Molecular evidence for occurrence of Tuberose mild mottle virus infecting tuberose (*Polianthes tuberosa*) in India

Saurabh Kulshrestha, Arpna Mehra, Vipin Hallan, Gaurav Raikhy, Raja Ram and A. A. Zaidi*

Plant Virus Lab, Floriculture Division, Institute of Himalayan Bioresource Technology, Palampur 176 061, India

Tuberose (*Polianthes tuberosa*) in the grower's field of Kangra valley, Himachal Pradesh, India showed presence of mosaic symptoms on leaves and flower peduncles. Tuberose plantations were heavily infected, indicating its widespread nature. Causal agent could only be transmitted mechanically to tuberose, indicating a narrow host range. Further, the virus could be transmitted by aphid vector in non-persistent manner. Positive results with ELISA employing potyvirus group-specific antibody, RT–PCR using group-specific primer for its detection and non-persistent insect transmission confirmed the presence of potyvirus. An ~ 750 bp RT–PCR amplified fragment was then cloned and sequenced. Sequence analysis confirmed identity of the virus infecting tuberose in India as Tuberose mild mottle virus (TuMMoV). The 453 bp partial coat protein gene was found to be 100% homologous with TuMMoV isolate from China and 68–88% homologous with other established potyviruses. Phylogenetic analysis also confirmed the results of sequence alignment. The sequence of TuMMoV was found to be unique and when analysed by recombination detection programme, did not show any recombination with the studied potyvirus sequences. The present communication reports occurrence of TuMMoV infecting tuberose from India. Given their specificity, sensitivity and reliability, ELISA and RT–PCR based diagnostics should be of benefit to phytosanitary/quarantine services related to the tuberose industry and also as a decision management tool for the growers.

Keywords: ELISA, *Polianthes tuberosa*, RT–PCR, Tuberose mild mottle virus.

TUBEROSE (*Polianthes tuberosa* Linn.) is an ornamental bulbous plant native to Mexico. In India it occupies a prime position in the floriculture industry, being equally suitable for the cut flower trade, buttonholes, etc. as well as in the essential oil industry[1]. The single flowered tuberose has high essential oil content ranging from 0.08 to 0.10%.

Viral disease decreases quality of flowers and bulbs, which leads to decreased profits to growers and propagators. A survey of tuberose plantations in floriculture field of the Institute of Himalayan Bioresource Technology (IHBT), Palampur and in the grower's field, showed mild mosaic and mottling symptoms on leaves of the plants. Such symptoms on tuberose have been described from a few places like New Zealand, China and Taiwan. These symptoms have been shown to be the result of infection by a potyvirus. Pearson and Horner[2] reported the presence of a potyvirus in tuberose from New Zealand. Chen *et al.*[3], and Chen and Chang[4] reported that the potyvirus infecting tuberose in Taiwan is *Tuberose mild mosaic virus* (TuMMV). Later on Lin *et al.*[5], found that the potyvirus infecting tuberose in China is a distinct one and they have tentatively named it Tuberose mild mottle virus (TuMMoV). The present study is an attempt to identify and characterize the virus causing mosaic symptoms on tuberose in the Indian scenario. The leaf sample of tuberose was tested by enzyme linked immunosorbent assay (ELISA) and reverse transcription–polymerase chain reaction (RT–PCR) for the presence of potyvirus by group-specific antibody and group-specific primers respectively. The RT–PCR amplified fragment was cloned and sequenced. The sequence was compared with other established potyviruses for homology, differences and recombination.

Plant material of double tuberose was collected from grower's field and experimental farm of Floriculture Division, IHBT. Inoculums for host range study were prepared by homogenizing the leaves in 0.1 M phosphate buffer, pH 7.2. The hosts inoculated mechanically for host range study were *Antirrinum majus*, *Chenopodium album*, *C. amaranticolor*, *C. quinoa*, *Cucurbita pepo*, *Cucumis sativus*, *Datura metel*, *D. stramonium*, *Lycopersicon esculentum*, *Nicotiana debenyii*, *N. glutinosa*, *N. rustica*, *N. tabacum*, *Petunia hybrida*, *Pisum sativum* and *Polianthes tuberosa*. Insect transmission was carried out using *Myzus persicae* as a vector in order to confirm the identity of the virus infecting tuberose as potyvirus.

ELISA was performed as described by Clark and Adams[6] with minor modifications. Potyvirus group-specific antibodies were procured from Agdia Inc., USA and buffers required for ELISA were prepared as described by the manufacturer.

RNA was extracted using QIAGEN RNeasy plant mini kit. RT–PCR was performed as described by Van der Vlugt *et al.*[7], using degenerate primer pair p9502 (5′-GCGG ATCCTTTTTTTTTTTTTTTTT-3′) and cpup (5′-TGAG GATCCTGGTGYATHGARAAYGG-3′, where Y = C/T, H = A/T/C, R = A/G), specific for potyvirus group. For reverse transcription (RT) reaction, 8 µl RNA (1–2 µg) was used with 0.2 µg downstream primer (p9502), 2 µl of 40 mM dNTP mix, 10 µl human placental RNase inhibitor, 1 µl 0.1 M DTT, 4 µl 5x RT buffer and 7.5 units AMV RT. The reaction mixture was incubated at 42°C for 1 h. PCR amplification was carried out in GeneAmp PCR9700 system (Applied Biosystems, USA) with 50 µl amplification mixture containing 5 µl cDNA product, 0.2 µg upstream primer, 0.2 µg downstream primer, 5 µl 10x PCR buffer, 1 µl 30 mM dNTP mix and 1.5 units Taq DNA polymerase.

The nucleotide sequence data reported here have been submitted in the EMBL Nucleotide Sequence Database under the accession number AJ888228.

*For correspondence. (e-mail: zaidi_aijaz@yahoo.com)

Amplification was carried out for 40 cycles with a denaturation step of 1 min at 94°C, an annealing step of 2 min at 58°C, an elongation step of 1 min at 72°C (the final elongation step of 10 min at 72°C). The amplified PCR product was electrophoresed in 1% agarose gel and visualized after staining with ethidium bromide. The amplified fragment was cloned in pGEM-T easy vector system (Promega, USA) and several recombinant clones containing the amplified fragment of interest were sequenced.

The sequence was aligned with corresponding sequences of other established potyviruses from the database using BLAST[8]. The program BLASTP was used to search the amino acid sequence database. Pairwise comparisons were performed by the ALIGN-2 program utilizing the DOTHELEX algorithm[9]. Multiple alignments were generated by the MULTALIN program[10]. Phylogenetic tree was constructed with the help of ClustalW[11] (www.ddbj.nig.ac.jp). The tree was subjected to bootstrap (using 1000 replicates) and viewed with the help of TREEVIEW[12]. Recombination Detection Program[13], which will identify recombination breakpoints using various published recombination detection programs, including GENECONV, BOOTSCAN, MAXIMUM, CHIMAERA and SISTER SCANNING was used in order to identify any possible recombination events in the Indian isolate of TuMMoV with other established potyviruses.

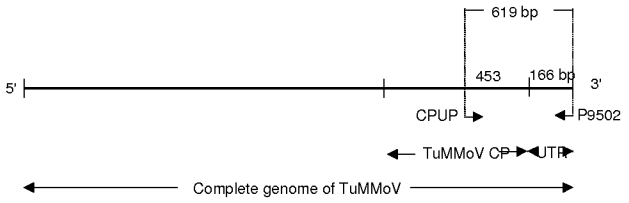

Figure 1. Ray diagram showing position of primers (P9502 and CPUP) used for RT–PCR in complete genome of Tuberose mild mottle virus.

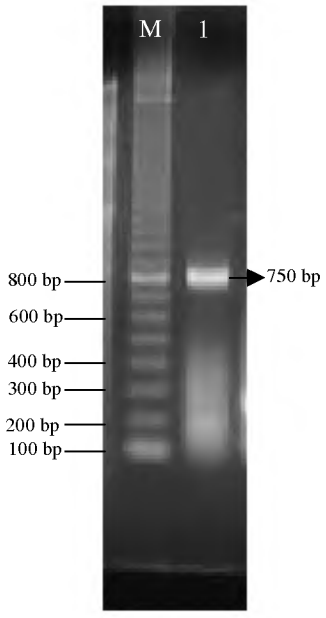

Figure 2. Amplification of a portion of Tuberose mild mottle virus (TuMMoV) genome. Lane M, 100 bp marker; Lane 1, ~750 bp amplification from TuMMoV genome.

Naturally infected plantations of tuberose showed mosaic symptoms on leaves and peduncles of the plant. Mechanical inoculation test revealed no symptoms on any of the above-mentioned plants even after one month of inoculation, except on tuberose. The data confirm the earlier findings[2,4,5] that tuberose was the only host identified for tuberose potyvirus. *Myzus persicae* successfully transmitted the virus non-persistently to healthy tuberose plants, which indicates that the virus infecting tuberose could be a potyvirus.

Potyvirus group-specific antibodies were used to confirm the identity of virus causing mottling in tuberose plants using ELISA. When done in triplicate, ELISA showed strong positive signals, infected samples showed A405 value of 0.549, negative control 0.046 and positive control 0.680, which confirm that tuberose is infected by a potyvirus. RT–PCR using potyvirus group-specific primers p9502 and CPUP[7] (Figure 1) gave an amplification of expected size, i.e. ~750 bp in tuberose leaves (Figure 2). This further confirmed that the virus infecting tuberose is a potyvirus. The amplified fragment was cloned in pGEM-T easy vector system and sequenced. On sequencing, it was found that the amplified fragment is 619 bp of extreme 3′-terminal region of TuMMoV. This sequence was submitted to EMBL database under accession number AJ888228. This 619 bp sequence contains 453 bp of coat protein (*CP*) gene of TuMMoV and 166 bp of 3′-untranslated region. The sequence of Indian isolate of TuMMoV was aligned with the corresponding sequences of other established potyviruses at both nucleotide and amino acid levels. The sequence of *CP* gene of Indian isolate of TuMMoV is 97% homologous with TuMMoV reported from China at nucleotide level and 100% homologous at amino acid level. It is 78 and 88% homologous with TuMMV reported from Taiwan at nucleotide and amino acid level respectively. It is also 68–75% homologous at amino acid level with other established potyviruses in the database (Table 1). The 166 bp 3′-terminal region of Indian isolate of TuMMoV is 98% homologous with the Chinese isolate of TuMMoV. Phylogenetic analysis also revealed that the Indian isolate is closely related to the Chinese isolate of TuMMoV and Taiwanese isolate of TuMMV. Rest of the potyviruses are clustered separately, showing their distant relationship (Figure 3). The 619 bp amplified fragment was used to identify the possible recombination events with the corresponding sequences of other established potyviruses in the database. The programs did not identify the isolate to be involved (either as daughter or as parent) in recombination with other studied potyviruses. It showed that the genome of TuMMoV is unique and has not evolved from any other potyvirus used in the study.

Table 1. Per cent amino acid (below diagonal) and nucleotide (above diagonal) sequence similarities between partial CPs of different potyviruses

	TuMMoV-I	TuMMoV-C	TuMMV-T	PMoV-K	LMoV-C	NPV-I	TBBV-J	LYSV-B	SuMV-Th	ZMV-Is	BCMV-US	TurMV-US
TuMMoV-I	X	97	78	67	66	68	65	67	69	66	63	66
TuMMoV-C	100	X	79	67	67	68	67	68	69	67	64	67
TuMMV-T	88	88	X	67	70	69	70	66	68	67	63	68
PMoV-K	75	75	76	X	64	68	64	62	63	65	63	71
LMoV-C	73	73	75	70	X	67	97	67	65	62	67	68
NPV-I	76	76	77	75	75	X	68	66	68	66	68	80
TBBV-J	73	73	75	70	99	75	X	67	66	63	67	68
LYSV-B	70	70	76	70	76	76	76	X	64	64	63	63
SuMV-Th	70	70	70	73	69	72	70	70	X	78	64	69
ZMV-Is	70	70	71	71	67	72	68	72	84	X	67	68
BCMV-US	68	68	70	72	69	71	69	72	68	72	X	65
TurMV-US	74	74	76	77	74	87	74	72	76	72	71	X

TuMMoV-I, Tuberose mild mottle virus Indian isolate AJ888228; TuMMoV-C, Tuberose mild mottle virus China isolate AJ581528; TuMMV-T, *Tuberose mild mosaic virus* Taiwan isolate AF062926; PMoV-K, *Pepper mottle virus* Korea isolate AY748921; LMoV-C, *Lily mottle virus* China isolate AJ874696; NPV-I, *Narcissus potyvirus* Indian isolate AJ871400; TBBV-J, *Tulip band breaking virus* Japan isolate AB090385; LYSV-B, *Leak yellow stripe virus* Brazil isolate AF228415; SuMV-Th, *Sugarcane mosaic virus* Thailand isolate AY629311; ZMV-Is, *Zucchini mosaic virus* Israel isolate CP AF228693; BCMV-US, *Bean common mosaic virus* USA isolate AY863025; TurMV-US, *Turnip mosaic virus* USA isolate NC_002509.

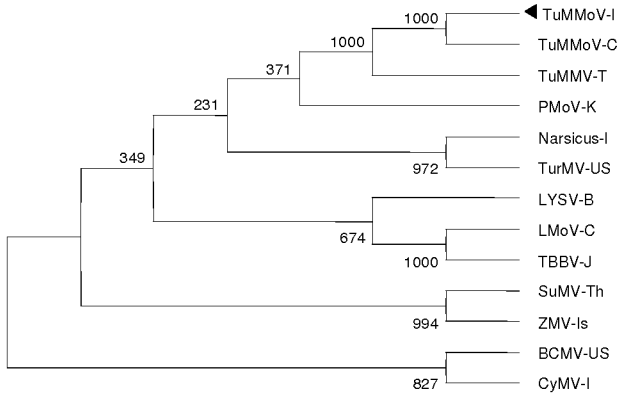

Figure 3. Phylogenetic relationship of newly characterized (Indian isolate of TuMMoV) virus (indicated by internal arrow) with corresponding sequences of other potyvirus isolates listed in Table 1. Branch lengths are proportional to the number of inferred character state transformations. Vertical branches are arbitrary. Bootstrap values (% replication) are shown at all the major branches.

Chen and Chang[4] and Lin *et al.*[5] have also reported the use of group-specific primers for the detection of tuberose potyvirus. Chen and Chang[4], and Chen *et al.*[3] first reported the presence of TuMMV in tuberose from Taiwan. Lin *et al.*[5] reported the presence of a distinct potyvirus on tuberose, i.e. TuMMoV. We have detected the presence of potyvirus infecting tuberose in India using various detection methods and sequenced a portion of its genome. Sequence analysis revealed that the potyvirus infecting tuberose is also a strain of TuMMoV. This is a report on the presence of TuMMoV infecting tuberose in India.

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