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Occurrence and coat protein sequence-based characterisation of *Bean yellow mosaic virus* (BYMV) associated with vanilla (*Vanilla planifolia* Andrews) in India

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Occurrence and coat protein sequence-based characterisation of *Bean yellow mosaic virus* (BYMV) associated with vanilla (*Vanilla planifolia* Andrews) in India

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Thirty vanilla plants showing virus-like symptoms were subjected to reverse transcription-polymerase chain reaction (RT-PCR) using *Bean yellow mosaic virus* (BYMV) specific primers. The plants showing severe yellow mosaic and leaf distortion collected from Madikeri, Karnataka were found to be infected with BYMV. In RT-PCR, the BYMV specific primers amplified a ~ 950 bp expected product that was cloned and sequenced. The sequenced region contained 957 bases spanning nuclear inclusion b (NIb) and coat protein (CP) genes. Sequence analyses and phylogenetic studies based on the CP region confirmed the identity of the virus as a strain of BYMV. This is the first report on the occurrence of BYMV infecting vanilla in India.

Keywords: *Bean yellow mosaic virus*; coat protein; RT-PCR; sequence analyses; phylogenetic relationships; *Vanilla planifolia*

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Introduction

Bean yellow mosaic virus (BYMV) infects field legumes such as beans, peas, clover and monocotyledonous and dicotyledonous ornamental crops including some orchids and trees (Winter and Nienhaus 1989). The virus induces mosaic, leaf distortion and sometimes necrosis of stem and veins, and stunting in many crops. BYMV has flexuous filamentous particle morphology (about 750 nm in length), transmitted by many aphid species in the non-persistent manner with a single species of linear positive sense single stranded RNA. The N-terminal region of the coat protein (CP) was found highly variable among BYMV isolates (Hammond and Hammond 2003).

Vanilla (*Vanilla planifolia* Andrews) is a valuable orchid spice cultivated for its highly priced beans. Viruses infecting vanilla were reported from most of the vanilla-growing countries across the world. Occurrence of BYMV on vanilla was first reported from Reunion Islands based on polymerase chain reaction (PCR) amplification of a 327 bp region corresponding to core region of the CP gene (Grisoni et al. 2006). In India, large-scale intensive cultivation of vanilla started only recently in the states of Karnataka, Kerala and Tamil Nadu. Since vanilla is

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propagated vegetatively, the spread of viral diseases has become increasingly serious due to the use of infected stem cuttings. So far the occurrence of two viruses namely, *Cucumber mosaic virus* (CMV) (Madhubala et al. 2005) and *Cymbidium mosaic virus* (CymMV) (Bhat et al. 2006) were reported from India. Electron microscopy of the infected vanilla samples from India also revealed the presence of *Potyvirus*-like particles (Bhat et al. 2004). However, the exact identification of the causal virus remained unaddressed. In this article, we report the occurrence and CP-based molecular characterisation of BYMV infecting vanilla in India.

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Materials and methods

A total of 30 vanilla plants exhibiting virus-like symptoms such as mosaic, necrosis, leaf distortion, stunting and drying of aerial adventitious roots collected from the states of Karnataka and Kerala in India were used in this study. Total RNA from these plants was following the protocol of Siju et al. (2007). A healthy vanilla plant raised in the greenhouse was included as a negative control in all the tests. Total RNA from each of the plants was subjected to RT-PCR using primer pair (5′GCACCATATAGTCAATTGAG3′) and (5′GACATCTCCTGCTGTGTG3′). Primers were designed based on multiple sequence alignment (MSA) of nuclear inclusion b (NIb) and CP gene sequences of BYMV isolates available in GenBank. One-tube one-step RT-PCR reaction was carried out in a 50 μl reaction volume containing 50 ng each of the sense and antisense primers, 10 U Ribonuclease inhibitor (Fermentas, USA), 20 U M-MuLV reverse transcriptase (Fermentas, USA), 1.5 U Taq polymerase (New England Biolabs, USA), 1 × PCR buffer (Genei, Bangalore), 10 mM dithiothreitol (Genei, Bangalore) and 10 μM each of the dNTPs (Sigma, USA). PCR mix (46 μl) containing the above components was added to the tubes containing the template RNA (4 μl) resulting in a final reaction volume of 50 μl. Amplifications were performed in an automated thermal cycler (Eppendorf Master Cycler Gradient) and the program consisted of one cycle at 42°C for 45 min for cDNA synthesis followed by a 40-cycle reaction profile involving 30 s of denaturation at 94°C, 1 min of annealing at 50°C and 1 min of extension at 72°C and a single cycle of final extension at 72°C for 10 min. The reaction products were then analysed on a 1% agarose gel along with 500 bp DNA ladder (Fermentas, USA). The resulting PCR products were visualised and photographed using a UV transilluminator and a gel documentation apparatus (Alpha Innotech Corporation, CA, USA).

The PCR products were purified using Perfectprep Gel Cleanup Kit (Eppendorf, Germany), cloned into pTZ57R/T cloning vector (Fermentas, USA) and transformed into competent *Escherichia coli* strain JM-109 using InsTAclone PCR cloning kit (Fermentas, USA) following manufacturers' instructions. Three recombinant clones identified by PCR and restriction endonuclease digestion were sequenced (from both the ends) at the automated DNA sequencing facility available at Genei, Bangalore, India. MSA were made using Clustal X (1.81) and per cent sequence identities were determined using Bio-Edit program version 5.0.9. Sequence phylogram was constructed by Neighbor-Joining (NJ) method (Bootstrap analysis with 1000 replicates) in Clustal X (1.81) and rooted trees were generated using TREEVIEW software (Win 32) version 1.6.6 (Page 1996). The CP nucleotide and their deduced amino acid sequences of BYMV strains under comparison were obtained from GenBank (Benson et al. 1999). BLAST program, Altschul et al.

(1997), was used to identify related sequences available from the GenBank. Strains of BYMV infecting vanilla in Reunion were not included in study as they contained sequences pertaining only to the core region of CP.

Results and discussion

Of the 30 infected vanilla plants tested by RT-PCR, two plants from Madikeri, Karnataka, exhibiting severe yellow mosaic and dark green islands on the leaves followed by leaf distortion were found positive for BYMV. The mosaic seen on young leaves of infected plants eventually turned yellow leading to the necrosis (Figure 1). The ~950 bp product corresponding to partial NIb and CP of this isolate was cloned, sequenced and deposited in GenBank (Accession No. EJ752701). The sequence contained 957 bases of which the first 174 bases correspond to the 3′ end of NIb while the remaining 783 bases belonged to the CP gene which potentially codes for 261 amino acids. In general, the full length CP gene of BYMV consists of 818 nucleotides coding for 272 amino acids. Thus, the present sequence lacks 35 bases coding for 11 amino acids at the C-terminus of the CP. The corresponding partial CP sequence was compared with the available BYMV strains from India and a few other strains from other parts of the world.

The per cent nucleotide and deduced amino acid identities of the present isolate with strains of BYMV from different geographical locations under comparison ranged from 79.8 to 98.5% and 85 to 97.7%. Maximum nucleotide (98.5%) and amino acid (97.7%) identities were shared by the strain S22N (AB029435) infecting gladiolus from Japan while the least nucleotide (79.8%) and amino acid (85%) identities were observed with two isolates infecting pea (S71232) and *Melilotus indicus* (X81124) from Australia. The percent nucleotide and amino acid sequence identities of the present isolate with BYMV sequences from India ranged from 80 to 97% and 86.9 to 95.4%. Maximum nucleotide and amino acid sequence identity of 97% and 95.4% was observed with VM0 (AY845011) and VM23 (AY845012) isolates of BYMV both infecting *V. planifolia* (unpublished) while the least identity of 80% and 86.9% was with P242 (AB041971) infecting gladiolus.

Figure 1. Vanilla plant infected with BYMV showing severe yellow mosaic and leaf distortion.

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Results on partial CP-deduced amino acid MSA revealed a highly variable N-terminus region that had maximum sequence heterogenicity while the core region was much conserved (Figure 2). The stretch of 261 amino acids contained a conserved 'NAG' motif (associated with aphid transmissibility) at positions 7–9. The sequence also contained two other conserved motifs, 'MVWCIEN' at positions 123–129 and 'QMKAAA' at positions 226–231. Except for the isolate/strains MI, M-1 and Lcbk, the 'MVWCIEN' was found to be conserved in all the BYMV strains taken for comparison while the 'QMKAAA' motif was seen in all the strains of BYMV under comparison. The deduced CP amino acid distances of 23 strains of BYMV and the present isolate (VP) were used to generate phylogenetic tree. BYMV infecting vanilla in India (VP) formed a cluster with strains 'S22N' and 'Msdvl' followed by the other strains such as 'B33', 'No.4', 'Hgz', 'VM0' and 'VM23' (Figure 3) while strains 'Palm' and 'Frs' from India were distantly related.

Grisoni et al. (2006) reported the occurrence of BYMV on *V. tahitensis* from Re Union Islands based on sequence relationship of a 327 bp fragment of the core region of CP gene. Sequence of N-terminal domain of CP differs markedly between individual viruses of Potyviridae, whereas the central core and C-terminal domain

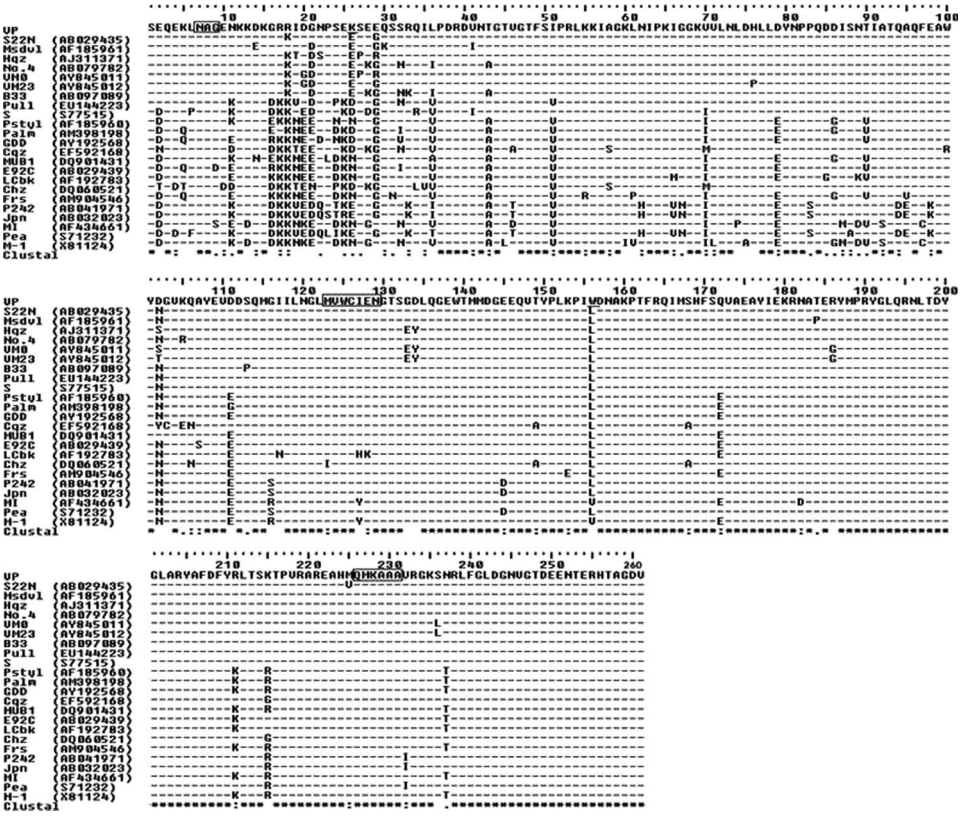

Figure 2. Multiple sequence alignment of the sequence of partial CP gene of BYMVinfecting vanilla in India (VP) with isolates/strains of BYMV from different geographical locations of the world. The sequences were aligned using Clustal X algorithm and the alignments were viewed using BioEdit Program V. 5.0.9. Nucleotide identities with reference to VP (BYMV-VP) are indicated by a '*'. Conserved sequences are shown in rectangular box.

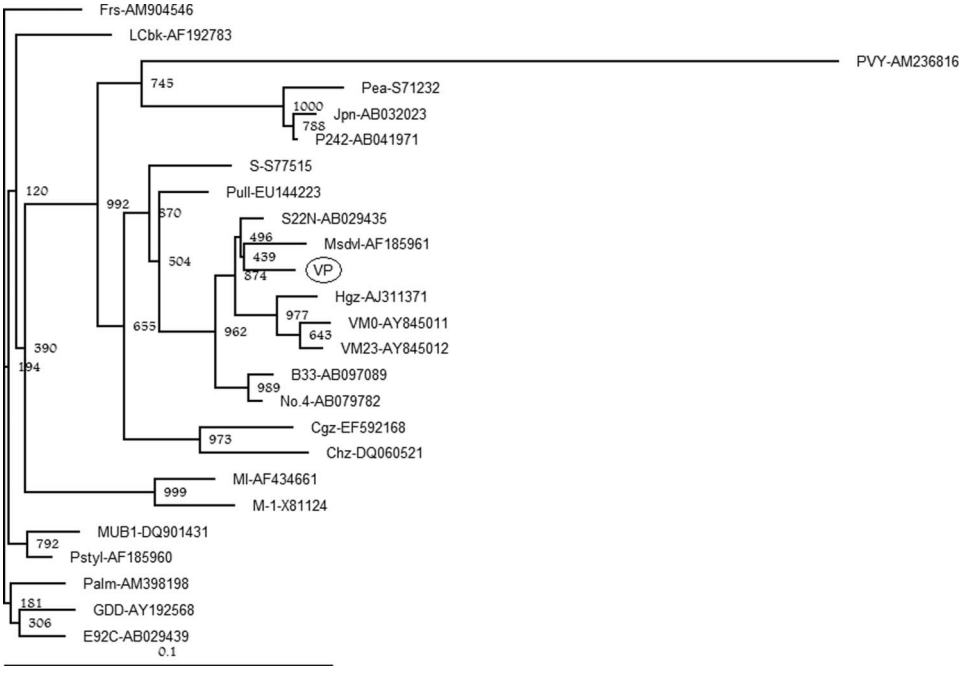

Figure 3. Bootstrapped Neighbor Joining (NJ) phylogenetic tree constructed based on the deduced partial coat protein (CP) sequence of BYMV isolate on vanilla in India (BYMV-VA) (shown in a circle) and strains/isolates of BYMV from different geographical locations of the world. Bootstrap values are given at the nodes and PVY was used as an outgroup.

were highly conserved (Shukla and Ward 1988). Hence, the exact identification of the potyvirus based on sequence of core region of CP could be unreliable (Bos 1992; Zettler 1992). In the present study, sequence of CP including N-terminus could identify the virus as a strain of BYMV. The sequence analyses of the present isolate showed its closeness to BYMV isolate infecting gladiolus from Japan rather than BYMV infecting vanilla in India indicating the existence of variations in the BYMV isolates infecting vanilla. The results also revealed that the clustering of present BYMV isolate was independent of the host and geographical location. Analyses of CP gene sequence of large number of BYMV isolates from different hosts and regions and geographical locations underscore the importance to study the origin and variability that exists in BYMV isolates from India and elsewhere.

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