primer paired with specific TBSV full-length oligonucleotide primers (TBSVF, 5'-AAA TTC TCC AGG ATT TCT CGA CC-3';TBSVR, 5'-GGG CTG CAT TTC TGC AAT GTT CC-3'). RT-PCR-amplified DNA fragments were cloned into the TA-cloning vectors pCR 2.1-TOPO (Invitrogen, Carlsbad, CA, USA). Recombinant plasmids containing cloned cDNAs were purified and sequenced by Macrogen Inc., Seoul, South Korea. DNA sequencing was done using universal M13-forward and M13-reverse primers and virus-specific primers. Nucleotide sequence analyses were aligned using ClustalW. Phylogenetic trees were calculated according to the Workbench CLC program. Bootstrap analysis was performed using the same program (Thompson *et al*., 1997).

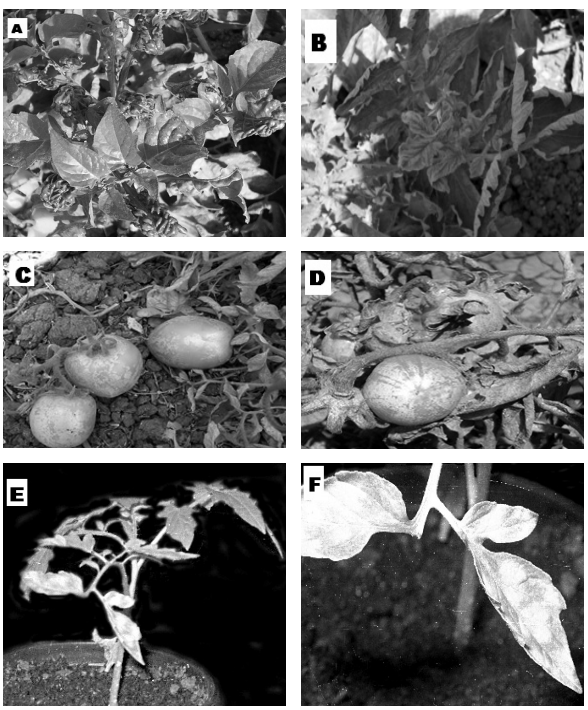

Fig. 1. Diagnostic symptoms of TBSV infection in surveyed fields in different governorates of Egypt in 2007–2008 during the growing season. TBSV-infected tomato leaves showing (A) malformation and bushy growth in the field, (B) stunting, vein necrosis, bushy growth and chlorotic spots on the leaves, (C, D) fewer smaller fruits with chlorotic blotching, rings and line pattern. (E, F) The isolate caused chlorotic ring local lesions on inoculated leaves, followed by stunt and necrosis of lower leaves in the greenhouse.

Results

Incidence of TBSV

Symptoms indicative of TBSV infection of *Lycopersicon esculentum* were observed in some surveyed fields in seven different governorates of Egypt (Alexandria, El Beheira, El Gharbeya, El Dakahlya, El Kalyoubia, El Monofia, and El Fayioum). The characteristic symptoms detected were in the form of malformation and bushy growth of the young leaves (Fig. 1A), bushy growth with stunting and chlorotic spots on the leaves (Fig. 1B), fewer smaller fruits with chlorotic blotching, rings, and line pattern (Figs. 1C, D). Artificially inoculated tomato plants under greenhouse conditions showed chlorotic ring local lesions followed by bushy growth symptoms in addition to vein necrosis, stunt and necrosis of lower leaves (Figs. 1E and F). The major observation in the survey was that TBSV-characteristic symptoms were not detected in large areas across surveyed fields, but rather in small loci in an irregular pattern. During the survey the virus has also been identified by ELISA in other host crops namely *Cucurbita pepo*, *Solanum melongena*, and *Capsicum annum* (results not shown). Overall incidence of 175 TBSV infections in 600 randomly collected samples assayed by DAS-ELISA was estimated. El Fayioum governorate showed the highest incidence of TBSV infection in randomly selected samples, about 84.7%, whereas Alexandria showed the lowest incidence of 2.17%.

Structure and physicochemical properties of TBSV Egh isolate

Purified virus particles stained with 2% uranyl acetate negative stain and examined by a TEM revealed isometric virus particles of approx. 30 nm in diameter (Fig. 2). Electrophoresis results of the viral protein of TBSV Egh 12% SDS-polyacrylamide gel are shown in Fig. 3 indicating the presence of a single protein band, *i.e.* the TBSV Egh coat protein of approx. size 41 kDa.

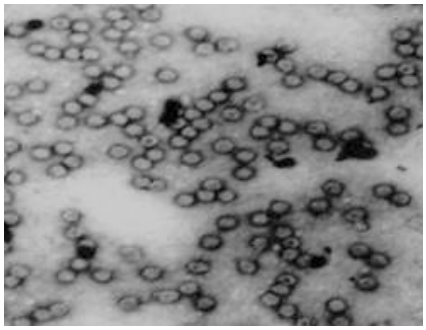

Fig. 2. Electron micrograph of purified TBSV Egh virus particles. Grids were negatively stained with 2% uranyl acetate.

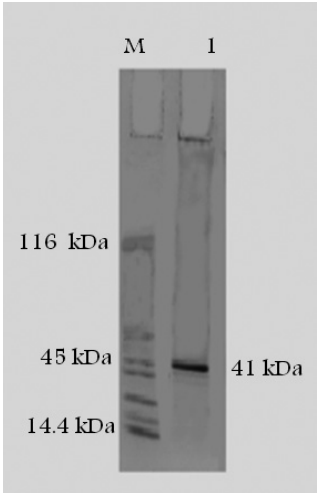

Fig. 3. Electrophoretic mobility of TBSV Egh isolate coat protein. Approx. 10 μl of TBSV Egh isolate were boiled in 1× SDS gel-loading buffer, electrophoresed on 12% SDS-polyacrylamide gel and stained with Coomassie blue. Lane M, low-molecular weight standard (14.4 kDa to 116 kDa); lane 1, TBSV Egh isolate coat protein.

Estimation of the size of genomic RNA nucleotide sequence and phylogeny of the TBSV Egh isolate

Total RNA was extracted from TBSV Egh isolate and run on formaldehyde agarose gel. The molecular weight of RNA with reference to the marker used was estimated to be approx. 4,800 nt, as shown in Fig. 4. Total genomic TBSV Egh RNA was used as a template for synthesis of a first-strand cDNA by the use of a forward specific TBSV full-length oligonucleotide primer to be subsequently used for the synthesis of viral genomic corresponding DNA. As shown in Fig. 5A a PCR-amplified fragment of 4,800 bp molecular weight was detected representing the synthesized full-length DNAs. A region at the 5' end of the genomic RNA corresponding to nucleotides 1 to 2401 of the TBSV Egh isolate was sequenced and used to determine the phylogenetic relationship of this isolate to previously described tombusviruses. These sequences included the complete coding region for the P33 and P92 viral proteins. The nucleotide sequence of TBSV Egh isolate generated in this study could be found in the EMBL database under the accession number GQ206144.1.

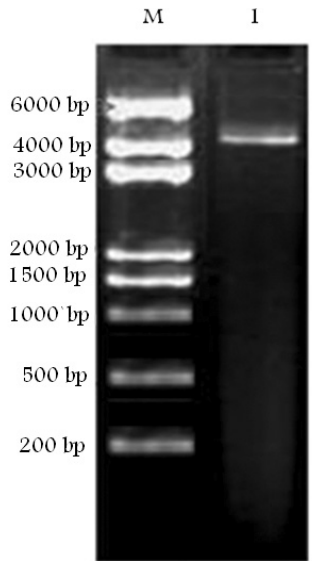

Fig. 4. Electrophoretic mobility of TBSV Egh isolate RNA. Approx. 5 μl of TBSV Egh isolate RNA were run on 1.2% formaldehyde agarose gel in 1× formaldehyde running buffer, stained with ethidium bromide, and photographed. Lane M, molecular weight standard RiboRuler (200–6000 bp) plus RNA ladder (Fermentase, Glen Burnie, MD, USA); lane 1, TBSV Egh isolate genomic RNA.

A phylogenetic tree for the nucleotide sequences is presented in Fig. 6. Comparison of the deduced amino acid sequences with corresponding ORFs of other tombusviruses is shown in Fig. 7. It indicates that the amino acid sequence of P33 has a high sequence identity (>95%) with TBSV nipplefruit (Nf) and cherry (Ch) isolate. The deduced sequence of gene P92 showed no

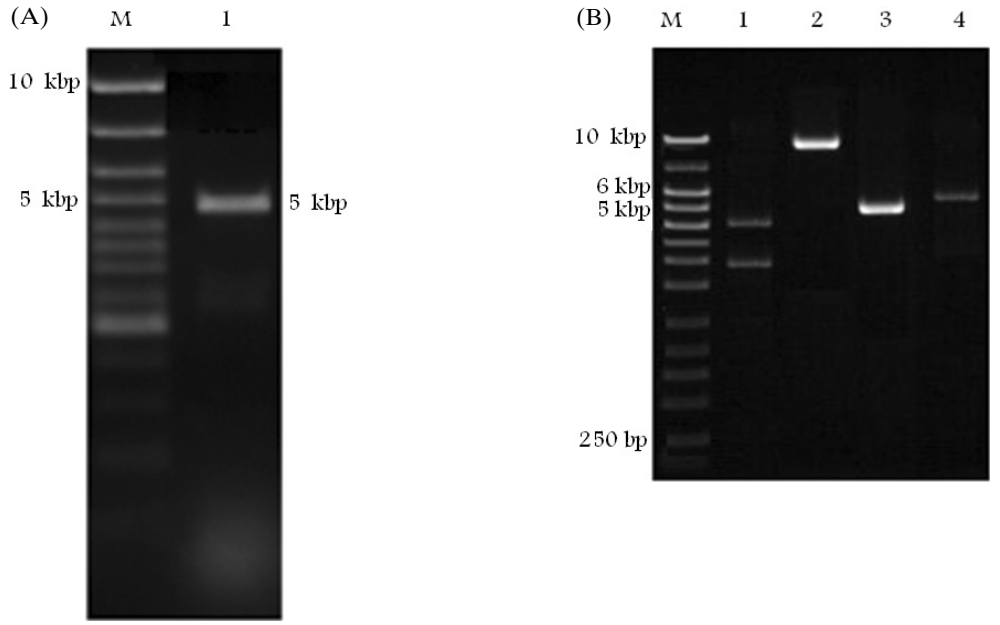

Fig. 5. (A) Electrophoretic mobility of TBSV Egh isolate purified PCR products amplified from cDNA using specific synthetic oligonucleotide primers. Approx. 5 μl of TBSV Egh isolate DNA were run on 1% agarose gel in 1× TBE buffer, stained with ethidium bromide and photographed. Lane M, molecular weight standard 1 Kb plus DNA ladder (SibEnzyme Ltd, Novosibirsk, Russia); lane 1, full-length genomic DNAs. (B) Electrophoretic mobility of TBSV Egh isolate full-length cDNA cloned in pCR 2.1-TOPO vector. Approx. 5 μl of recombinant plasmid DNA, Puc 19, and the TBSV Egh insert amplified by specific M 13 oligonucleotide primers and TBSV Egh insert amplified by specific TBSV full-length oligonucleotide primers were run on 1% agarose gel in 1× TBE buffer, stained with ethidium bromide, and photographed. Lane M, molecular weight standard 1 Kb plus DNA ladder (SibEnzyme Ltd.); lane 1, pUC19 vector; lane 2, recombinant plasmid carrying TBSV Egh insert; lane 3, TBSV Egh DNA amplified by specific TBSV full-length oligonucleotide primers; lane 4, TBSV Egh DNA amplified by specific oligonucleotide M 13 primers.

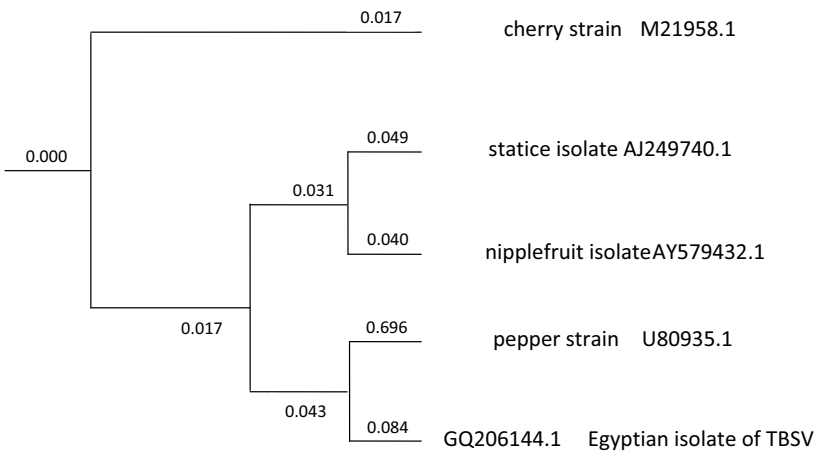

Fig. 6. Phylogenetic analysis of partial genome sequence of TBSV Egh isolate with aligned nucleotide sequences.

P92		TBSV-Ch	TBSY-NF	TBSV-S	TBSV-Egh	TBSV-P
		1	2	3	4	5
TBSV-Ch	1		97.80	95.84	90.22	96.58
TBSV-Nf	2	18		97.19	89.73	95.72
TBSV-S	3	34	23		88.26	94.25
TBSV-Egh	4	80	84	96		93.52
TBSV-P	5	28	35	47	53	

P33		TBSV-Nf	TBSY-Ch	TBSV-S	TBSV-Egh	TBSV-P
		1	2	3	4	5
TBSV-Nf	1		97.64	95.61	95.61	95.61
TBSV-Ch	2	7		94.26	95.61	95.61
TBSV-S	3	13	17		92.57	92.57
TBSV-Egh	4	13	13	22		100.00
TBSV-P	5	13	13	22	0	

Fig. 7. Pairwise amino acid sequence comparison of protein encoded by the genome of the TBSV Egh isolate.

such high sequence identity. The highest identity (>90%) was with TBSV cherry isolate.

Discussion

TBSV has been reported in many parts of the world (Ohki *et al*., 2005), yet this is the first report for its occurrence in Egypt. Based on symptomatology and serological identification the virus was detected in seven governorates (Table I). Incidence of the virus on other host crops has been also detected. The observations during the survey that indicated occurrence of infection only in separate clusters and not evenly spread could be correlated with the fact that virus transmission under natural conditions is known to be mainly through seed and soil and not by involving vectors (Kim *et al*., 2007). As the use of virus is the most likely explanation free seed certification is not a common practice. Embracement quarantine procedure for imported seeds and implementation of regulations with regards to seed certification practices may be successful strategies to prevent the settlement of TBSV in Egypt. All physicochemical properties of the TBSV Egh isolate with respect to structure, coat protein, and genomic RNA size were identical to previously known characteristics of the tombusviruses (Russo *et al*., 1994). Partial sequences are available for several tombusviruses isolates (Luis-Arteaga *et al*., 1996), which has

Table I. Incidence of TBSV infection in *Lycopersicon esculentum* in different Egyptian governorates tested by DAS-ELISA.

Governorate	Number of TBSV positive samples
Alexandria	2/92
El Beheira	16/92
El Gharbeya	20/92
El Dakahlya	11/44
El Kalyoubia	14/92
El Monofia	12/92
El Fayioum	78/92

Nominator number of TBSV positive samples as tested by ELISA, denominator total number of random samples collected from each governorate. Positive samples had twice the absorbance of the negative control at 405 nm.

been useful in classification and strain differentiation of tombusvirus isolates. We designed primers from the sequence data available in the GenBank for cloning and sequencing of the genome of the Egyptian isolate TBSV Egh. Sequencing of the first 2,401 nucleotides from the 5' end of TBSV Egh was done by the use of primers designed specifically for the 5' end. This is the non-conserved region of the Tombusvirus genome, thus would detect only identical or very closely related TBSV viruses. The TBSV genome organization revealed 5 ORFs encoding 5 viral proteins (Russo *et al.*, 1994). The TBSV Egh partial sequence included that of ORF1 encoding the 33-kDa protein required for duplication and ORF2 encoding the 92-kDa protein translated by a read through of the amber stop codon of ORF1 and possessing the conserved motif of RNA-dependent RNA polymerase carrying positive-strand RNA viruses (Russo *et al.*, 1994). The 5'-terminal nucleotide was AG as expected for the genera Tombusvirus, Necrovirus, Carmovirus, Machlomovirus, and Dranthovirus in the family Tombusviridae (Russo *et al.*, 1994). Phylogenetic analyses of the 5'-terminal sequences showed that TBSV Egh was yet closer related to the pepper strain (TBSV-P) (Szittya *et al.*, 2000) and cherry strain (TBSV-Ch) (Allan and Davidson, 1967) and more distant to nipplefruit (TBSV-Nf) (Ohki *et al.*, 2005) and statice isolate (TBSV-S) (Galezka *et al.*, 2000), respectively. The deduced amino acid sequences of P92 indicated high sequences identity only to TBSV nipplefruit and cherry isolate. However, P33 showed a relatively higher sequence identity to the cherry isolate. It is shown from our data to be a distinct cluster to the above isolates of the virus. Thus, the obtained partial sequence of the isolate indicates that it could be a new strain of TBSV.

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