European Journal of Plant Pathology **107:** 833–837, 2001.

Short communication

Molecular evidence that a lily-infecting strain of *Tulip breaking virus* from Japan is a strain of *Lily mottle virus*

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Accepted 10 June 2001

Key words: coat protein gene, nucleotide sequence, phylogenetic analysis, taxonomy

Abstract

The sequence of the 3′-terminal 2074 nucleotides (nts), excluding the 3′-poly (A) tail, of RNA of a potyvirus isolated from lily (*Lilium* Asiatic hybrid cv. Enchantment) in Japan, currently tentatively designated as *Tulip breaking virus*-li (TBV-li), was determined. The sequence started within a single open reading frame (ORF) that encoded the carboxyl terminus of the large nuclear inclusion protein (NIb) and the complete 275-amino-acid coat protein (CP), followed by a 3′-untranslated region (3′-UTR) of 204 nts. The CP of TBV-li shared 91% amino acid (aa) sequence identity with that of TBV lily strain Dutch isolate (TBV-lily). The nt sequences of their 3′-UTR were 94% identical. However both viruses shared only 60–65% sequence identities with TBV tulip strain Niigata isolate in the corresponding regions. The results suggest that TBV-li is closely related to TBV-lily, and that these two TBV lily strains should be classified into a species different from TBV tulip strains. We therefore support a proposal to rename TBV-lily *Lily mottle virus* (LMoV), and suggest that TBV-li is another strain of LMoV (LMoV-J).

Flower color-breaking in tulips is a unique symptom caused by viruses, and was first reported in 1576 by Clusius (Mckay and Warner, 1933). These 'broken tulips' were highly appreciated in the Netherlands during the 16th and 17th centuries (Van Slogteren and De Bruyn Ouboter, 1941). McWhorter (1938) distinguished two tulip breaking viruses in the Netherlands by their symptoms: color-removing and color-adding viruses, causing light and dark flower breaks, respectively. Since mottle or mosaic viruses isolated from lily in the United States of America (Brierley, 1940; Brierley and Smith, 1944a,b, 1948) also induce flower-breaking in tulips, Brierley and Smith (1944b) compared these lily viruses with the tulip viruses reported by McWhorter and postulated that all these virus isolates were different strains of the same virus, *Tulip breaking virus* (TBV). In addition to the Netherlands and the USA, viral color-breaking in tulip flowers was also recognized in Japan (Yamaguchi and Matsui, 1963; Yamaguchi, 1964). TBV was classified as a member of the potyviruses (Brandes and Wetter, 1959). Successful purification of virus particles enabled production of a specific antiserum against each TBV isolate, and it was revealed that there were some serological differences among TBV isolates (Derks et al., 1982).

Seven TBV strains have been isolated worldwide: one American isolate, *Lily mottle virus* (LMoV) (Brierly and Smith, 1944b); two Dutch isolates, TBV-lily and TBV-tulip (Langeveld et al., 1991); four Japanese isolates consisting of two Niigata isolates, TBV-li and TBV-T (Miyagawa, 1997; Miyagawa et al., 1997) and two Okayama isolates, TBV-L and TBV-T (Maeda and Inoue, 1981; Maeda et al., 1984). In order to discriminate TBV-T Niigata and Okayama isolates, in this paper we designate them as TBV-TN and TBV-TO, respectively.

As for TBV isolates from lily, TBV-lily was first isolated from *Lilium longiflorum* cv. Flevo in the Netherlands (Alper et al., 1982) and identified as an

aphid-borne potyvirus. Although TBV-lily is endemic in lily plants and does not occur in tulips in the field, it also causes symptoms on tulips which are nearly the same as those caused by TBV-tulip (Asjes et al., 1973). TBV-L which was once named as Lily mild mottle virus was isolated from lily in Japan and was later classified as a strain of TBV because it is closely related to TBV-TO serologically and complete cross protection was observed between them (Maeda and Inoue, 1981; Maeda et al., 1984). Later, TBV-li was isolated from lily (*Lilium* Asiatic hybrid cv. Enchantment) (Miyagawa, 1997). However, it was found that the antisera against TBV-li did not react with TBV-TN and the antisera against TBV-tulip (Asjes et al., 1973) did not react with TBV-li (Miyagawa, 1997).

In addition, there are reports of potyviruses, other than TBV, which cause flower color-breaking in tulips. The relationships between these color-breaking viruses including TBV, are complex. To understand their taxonomic relationships better, the sequences of their CP have been determined. At first, the 3′-terminal sequence of genomic RNA including the complete sequence of the CP of TBV-lily (Dutch; lily) was reported (Langeveld et al., 1991). Later, the sequences of amplified DNA fragments (277 nts, 92 aa) spanning a conserved area of the CP cistron of potyviruses isolated from tulips that cause color-breaking in tulip flowers, such as TBV-tulip (Dutch; tulip), Tulip top breaking virus (TTBV), Tulip band breaking virus (TBBV) and *Rembrandt tulip breaking virus* (ReTBV) were determined. In addition the 3′-terminal sequence including the complete sequence of the CP of TBV-TN (Niigata; tulip) was determined (Ohira et al., 1994). Here we present the 3′-terminal sequence including the complete sequence of the CP of TBV-li (Niigata; lily) and discuss the relationships among TBV isolates both from Netherland and Japan and from tulips and lilies.

Virus particles of TBV-li were purified using the method described by Miyagawa (1997) from infected leaf tissues of lily plants (*Lilium* Asiatic hybrid cv. Enchantment) in which the virus was propagated by mechanical inoculation. Viral RNA was isolated from purified virus particles as described by Kashiwazaki et al. (1989) and suspended in RNase-free water. cDNA was synthesized from 2 μg of viral RNA following the manufacturers' protocol with a cDNA synthesis kit (cDNA Synthesis Module, Amersham Pharmacia Biotech, UK). Double-stranded cDNA was cloned into the *Sma*I site of dephosphorylated pUC18 plasmid vector. A clone with a 2.2 kb insert was selected for the following sequence analysis.

The nucleotide (nt) sequence was determined by the dideoxynucleotide chain termination reaction (Sanger et al., 1977) using universal primers and internal primers designed from determined sequences using an ABI PRISM™ 377 DNA Sequencer (Applied Biosystems, USA). The cDNA was sequenced in both orientations. The nt and deduced aa sequences were analyzed using DNASIS software (Ver. 3.7 Hitachi Soft Engineering, Yokohama, Japan). The phylogenetic analyses using bootstrap option (100 replicates) were performed with Clustal W Multiple Alignments and Tree-making, available at DNA Data Bank of Japan (DDBJ) through the Internet.

The nt sequence of the cDNA for 3′-terminus of TBV-li provided 2074 nts excluding the 3′-poly(A) tail. The sequence started within a long open reading frame (ORF) that encoded a polypeptide of 622 aa and terminated at position 1868 with an ochre codon, leaving 204 untranslated nts at its 3′-terminus (3′-UTR). Comparisons with the sequences of TBV-lily, TBV-TN, and other related potyviruses suggested that this polypeptide represents the C-terminal part of a polyprotein, including a part of the large nuclear inclusion protein (NIb) followed by CP. The cleavage site between the NIb and CP was expected to be Q/A at aa position 347/348. A consensus motif of GDD was reported to be conserved in viral RNA dependent RNA polymerase (RdRp) (Koonin and Dolja, 1993), and it was also present in the NIb, putative RdRp of potyviruses, of TBV-li at aa position 181–183. The CP of TBV-li consisted of 275 aa with a predicted *Mr* of 30.9 kDa, nearly the same as those of TBV-lily (30.7 kDa) and TBV-TN (30.1 kDa).

The aa sequence of the CP of TBV-li determined in this work was one aa longer than that of TBV-lily. The identity of the CP aa sequence of the two viruses was 91%. The 3′-UTR of TBV-li showed 94% nt sequence identity with that of TBV-lily and was two nts shorter than that of TBV-lily. In contrast, TBV-li and TBV-TN had 65% aa sequence identity in their CPs, and 60% nt sequence identity in the 3′-UTR. These scores were very close to those between TBV-lily and TBV-TN, and were significantly lower than those between isolates of the same potyvirus species: *Plum pox virus* (PPV)-D and -NAT, *Potato virus Y* (PVY)-N and -H, and *Tobacco etch virus* (TEV)-HAT and -Non-Wilting.

TBV is recognized in tulips and lilies wherever these plants are grown. The symptom in tulip of TBV isolated from lily has been reported to be similar to those of TBV isolated from tulip. However, the mosaic patterns on the leaves are slightly different. TBV isolated

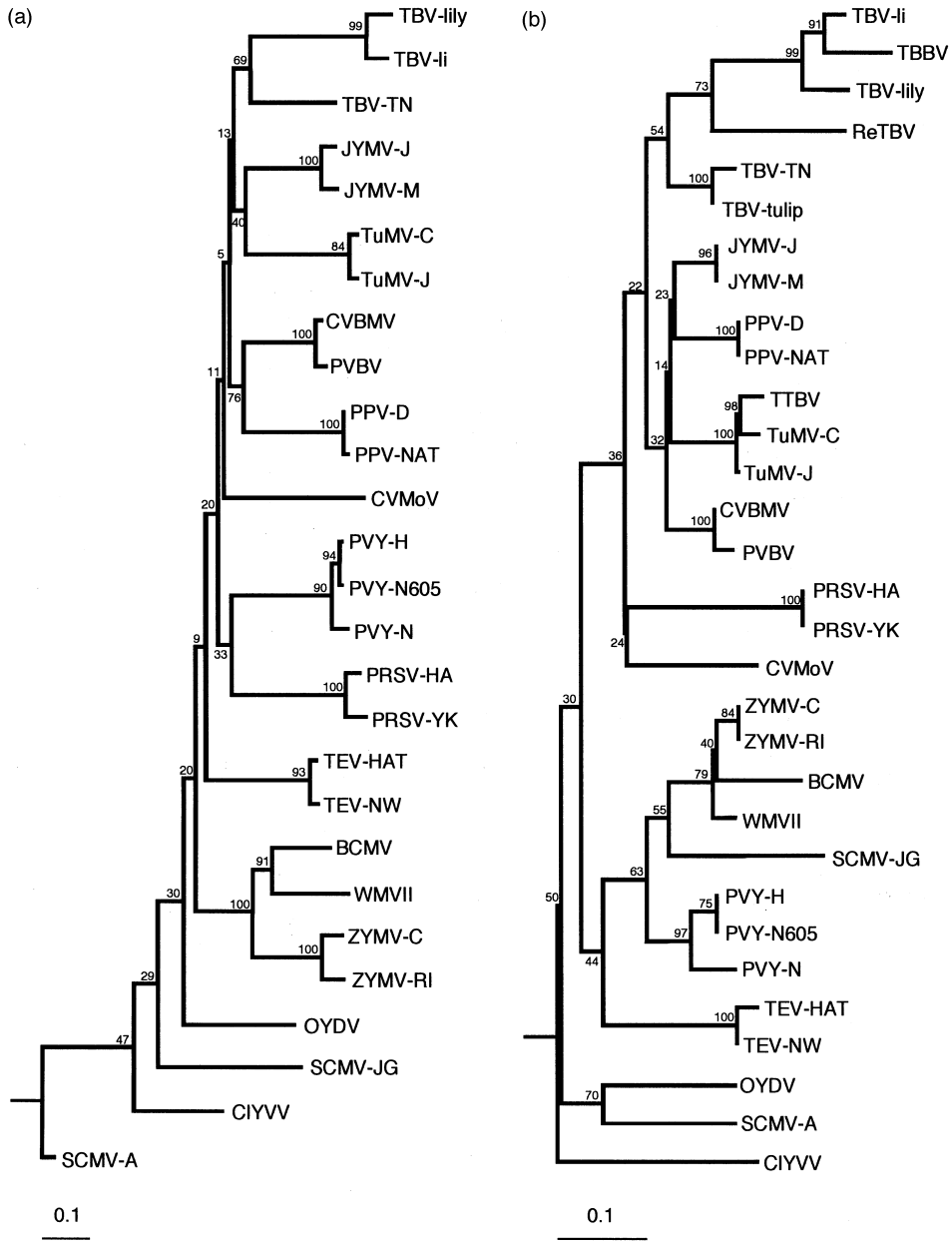

Figure 1. Phylogenetic trees based on (a) the complete aa sequence of CP and (b) the 92 aa sequence of CP core region of 31 different potyviruses. Each sequence data set was computed by Clustal W Multiple Alignments and Tree-making, available at DNA Date Bank of Japan (DDBJ) through Internet. Outgroup used in both trees was *Potato virus X* (PVX): D00344. Corresponding region of PVX CP aa sequence was used for each analysis. The sequence data of viruses used for analysis were obtained from GenBank: *Bean common mosaic virus* (BCMV): U19287, *Clover yellow vein virus* (C1YVV): S77521, *Chilli vein bandng mottle virus* (CVBMV): AB012221, *Carnation vein mottle virus* (CVMoV): AB017630, *Japanese yam mosaic virus* (JYMV)-J: AB016500, -M: AB027007, *Onion yellow dwarf virus* (OYDV): D73378, *Plum pox virus* (PPV)-D: X16415, -NAT: D13751, *Papaya ringspot virus* (PRSV)-HA: S46722, -YK: X97251, *Pepper vein banding virus* (PVBV): AJ237843, *Potato virus Y* (PVY)-H: M95491, -N: D00441, -N605: X97895, *Rembrandt tulip breaking virus* (ReTBV): S60808, *Sugarcane mosaic virus* (SCMV)-A: U57354, -JG: D00094, *Tobacco etch virus* (TEV)-HAT: M11458, -NW: L38714, *Tulip breaking virus* (TBV)-TN: X63630, -tulip: S60804, -lily: S44147, Tulip band breaking virus (TBBV): S60805, Tulip top breaking virus (TTBV): S60806, *Turnip mosaic virus* (TuMV)-C: D10927, -J: D83184, *Watermelon mosaic virus* 2 (WMVII): D13913, *Zucchini yellow mosaic virus* (ZYMV)-C: L31350, -RI: L29569 and -S: AF014811.

from tulip, such as TBV-tulip and TBV-TN, causes dark red breaks on tulip petals and mild mosaic patterns on the leaves, whereas TBV isolated from lily, such as TBV-lily and TBV-li, causes breaks similar to those caused by TBV tulip strains on tulip petals but clear mosaic patterns on leaves. In turn, as for TBV-lily and TBV-li, the symptoms in tulip caused by them are quite similar in both petals and leaves, but the host ranges are different. TBV-lily infects *Chenopodium quinoa* and *Tetoragonia expansa* locally, and *Nicotiana benthamiana* systemically, as well as lilies and tulips, on the other hand TBV-li infects lilies and tulips only. In these ways, the relationships among TBV isolates both from Netherland and Japan and from tulips and lilies are slightly elaborated, but, for a long time, they were still classified as the same potyvirus species, TBV, due to a lack of enough sequence data.

According to the serological properties and sequence analysis of amplified DNA fragments (277 nts, 92 aa) spanning a conserved area of the CP cistron, about one-thirds of whole coding region of CP, of potyviruses isolated from tulips that cause color-breaking in tulip flowers, it was suggested that TBV-tulip and TBV-lily should be distinct viruses and TBV-lily should be named *Lily mottle virus* (LMoV) (Dekker et al., 1993). In addition the complete CP sequence of TBV-TN (Niigata; tulip) was reported and it was suggested that TBV-TN and TBV-tulip were quite closely related because they were identical in the corresponding region. At the same time it was suggested that TBV-TN and TBV-lily were distinct species because only 64% aa identity was found between their CPs.

There are many reports concerning the classification of potyviruses based on the CP sequences (Shukla et al., 1988, 1994; Ward and Shukla, 1991). Here, we determined the 3′-terminal sequence of TBV-li, including the complete sequence of the CP, and performed phylogenetic analyses using the sequences of both the complete CP and the 92 aa CP core region of related potyviruses. As shown in Figure 1(a), TBV-li clustered with TBV-lily, and was separated from TBV-TN. Figure 1(a) clearly indicates that the distance between TBV-li and TBV-lily was as small as the distances between strains of the same species, and that the distance between TBV-li plus TBV-lily and TBV-TN was as large as those between different virus species. Figure 1(b) also shows that TBV-TN together with TBV-tulip were quite distant from TBV-lily and TBV-li, which confirmed the distant relationship between TBV from tulips and TBV from lilies. Nevertheless, TBV-li was located closer to TBBV than TBV-lily. TBBV shared a high identity (94%) with TBV-li for the 92 aa, and the identity was as high as between TBV-lily and TBV-li (93%). This suggests that these three viruses are strains of the same species. Unfortunately, since the sequences of the rest of the CP region and the 3′UTR were not determined, we cannot propose the taxonomic status of TBBV here.

The 7th edition of Virus Taxonomy discusses how different virus isolates can be classified as the same virus species when they have more than ∼85% identity in the CP sequence or ∼75% identity in the 3′UTR (Van Regenmortel et al., 2000). Here, the sequence identities of the CP aa sequence and the 3′UTR nucleotide sequence between TBV-lily and TBV-li were 91% (>85%) and 94% (>75%), respectively. TBVs from tulips and lilies differ in serology (Asjes et al., 1973; Miyagawa, 1997). Moreover, in the field, TBV-li has been detected in lilies, but not in tulips, and TBV-TN has been detected in tulips, but not in lilies (Miyagawa, 1997). Combining the results of the sequence identity, serological analysis, and host specificity with the phylogenetic analyses strongly suggests that TBVs from lilies and tulips are different species and that TBV-lily (LMoV) together with TBV-li should be classified as strains of the same virus species. We suggest that TBV-li should be renamed LMoV-J, to match the name LMoV Dekker et al. (1993) proposed for TBV-lily. Concerning TBV-L, which was serologically related to TBV-TO, since no sequence data are available, we cannot propose its taxonomic situation here.

The nt sequence data reported here will appear in DDBJ Nucleotide Sequence Databases under the accession number (AB054886).

Acknowledgements

We are gratefully appreciated to Mr. M. Miyagawa for providing TBV-li isolate, and to Mr. S. Hatano for excellent technical assistance.

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