

Further Characterization of *Tulip Severe Mosaic Virus* Supports Classification as a Member of Genus *Ampellovirus* in the Family *Closteroviridae*

K.T.K. Pham, G.J. Blom-Barnhoorn, V.P. Bijman, M.E.C. Lemmers and A.F.L.M. Derks
Wageningen UR, Applied Plant Research
Section Flower bulbs and Nursery stocks, P.O. BOX 85, 2160 AB Lisse
The Netherlands

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Abstract

Tulip bulb and flower production greatly contribute to the economy in The Netherlands. However, severe loss in this industry is caused by different viruses. In recent years, diagnostic tools were developed for most of these viruses with *Tulip severe mosaic virus* (TSMV) as one of the few exceptions. To further characterize TSMV, the virus was amplified by reverse transcription-polymerase chain reaction (RT-PCR) with degenerate primers targeted to conserved sequences of the Heat shock protein 70 gene of viruses from the family *Closteroviridae*. Phylogenetic analysis of the 0.5 kb HSP70 gene fragments showed that TSMV is closely related to *Plum bark necrosis stem pitting-associated virus* (PBNSPaV), *Apricot stem pitting-associated virus* (ASPaV) and *Pineapple mealybug wilt-associated virus 1* (PMWaV-1), all three members of the genus *Ampellovirus*. In addition, a set of primers amplifying a TSMV-specific fragment was designed that enables fast and specific detection of the virus.

Additionally to the molecular experiments, two possible modes of virus transmission were tested, viz. by aphids and by soil-borne organisms. However, no transmission was recorded by both methods.

INTRODUCTION

Tulip bulb and flower production greatly contribute to the economy in The Netherlands. However, economic losses due to viral infection of tulips (*Tulipa* L.) can be extensive. In 2007, the total loss related to the prevention of viral infections was estimated at 23.5 million € (S. Langeslag, person. Commun.). In the past decades the different viruses that can infect tulips in The Netherlands have been studied extensively (Asjes, 1994). Most of these viruses were molecularly characterized, including the viruses with filamentous particles: viz. *Tulip breaking virus* (TBV) (Langeveld et al., 1991; Dekker et al., 1993), *Lily symptomless virus* (LSV) (Memelink et al., 1990), *Tulip virus X* (TVX) (Yamaji et al., 2001) and *Turnip mosaic virus* (TuMV) (Dekker et al., 1993). As a result of their economic importance, both serological detection by ELISA and molecular diagnostic tools became available. One of the few uncharacterized viruses in tulip is *Tulip severe mosaic virus* (TSMV), a filamentous virus measuring 1600-2400 nm that was classified as a member of the genus closteroviruses (Nagao et al., 1988). Virus infection results in a mosaic of irregularly formed light- and dark-green lesions. Within these lesions clearly bordered ringspots can be observed on both sides of the leaves. These ringspots are characteristically large, oval and elongated; the leaf surface may also feel roughened. Flowers show tiny streaks at the edge of the petals in the final stage of flowering. Plants may be dwarfed and die prematurely (Asjes, 1994). Currently, antisera are not available for identification or detection of TSMV. The only way to confirm the presence of this virus is by electron microscopical analysis of samples on the basis of particle morphology.

During the past decades, nucleic acid-based diagnostics (mainly RT-PCR) were developed to identify filamentous plant viruses, including trichoviruses, i.e. *Apple chlorotic leaf spot virus* (ACLSV), vitiviruses, i.e. *Grapevine virus A* (GVA), capilloviruses, i.e. *Citrus tatter leaf virus* (CTLV), foveaviruses, i.e. *Apple stem pitting virus* (ASPV) and closteroviruses (Tian et al., 1996; Saldarelli et al., 1998; Foissac et al., 2005).

Based on these molecular and biological data, the taxonomic structure of the family of *Closteroviridae* was revised (Martelli et al., 2002). Within the family *Closteroviridae*, viruses were reclassified in 3 genera: (i) *Closterovirus*, (ii) *Ampellovirus*, and (iii) *Crinivirus*.

It is important for the bulb industry to be able to confirm viral infections with TSMV, thus we developed a procedure for identification of TSMV and a RT-PCR assay for diagnostic purposes. Furthermore, we analyzed whether TSMV can be transmitted by aphids or soil-borne organisms.

MATERIALS AND METHODS

Viral Isolates

Based on characteristic symptoms in diseased tulip leaves (Asjes, 1994) (Fig. 1), viral isolates used in this study were collected from different fields in The Netherlands. The presence and morphology of virus particles in tulip leaf extracts was confirmed by electron microscopy.

Freeze-dried leaf material of sugar beet infected with *Beet yellows virus* (BYV) was used as a positive control for the presence of closteroviruses in the RT-PCR tests with degenerate primers. Tulip material infected with *Turnip mosaic virus* (TuMV), *Tulip breaking virus* (TBV), *Tobacco rattle virus* (TRV) and *Tobacco necrosis virus* (TNV) was used as a negative control.

RNA Extraction and RT-PCR with Degenerate Primers

RNA was extracted from infected leaf samples (10 mg) using the plant tissue protocol of the Purescript RNA isolation kit (Gentra systems, Minneapolis, USA). The degenerate primers used (HSP-P-1 and HSP-P-2) were designed by Tian et al. (1996) based on the conserved phosphate 1 and 2 motifs of the HSP70 gene for members of the family *Closteroviridae*. A third primer, HSP-P-3 (5'-GCN GCH GCN GAN GGT TCR TTH A-3'), based on the conserved connect-1 motif was designed in this study. Reverse transcription was performed on 1 to 2 µg total RNA in 20 µl at 37°C for 1 hour, supplemented with 200 ng primer HSP-P-2 or HSP-P-3 and 200 units MLV reverse transcriptase (Invitrogen, California, USA). 5 µl of the reverse transcription mixture was added to 45 µl PCR-amplification mixture containing 200 ng of each primer (HSP-P-1 and HSP-P-2 respectively HSP-P-3) and 25 µl PCR Mastermix (Promega, Madison, USA). PCR reactions were started by incubating at 94°C for 4 min, followed by 36 cycles of 94°C for 30 s, 42°C for 30 s, and 72°C for 1 min. The reaction was terminated by a final incubation at 72°C for 10 min. The cycle conditions used with the primer pair HSP-P-1/HSP-P-3 was identical except for the annealing temperature 48°C was used. All PCR products were analyzed by agarose gel electrophoresis.

Cloning and Sequencing of the TSMV Heat Shock Protein

PCR-products, obtained with degenerate primers HSP-P-1/HSP-P-2 were ligated into the pCR2.1TOPO vector (TA-TOPO Cloning kit, Invitrogen, California, USA). Recombinant plasmids were selected, and those containing inserts were identified by digestion with *EcoRI*. Plasmids with the appropriate inserts were sequenced (BaseClear, Leiden, The Netherlands). Nucleotide and protein sequences were analyzed using the BLAST algorithms of the National Center for Biotechnology Information (NCBI). Protein sequences were aligned by CLUSTAL W (version 1.82) of the European Bioinformatics Institute (EMBL-EBI).

TSMV-Specific PCR-Based Detection

Based on the obtained sequences of TSMV, virus-specific forward and reverse primers, denoted as TSMV-For (5' TGT ACC GAG ATA TCA AAA GGT GGG 3') and TSMV-Rev (5' GAC GGT TCA TTC ACA ACG GCT T 3') were designed using software program Primer3 (Whitehead Institute, Cambridge, USA). The RT-PCR was performed

using TSMV-For/TSMV-Rev following the protocol described above, with the exception that an annealing temperature of 58°C was used.

Transmission of TSMV

Members of the genus *Closterovirus* are transmitted by aphids. Because TSMV is presumed to be a member of the genus closterovirus (Nagao et al., 1988; Asjes, 1994), we tested the transmission of TSMV by aphids. Healthy tulips ('King's Orange' and 'Sevilla'; 64 plants with 4 repeats) were cultured under aphid-proof gauze and interplanted with TSMV-diseased plants (32 plants in each cage). In these cages, tulips or maple seedlings colonized with aphids, *Myzus persicae* or *Drepanosiphum platanoides* respectively, were introduced in early May. After about 4 weeks, the aphids were killed by the application of insecticides. The progeny of tulips exposed to the aphids was checked for symptoms in the next season.

To check the soil-borne nature of TSMV as proposed by Asjes (1994), experiments were conducted on a field plot, which is known for TSMV infections. Approximately 500 healthy plants of each of the two tulip cultivars (King's Orange and Sevilla) were cultured in the soil in an aphid-proof gauze cage from April the 15th until the end of the growing season (July). The progeny of the plants was checked for virus symptoms in the following season in a TSMV-free area. Results were compared with those from surrounding stocks in the field with TSMV history and not covered with gauze. All transmission experiments were performed twice in successive years.

RESULTS

RT-PCR with Degenerate Primers

PCR-amplification using primer pair HSP-P-1/HSP-P-2 at low annealing temperature (40°C) resulted in poor resolution of PCR products. Only PCR-amplification using purified virions or high-titer *Beet yellows virus* infected leaf material yielded a distinct amplification product with these primers under these conditions. However, replacing primer HSP-P-2 by HSP-P-3 with a higher annealing temperature (48°C), DNA fragments of approximately 524 bp were amplified. As with other closteroviruses, two closely migrating DNA fragments were seen for TSMV (Fig. 2) (Tian et al., 1996). Unfortunately, a plausible explanation for the phenomenon still lacks.

Sequence Analysis

HSP-P-1/HSP-P-3 PCR-products of two TSMV isolates derived from cultivar Kees Nelis and cultivar Ad Rem were sequenced (GenBank accession number EF203674 and EF203673). The sequences of these cDNA fragments consist of 524 bp and encode a partial HSP70 polypeptide of 160 amino acids. Comparison with HSP70-like proteins of viruses in the family *Closteroviridae* by BLASTX showed that TSMV has the highest amino acid sequence homology to three ampeloviruses, viz. *Plum bark necrosis stem pitting -associated virus* (PBNPaV; 50% amino acid sequence identity; 69% amino acid sequence similarity), *Pineapple mealy bug wilt-associated virus 1* (PMWaV-1; 47% amino acid sequence identity; 62% amino acid sequence similarity) and *Apricot stem pitting-associated virus* (ASPaV; 50% amino acid sequence identity; 69% amino acid sequence similarity). Multiple alignments of the amino acid sequences of two TSMV isolates and other sequenced members of the family *Closteroviridae* were generated. A phylogram of the region between the phosphate 1 and connect 1 motifs of HSP70 protein is shown in Figure 3.

Detection of TSMV by Specific RT-PCR

Based on the obtained TSMV sequences and the nucleotide alignment with closely related viruses, TSMV specific primers were designed (TSMV-For and TSMV-Rev). TSMV-specific RT-PCR with specific primers was tested on samples obtained from infected tulips, as confirmed by electron microscopy (not shown). In all cases, TSMV-

specific amplicons of 350 bp were obtained; no amplification was observed for samples obtained from healthy tulips or tulips infected with other viral infections TuMV, TBV, TRV and TNV (Fig. 4). TSMV was also detected in some tulip cultivars or plants with less or indistinct symptoms (results not shown).

Transmission of TSMV

The ability of the aphid species *Myzus persicae* and *Drepanosiphum platanoides* to transmit TSMV was tested. The latter species was found to colonize seedlings of maple growing in tulip fields in which TSMV was detected and *M. persicae* is known to be an efficient vector of many plant viruses (Ng and Falk, 1996). No virus symptoms were observed in the progeny from tulip plants which were exposed in the first year to the viruliferous aphid species.

In two successive experiments on a field plot with TSMV history also no symptoms were observed in the progeny of tulips covered with aphid-proof gauze. In the progeny of the surrounding non-covered stocks the TSMV incidence was approximately 5% on basis of characteristic symptoms.

DISCUSSION

RT-PCR with degenerate primers HSP-P-1 and HSP-P-2 has been used for characterization of many viruses in the family *Closteroviridae* (Tian et al., 1996). Replacing HSP-P-2 by HSP-P-3 for both the initial cDNA synthesis and the PCR, allowed to increase the annealing temperature, which increased the specificity of the assay.

The heat shock protein gene was detected in severe mosaic diseased tulips. This provides further evidence that TSMV indeed is a member of the family *Closteroviridae*. Multiple alignments showed highest homology with the members of the genus *Ampellovirus*, and therefore, support classification of TSMV as a member of this genus. Furthermore, also the fact that TSMV was not transmitted by aphids indicates that it should not be classified as member of the genus *Closterovirus*. However, TSMV would be the first member of the genus *Ampellovirus* infecting monocots. (Martelli et al., 2002).

RT-PCR using the specific primers TSMV-For/Rev detects and identifies TSMV whereas electron microscopy only allows detection of TSMV-like viruses. This is a major advantage especially for observation in tulips where mixed infections of filamentous viruses occur. Therefore, our results indicate that RT-PCR assay with TSMV-specific primers is a promising diagnostic tool for detection of TSMV in tulip.

Like the most closely related *Plum bark necrosis stem pitting-associated virus* (PBNSPaV) (Marini et al., 2002) and *Apricot stem pitting-associated virus* (ASPaV) (Abou Ghanem-Sabanadzovic et al., 2001), the vector of TSMV is still unknown. Based on this study, there was no evidence for virus transmission by aphids like *Myzus persicae* and *Drepanosiphum platanoides* or by a soil-borne vector such as a fungus or nematode. The molecular characterization of TSMV as an ampellovirus suggests to the possibility of mealy bugs as vector (Martelli et al., 2002). However, in tulips mealy bugs have never been observed in contrast to other ornamental bulbous crops such as iris, hyacinth, *Narcissus*, *Nerine*, *Eucharis* and *Hippeastrum*.

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Literature Cited

- Abou Ghanem-Sabanadzovic, N., Mahboubi, M., Di Terlizzi, B., Sabanadzovic, S., Savino, V., Uyemoto, J.K. and Martelli, G.P. 2001. Molecular detection of a closterovirus associated with Apricot stem pitting in southern Italy. *Journal of Plant Pathology* 83:125-132.
- Asjes, C.J. 1994. Viruses in tulip in the Netherlands. *Acta Hort.* 377:289-300.

- Dekker, E.L., Derks, A.F.L.M., Asjes, C.J., Lemmers, M.E.C., Bol, J.F. and Langeveld, S.A. 1993. Characterization of potyviruses from tulip and lily which cause flower-breaking. *Journal of General Virology* 74:881-887.
- Dovas, C.I. and Katis, N.I. 2003. A spot multiplex nested RT-PCR for the simultaneous and generic detection of viruses involved in the aetiology of grapevine leafroll and rugose wood of grapevine. *Journal of Virological Methods* 109:217-226.
- Foissac, X., Svanella-Dumas, L., Gentit, P., Dulucq, M-J, Marais, A. and Candresse, T. 2005. Polyvalent degenerate oligonucleotides reverse transcription-polymerase chain reaction: a polyvalent detection and characterization tool for trichoviruses, capilloviruses, and foveaviruses. *Phytopathology* 95:617-625.
- Langeveld, S.A., Dore, J-M., Memelink, J., Derks, A.F.L.M., van der Vlugt, C.I.M., Asjes, C.J. and Bol, J.F. 1991. Identification of potyviruses using the polymerase chain reaction with degenerate primers. *Journal of General Virology* 72:1531- 1541.
- Marini, D. B., Zhang, Y.P., Rowhani, A. and Uyemoto, J.K. 2002. Etiology and host range of a closterovirus associated with Plum bark necrosis stem pitting disease. *Plant Disease* 86:415-417.
- Martelli, G.P., Agranovsky, A.A., Bar-Joseph, M., Boscia, D., Candresse, T., Coutts, R.H.A., Dolja, V.V., Falk, B.W., Gonsalves, D., Jelkmann, W., Karasev, A.V., Minafra, A., Namba, S., Vetten, H.J., Wisler, G.C. and Yoshikawa, N. 2002. Virology Division news: The family Closteroviridae revised. *Archives of Virology* 147:2039-2044.
- Memelink, J., van der Vlugt, C.I.M., Linthorst, H.J.M., Derks, A.F.L.M., Asjes, C.J. and Bol, J.F. 1990. Homologies between genomes of a carlavirus (lily symptomless virus) and a potexvirus (lily virus X) from lily plants. *Journal of General Virology* 71:917-924.
- Nagao, N., Nishio, T. and Kobayashi, T. 1988. Fine structure of tulip leaves infected with tulip severe mosaic virus, occurring in the Netherlands. *Research Bulletin Plant Protection Japan* 24:57-61.
- Ng, J.C.K. and Falk, B.W. 2006. Virus-vector interactions mediating non-persistent and semipersistent transmission of plant viruses. *Annual Review of Phytopathology* 44:183-212.
- Saldarelli, P., Rowhani, A., Routh, G., Minafra, A. and Digiario, M. 1998. Use of degenerate primers in a RT-PCR assay for the identification and analysis of some filamentous viruses, with special reference to clostero- and vitiviruses of the grapevine. *European Journal of Plant Pathology* 104:945-950.
- Tian, T., Klaassen, V.A., Soong, J., Wisler, G., Duffus, J.E. and Falk, B.W. 1996. Generation of cDNAs specific to lettuce infectious yellows closterovirus and other whitefly-transmitted viruses by RT-PCR and degenerate oligonucleotide primers corresponding to the closterovirus gene encoding the heat shock protein 70 homolog. *Phytopathology* 86:11167-11173.
- Yamaji, Y., Kagiwada, S., Nakabayashi, H., Ugaki, M. and Namba, S. 2001. Complete nucleotide sequence of Tulip virus X (TVX-J): border between species and strains within the genus Potexvirus. *Archives of Virology* 146:2309-2320.

Figures



Fig. 1. Leaf symptoms of TSMV in tulip 'Spring Song': chlorotic diamond-shaped lesions and line patterns (left); symptoms in tulip 'Apeldoorn' consisting of chlorotic severe mosaic and stripes on the leaves and misshapen flower with faint stripes (right).

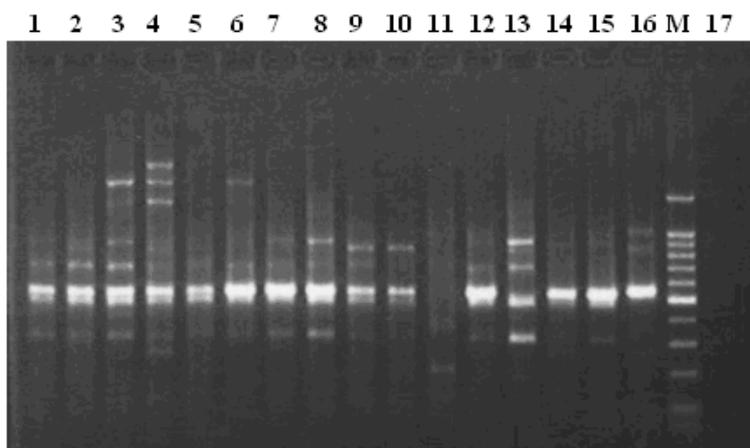


Fig. 2. RT-PCR products (524 bp) amplified with degenerate primers HSP-P-1 and HSP-P-3. Lanes 1-10, 12, 14, 15: various TSMV-infected tulip samples (the presence of viruses was confirmed by EM); lane 11: TuMV-infected tulip; lane 13: TVX-infected tulip; lane 16: BYV-infected sugar beet (positive control); M: 100 bp Ladder (Promega); lane 17: water (negative control).

Phylogram

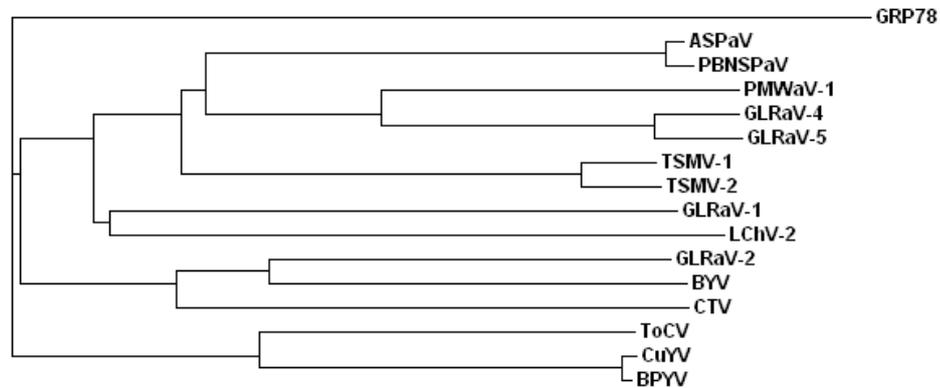


Fig. 3. A phylogenetic tree generated from an alignment of the amino acid sequences between phosphate1 and connect1 motifs of the HSP70 gene of TSMV isolates and other viruses of the family *Closteroviridae*. Accession numbers of the sequences used are: *Apricot stem pitting-associated virus* (ASPaV) CAC83741; *Plum bark necrosis stem pitting-associated virus* (PBNSPaV) AAF04602; *Pineapple mealybug wilt-associated virus 1* (PMWaV-1) AAL66711; *Grapevine leafroll-associated virus 4* (GLRaV-4) AAB96680; *Grapevine leafroll-associated virus 5* (GLRaV-5) AAK38608; *Little cherry virus 2* (LChV-2) AAP87788; *Grapevine leafroll-associated virus 1* (GLRaV-1) AAK38612; *Grapevine leafroll-associated virus 2* (GLRaV-2) CAA75811; *Beet yellows virus* (BYV) CAA51858; *Citrus tristeza virus* (CTV) AAC59627; *Tomato chlorosis virus* (ToCV) AAD01790; *Cucumber yellows virus* (CuYV) BAC66364; *Beet pseudo yellows virus* (BPYV) AAQ97386. Sequence of the GRP78 protein from tomato was used as an outgroup.

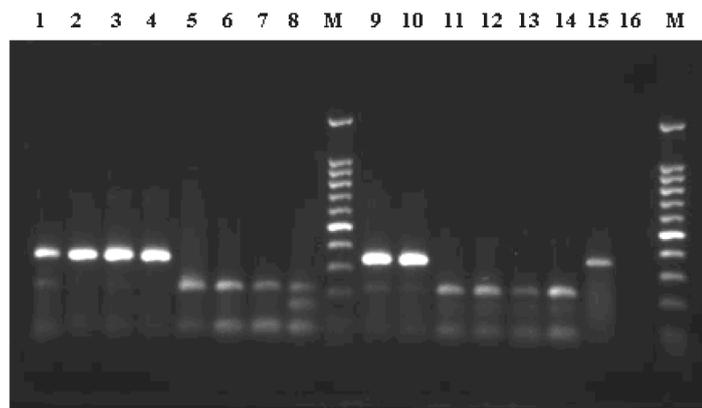


Fig. 4. RT-PCR products (350bp) amplified with specific primers for TSMV. Lane 1-4 and 9, 10, 15: severe TSMV infected tulips samples (the presence of viruses was confirmed by EM); lane 5-8: tulips infected with TuMV, TBV, TRV, TNV respectively; lane 11-14: healthy tulips; lane 16: water (negative control). M: 100 bp Ladder (Promega).

