ORIGINAL PAPER

Molecular phylogeny and secondary structure analysis of hop stunt viroid (HSVd) associated with Mulberry (*Morus alba*) in India

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Abstract

Hop stunt viroid (HSVd), a small, single stranded, circular, non-coding infectious RNA known to cause infection in various economically important crop plants. In the present investigation, a study was conducted in the southern part of Karnataka districts of India to detect the possible association of HSVd infection in mulberry plants. A total of 41 mulberry plants showing typical viroid-like symptoms along with asymptomatic samples were collected and screened using conventional Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) using a specific set of HSVd-Fw/ HSVd-Re primers. Out of 41 samples, the study confirmed the presence of HSVd in six samples of mulberry collected from Ramanagara (1 sample), Chikkaballapur (3 samples) and Doddaballapura (2 samples) regions with an expected HSVd amplicon size of ~290–300 nucleotides. The mechanical transmission of HSVd was also confirmed on cucumber (cv. Suyo) seedlings through bioassay, which was reconfirmed by RT-PCR. The amplicons were cloned, sequenced, and the representative nucleotide sequences were deposited in the NCBI GenBank. Subsequently, molecular phylogenetic analysis showed that HSVd mulberry isolates from this study were most closely related to grapevine isolates, indicating a common origin. On the other hand, it was shown to belong to a different group from mulberry isolates so far reported from Iran, Italy, Lebanon, and China. The secondary structure analysis of HSVd mulberry Indian isolates exhibited substitutions in the terminal left, pathogenicity, and variable regions compared to those of the Indian grapevine isolates. As far as this study is concerned, HSVd was detected exclusively in some mulberry plants with viral-like symptoms, but the pathogenesis and symptom expression needs to be further investigated to establish the relationship between HSVd and the disease symptoms in the mulberry plants.

Keywords Reverse transcription PCR · Hop stunt viroid · Mulberry · Bioassay · Secondary structure analysis

Introduction

Viroids are the smallest known autonomously replicating infectious plant pathogens, consisting of circular, covalently closed, single-stranded small RNA molecules whose genome length varies between 220 and 450 nucleotides (nt) (Lee et al. 2022). Some viroids have a wide host range that systemically induce disease on many agricultural and horticultural crops (Diener 2001; Ding 2009). *Hop stunt viroid* (HSVd) is a member of the family *Pospiviroidae* and belongs to the genus *Hostuviroid*, consisting of 294–309 nt and was first reported from hops in Japan (Sasaki and Shikata 1977b; Hataya et al. 2017; Di Serio et al. 2021). HSVd has the most diverse host range, having been detected in a wide variety of woody and herbaceous plants and has been frequently pathogenic to a certain sensitive host varieties (Sasaki and Shikata 1977a; Yang et al. 2007; Hataya et al. 2017). The symptomology of HSVd infection on different host plants include leaf curling, vein thickening, clearing and banding, leaf and fruit deformation, yellow speckle or dappling on fruit and stunted growth (Sano et al. 1989; Yang et al. 2007; Hataya et al. 2017). The infection caused

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by HSVd appears to be latent in most of the hosts studied (Polivka et al. 1996; Sano 2013). Several viroid infections have been reported in India, of which PSTVd on potato and tomato, CEVd on citrus, CSVd and CChMVd on chrysanthemum, CLVd on *Nematanthus* and grapevine associated AGVd, HSVd and GYSVd (Mishra et al. 1991; Singh et al. 2010; Adkar-Purushothama et al. 2013; Singh and Kaur 2014; Jeevalatha et al. 2015; Kumar et al. 2015; Walia et al. 2021; Shilpa et al. 2022).

Hop stunt viroid is known to infect a broad range of natural hosts and thus, pose a potential threat to agricultural crops worldwide. It was first identified in hop plants showing abnormal dwarfing of bines and hence termed as hop stunt disease (Sasaki and Shikata 1977b). The disease was first emerged in Japan during the 1950s and became the biggest concern for hop growers in the 1970s, causing huge economic losses (Sano 2013). Since then, hop stunts have also been epidemic in the United States (Eastwell and Nelson 2007), China (Guo et al. 2008) and Slovenia (Radišek et al. 2012). Apart from hops, HSVd has a wide host range, infecting cucumber, fruit bearing citrus, malus, prunus, plum, peach, apricot, sweet cherry, apple, fig and *Vitis* spp. including flower bearing *Hibiscus* spp. (Hataya et al. 2017; Marquez-Molins et al. 2021) and recently reported on economically important mulberry plants from Italy, Lebanon, Iran and China (Elbeaino et al. 2012; Lu et al. 2023). Almost symptomless in many of these host species, HSVd infection causes specific diseases on wide varieties of cucumber, some sensitive cultivars of plum and peach, and specific variants have been reported to cause cachexia disease on some sensitive citrus varieties (Semancik et al. 1988).

Mulberry (*Morus alba*) is one of the most economically important perennial cash crops cultivated in India and is cultivated in 242 thousand hectares with an annual production of 24,000 MT in 2021 as the source of silkworm feed (Anonymous 2021). In India, the state of Karnataka is the leading raw silk producer, with 11,000 MT of raw silk produced in fiscal year 2022 to feed silkworms (Anonymous 2022). Recently, HSVd infection has been reported from mulberry in Italy, Lebanon, Iran and China (Elbeaino et al. 2012; Amiri Mazhar et al. 2014; Lu et al. 2023). In our preliminary investigations on mulberry plants showing typical viral-like symptoms found in different mulberry growing regions of Karnataka state of India during 2020–2022, HSVd was successfully detected from some symptomatic plants (Shilpa et al. 2023). Hence, the present investigation was undertaken to characterize HSVd in Indian mulberry by employing molecular phylogenetic and secondary structure analyses along with the sequencing of a new isolate.

Materials and methods

Chemicals

All the chemicals used during the study were of molecular biology grade, which were procured from Hi-Media, India, viz., CTAB, lithium chloride, NaCl, EDTA, 2-mercaptoethanol, carborundum-600 meshes, agarose, ethidium bromide, TAE buffer, bentonite solution, phenol, chloroform, ethanol, nuclease-free water, DNase I, and DTT. dNTPs and random hexamer DNA were procured from Thermo-Scientific, India.

Collection of viroid samples

To investigate the presence of HSVd in mulberry plants, a field survey was conducted from December 2020 to April 2022 in many mulberry growing regions of Bangalore rural, Chamrajanagara, Chikkaballapur and Ramanagara districts of Southern Karnataka (India). Mulberry leaf samples (41 samples) displaying virus-like symptoms such as vein banding, leaf curling, yellow speckle and leaf deformation were collected along with asymptomatic samples. The collected samples were thoroughly screened for HSVd infection using RT-PCR and bioassay methods.

Total RNA extraction

To extract small RNA from infected and healthy leaf samples, 2× CTAB-based 4 M lithium chloride (LiCl) precipitation method was employed as described in our previous studies (Shilpa et al. 2022). About 100 mg of symptomatic and healthy mulberry leaf tissues were ground into a fine powder in a sterile mortar and pestle using liquid nitrogen and was homogenized with an appropriate volume of preheated 2× CTAB buffer [0.1 M Tris–HCl (pH 9.5), 1.4 M NaCl, 0.02 M EDTA (pH 7.0), 2% CTAB, and 0.05% 2-mercaptoethanol]. To the homogenate, an equal volume of phenol: chloroform (1:1) was added and nucleic acid was extracted, followed by centrifugation at 13,000 rpm for 5 min. at 4 °C. The aqueous phase was collected and total nucleic acids were precipitated by adding 2.5 volumes of 99.5% ethanol, incubated overnight at -30 °C, and centrifuged at 13,000 rpm for 10 min. The pellets were air-dried at room temperature and re-suspended in nuclease-free water, followed by the addition of an equal volume of 4 M LiCl with gentle shaking and allowed to stand on ice for 4 h. High molecular weight RNAs were precipitated by centrifugation at 13,000 rpm for 10 min., and 4 M LiCl soluble nucleic acids were recovered by ethanol precipitation from the resultant supernatant, followed by DNase I treatment for 30 min. at 37 °C. Low molecular weight- RNA

(LMW-RNA) was ethanol precipitated by centrifuging at 13,000 rpm, and the resultant residues were washed with 70% ethanol and air-dried. Finally, the obtained RNA pellets were suspended in 100 µl of nuclease-free water. Further obtained RNA was quantified using NanoDrop Lite Spectrophotometer (Thermo Scientific™, USA) and stored at -20 °C for further analysis.

Reverse transcription (RT)

The first-strand complementary DNA was synthesized as described in our previous studies in a 25 µl total reaction mixture volume (Shilpa et al. 2022). Where 3 µl (2 µg) of purified RNA sample containing 1 µl dNTPs (10 mM) and 1 µl random hexamer (100 µM) was prepared (total volume of 5 µl) and used for the preparation of RT-solution. The reaction mixture was thoroughly mixed and incubated for 5 min. at 95 °C on a Thermal Cycler (Applied Biosystems, USA) and immediately chilled on ice for 5 min. Approximately 5 µl of denatured RNA was added to cDNA synthesis reaction mixture containing 1 µl M-MuLV reverse transcriptase (200 U/µl) (Invitrogen, Carlsbad, CA, USA), 5× first strand buffer (2 µl), 0.1 M DTT (2 µl), 1 µl RNase inhibitor (20U/µl) (Thermo Scientific™, USA) and final volume was made up with nuclease free water (14 µl). The RT reaction was incubated for 60 min. at 37 °C. After incubation, the reaction mixture was heated at 72 °C for 5 min. on a Veriti 96-Well Thermal cycler (Applied Biosystems, USA), chilled on ice for 2 min. to inactivate the reverse transcriptase. The reaction mixture without template RNA served as a negative control.

RT-PCR amplification and cloning

To detect HSVd, a conventional PCR was performed using a resultant RT-solutions (2 µl) in a 25 µl PCR reaction mixture. The PCR master mixture containing 2 units of Taq DNA Polymerase (0.3 µl) (Takara Bio, Shiga, Japan), 10×Taq PCR buffer (2.5 µl), 2 mM dNTP (2.5 µl), 25 mM MgCl$_2$ (2.5 µl), and 20 pmol each (1 µl) of HSVdF1-5'-GGGGCAACTCTTCTCAGAATCC-3' and HSVdR1-5'-GGGGCTCCTTTCTCAGGTAAGTC-3' (Bernad and Duran-Vila 2006) and remaining volume was made up with nuclease free water (13.2 µl). Similarly, samples were also prepared from healthy and symptomatic mulberry plants. The reaction mixture was thoroughly mixed before carrying out PCR in a thermal cycler with the following PCR cycling profile: initial denaturation at 95 °C for 4 min., followed by 35 cycles of denaturation at 94 °C for 1 min., annealing at 55.3 °C for 1 min., and extension at 72 °C for 1 min., and final extension was performed at 72 °C for 10 min. The amplified PCR products were separated by 1.5% agarose gel

electrophoresis with ethidium bromide (0.5 µg/ml), in 1× TAE buffer at 100 V for 35 min., and visualized under UV transilluminator using Gel Documentation apparatus (Major Science, USA). The amplified PCR products were purified using a GeneJET Purification Kit (Thermo Scientific, USA) following manufacturer's instructions. Further, the purified DNA fragments were cloned into pGEM®-T Easy vector (Promega, Madison, USA), and a bacterial transformation process was performed following manufacturer's protocol.

Biological indexing of HSVd

Biological indexing was performed to confirm the pathogenicity of HSVd on cucumber (*Cucumis sativus*, cv. 'Suyo') indicator plants. The 100 ng of small RNAs (inoculum) from HSVd-positive and healthy mulberry plants were prepared by homogenizing with 0.1 M sodium phosphate buffer (pH 7.0) supplemented with bentonite (1 µg/ ml) (Sano et al. 2004). Inoculations were carried out on 25 days-old three cucumber seedlings at the two-leaf stage by gentle rubbing with 50 µl of the LMW-RNA inoculum on each leaflet, which was previously dusted with carborundum-600 meshes (HiMedia) in greenhouse conditions (Verhoeven et al. 2010). The inoculated leaflets were left for 2 min., and gently washed with sterile distilled water. Cucumber seedlings inoculated only with sterile distilled water served as control. All the inoculated seedlings were maintained under controlled conditions (25 -30 °C) to screen the infection by HSVd, and the experiments were repeated twice. The HSVd infections in all the symptomatic cucumber plants were further confirmed by RT-PCR product and sequencing.

Sequencing and phylogenetic analysis

The amplified PCR and cloned DNA products were sequenced in an automated DNA sequencing system (Applied Biosystems, ABI Prism 310, USA) by the Sanger sequencing method. The obtained sequences were aligned and analyzed using BioEdit sequence alignment editor (version 7.2.6.0) and ClustalW software. Further, the sequences were subjected to nBLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and analysis using the NCBI-BLAST program to compare with the related nucleotide sequences available in the NCBI GenBank. The obtained consensus nucleotide sequences were assembled and compared for similarities to identify the closest match. A phylogenetic tree was constructed with the representative homologous viroid sequences retrieved from the NCBI GenBank (https://www.ncbi.nlm.nih.gov/) employing the Neighbor-Joining method (NJ) of MEGA 11 version (Tamura et al. 2021) with 500 bootstrap replications to assess the reliability of the constructed evolutionary tree. The evolutionary distances were

calculated using the Kimura-2-parameter method (Kimura 1980).

Secondary structure analysis of HSVd

The sequences of isolated HSVd (Acc. No. OP313761) and representative HSVd available in GenBank (Acc. No. AB742225) were obtained, and multiple sequence alignments were performed using the ClustalW program. The possible secondary structure was predicted to identify the variations and mismatches at the central conserved, terminal, variable and pathogenicity regions. In addition, the base-paring probabilities were also predicated among the aligned sequences using RNA structure Version 6.3- Mathews Lab Computational Biology of RNA (https://rna.urmc.rochester.edu) and Mfold (Zuker 2003) with the lowest free energy at a folding temperature of 37 °C and MFOLD (circular version) program from the GCG package and RNA viz. (Zuker 1989) for prediction of the secondary structure of HSVd.

Results

HSVd symptoms and molecular detection through RT-PCR

During the study period, samples were collected from mulberry plants that showed typical virus-like symptoms, such as leaf curling, yellow speckle, yellow mottling, vein thickening, clearing and banding, leaf deformation and stunted growth (Fig. 1). Of the 41 symptomatic mulberry leaf samples from different locations (Fig. 2A and B), six from Ramanagara (1 sample), Chikkaballapur (3 samples) and Doddaballapura (2 samples) regions tested positive for HSVd with an expected amplicon size of ∼290-300 nt for HSVd (Fig. 2C). The HSVd positive samples showed severe symptoms like yellow spots, leaf curling with leaf rugosity, leaf thickening along with stunted growth. However, none of the other asymptomatic samples showed amplicons for HSVd. Three individual samples, each taken from a different location at the sample collection site, were selected, and the amplified full-length cDNA fragments were recovered from gel purification for further cloning and sequencing analysis.

Biological indexing

After 6-7 weeks post-inoculation, all three small RNA inoculated cucumber plants displayed typical HSVd symptoms such as leaf curling, stunted growth, yellowing, and thickening. Whereas water-inoculated cucumber plants remained asymptomatic and tested negative even after 7 weeks of post-inoculation under greenhouse conditions (Fig. 3). Bioassay results were confirmed by RT-PCR, samples inoculated using Ramanagra, Doddaballapura and Chikkaballapura LMW-RNAs showed an expected amplicon size of ∼290300 nt by confirming the presence of HSVd.

Sequencing and phylogenetic analysis

A clone from each positive isolate (∼290–300 nt) was selected and sequenced from both directions to confirm HSVd infection. The nBLAST analysis revealed the presence of HSVd in mulberry by showing 99.0% homology with HSVd sequence from *Vitis* spp. isolated from Russia (Acc. No. OL799308), China (Acc. No. AB219944), followed by 98.7% homology with the Indian isolate of grapevine (Acc. No. AB742225), Korean isolate of Japanese apricot (Acc. No. KY445746) and Chinese isolate of Japanese plum (Acc. No. EF076834). A similar co-efficient of 98.0% identity with HSVd infecting hop (Acc. No. AB039270) and peach fruit (Acc. No. D13765) in Japan was observed in the study. The assembled nucleotide sequences of HSVd isolates obtained in this study and also in our previous investigation (Shilpa et al. 2023) and the details of the accession numbers of the deposited sequences of the present work along with reference sequences, are provided in Suppl. Table 1. The obtained representative consensus sequences were deposited in NCBI GenBank with the Acc. No. OP313761, OP313762 (Shilpa et al. 2023) and OQ784657 (Suppl.Table 1). The sequence of OP313761 and OQ784657 was 100% matched with each other, but OP313762 was one nucleotide difference at position 86, however, this variation was considered to be an artifact because it was located within the PCR primers used.

The obtained representative nucleotide sequences (Acc. No. OP313761, OP313762 and OQ784657) were considered for constructing a phylogenetic tree. The HSVd sequences from various hosts among the world were selected from the NCBI database. To simplify the tree, closely related or representative sequences of the HSVd isolates were considered along with previously deposited sequences of HSVd from grapevine and citrus in India. The phylogenetic analysis among these HSVd sequences from mulberry, grapevine, hop, citrus, peach and others, including the present sequences, formed several distinct clades. Detailed inspection revealed that the Indian mulberry sequences belong to the grapevine group, which authenticates the close evolutionary relationship but differs from those isolated from mulberry in Italy, Lebanon, Iran, and China (Fig. 4).

Secondary structure analysis

The MultAlin analysis of HSVd (Acc. No. OP313761) sequence assembled with closely related previously reported
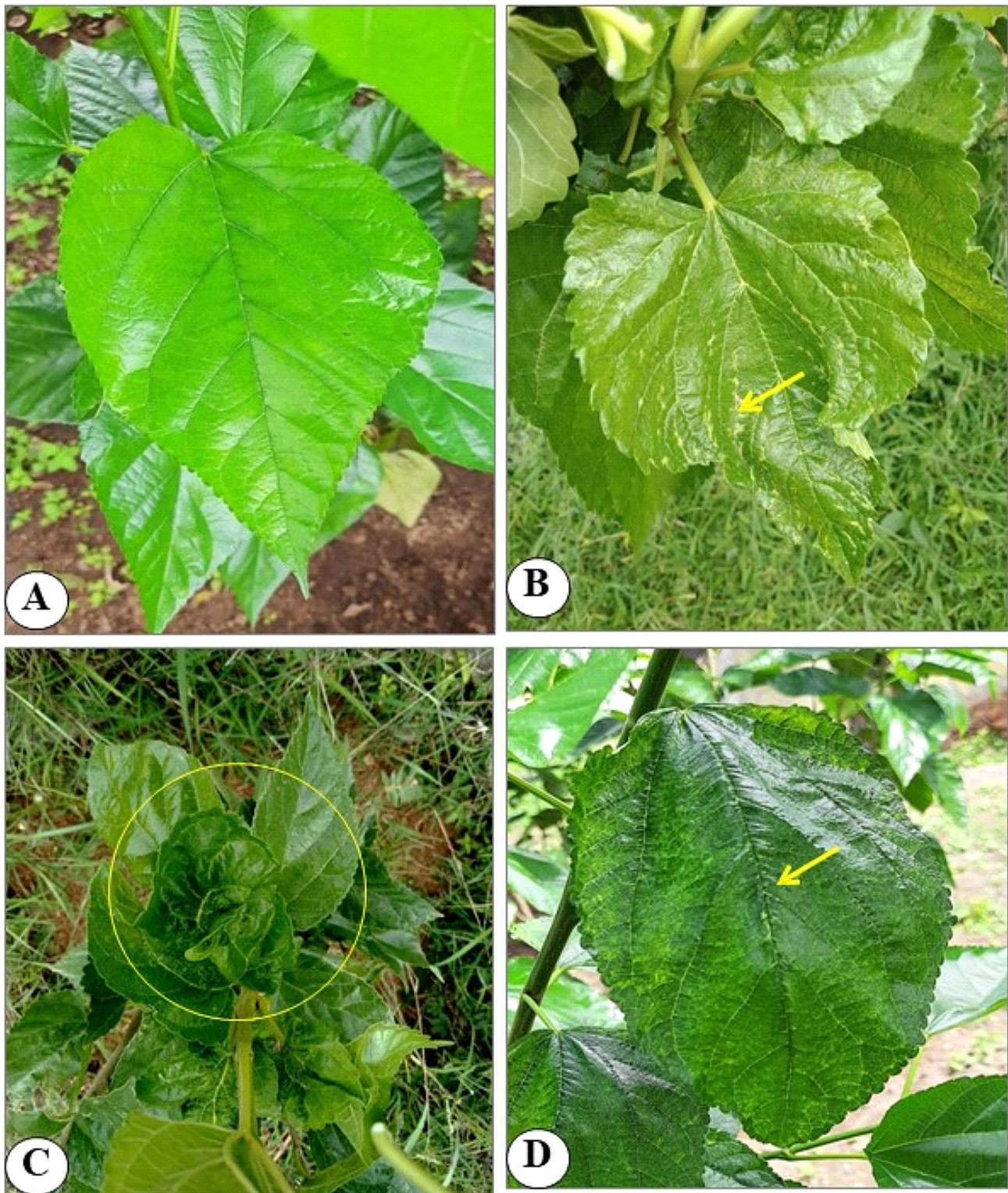
Fig. 1 Mulberry leaf samples showing viral-like symptoms. **A**: Healthy leaf; **B**: Yellow spots; **C**: Leaf curling with leaf rugosity; **D**: Leaf thickening

Fig. 2 Amplification of HSVd (∼290-300 nt) from mulberry samples showing amplified products on 1.5% agarose gel. **A–B**: Representative mulberry samples showing symptoms of yellow spots and vein banding; **C**: Lane M: 100 bp marker; Lane 1–4: Chikkaballapur sample; Lane 5–7: Dodaballapura sample; Lane 8–9: Ramanagara sample; Lane 2,5 and 8: Asymptomatic sample from represenrive regions (Control)
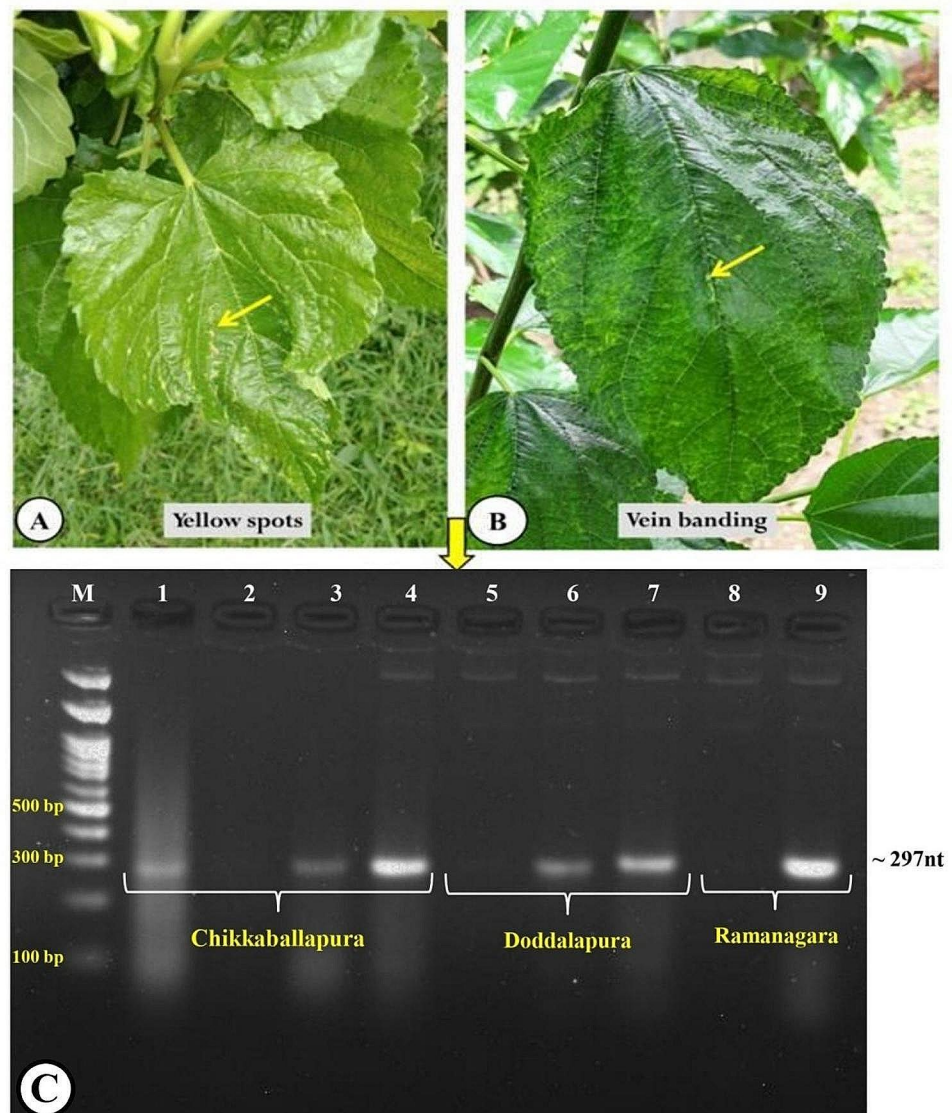

representative Indian isolate of HSVd sequences from grapevine species (Acc. No. AB742225) revealed that there were not many differences among the nucleotide sequences studied. The secondary structure analysis by mfold thermodynamically predicted a rod-shaped structure that fit well with a molecular model consisting of five structural domains (Fig. 5A). The predicted structure revealed the presence of 27 loops with a slight difference compared to the reference structure predicted from an Indian grapevine sequence (Acc. No. AB742225; Fig. 5B) (Sahana et al. 2013). It was also noted that the HSVd from mulberry isolate showed 4 substitutions in the positions 58 ($U_{58} \rightarrow G$), 126 ($C_{126} \rightarrow U$), 253 ($C_{253} \rightarrow U$) and 272 ($U_{272} \rightarrow C$) and shared PWIS of 98.7%. Of them, a $U58 \rightarrow G$ transversion closed an internal loop and a $U272 \rightarrow C$ transition enlarged the other internal loop in the predicted secondary structure. The other two substitution do

not seem to cause structural changes between HSVd isolate from mulberry and grapevine in India.

Discussion

Hop stunt viroid is known to infect a wide range of natural hosts, making it a potential hazard to agricultural crops globally. In India, HSVd has been detected in grapevine (Sahana et al. 2013) and citrus (Roy and Ramachandran 2003). However, HSVd infection on mulberry has not been diagnosed and reported until recently (Shilpa et al. 2023). During the study, the preliminary RT-PCR identified the HSVd infection for the first time in six mulberry samples collected from various fields of Karnataka state of India. Besides, the sequence data of the same confirmed the HSVd association with mulberry with a high percentage homology
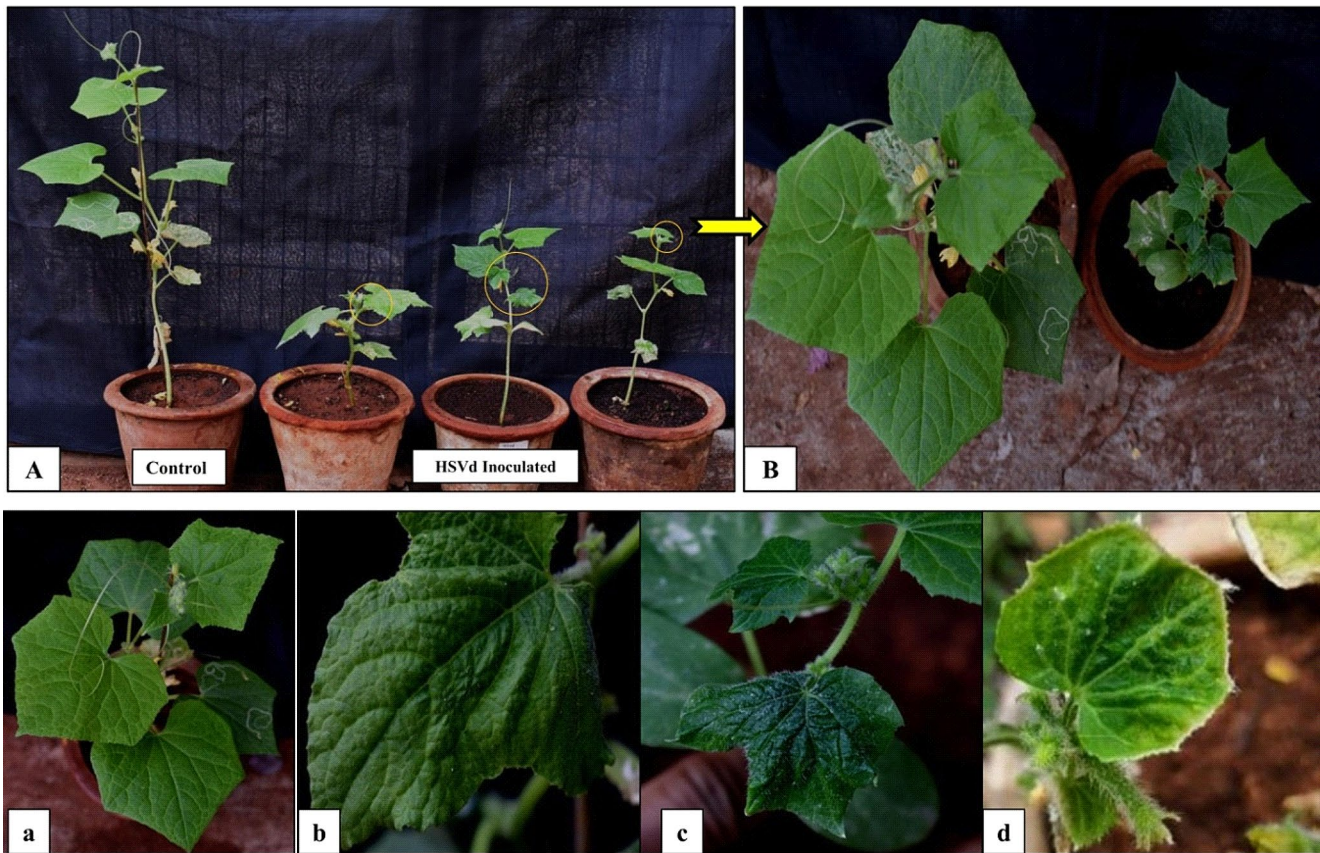
Fig. 3 Bioassay studies with mulberry-HSVd on indicator plant. **A–B**: Inoculated and healthy cucumber indicator plants under greenhouse conditions; **a**: Healthy plant; **b–d**: HSVd inoculated plants exhibiting symptoms of stunted growth, curling and small leaves with yellow spots

to HSVd-grapevine, isolated from Russia, China, India (Sahana et al. 2013; Singhal et al. 2019) and worldwide. The phylogenetic analysis of the HSVd Indian mulberry isolates showed an evolutionary relationship with those from the grapevine, plum, peach, and hop reported from various countries, including India; i.e.,"grapevine"or "grapevine-hop" group (Amari et al. 2001; Sano et al. 2001). In accordance, the results were found somewhat different from those reported from Italy–Lebanon, Iran, and China, wherein mulberry isolates which clustered together with those of "citrus" group, "plum" group, and "hop" group, respectively (Elbeaino et al. 2012; Amiri Mazhar et al. 2014; Lu et al. 2023). These results indicate that the HSVd mulberry isolates reported so far from Italy-Lebanon, Iran, China, and now from India belong to distinct groups depending on the region and do not show host specificity. Incidentally, while the PWIS between Indian mulberry and Indian grapevine was 99% (4 nucleotides differences), those between Indian mulberry and Italian-Lebanese mulberry, Iranian mulberry, and Chinese mulberry was ∼95.7% (13–16 nucleotides differences), 89.0% or less (33 or more nucleotides differences), and ∼94.6% (17–18 nucleotides differences), respectively (Fig. 4). In spite of the high similarlity of

the nucleotide sequence, the secondary structure analysis revealed the presence of 27 loops in the Indian mulberry isolate, which was apparently different in two loops located in the TL and P domains, both responsible for the pathogenicity, compared to those of Indian grapevine, suggesting a close relationship between the two isolates but might have a possible difference in the pathogenicity.

Amiri Mazhar et al. (2014) specifically detected HSVd from mulberry trees exhibiting vein-clearing and yellow speckle symptoms and showed that the mulberry disease in question is transmitted from mulberry to mulberry by grafting the buds of the diseased tree onto the mulberry seedlings and by mechanical inoculation of the mulberry seedlings with the sap (but not purified preparation) from the diseased leaves. Since HSVd was detected again from inoculated seedlings, they concluded that vein clearing and deformation of mulberry leaves is a transmissible disease associated with HSVd, the involvement of other viral infectious agents cannot be completely excluded. In this study, HSVd was also detected only from samples showing severe viral-like symptoms, although the detection frequency was not very high. The mechanical inoculation experiment confirmed HSVd infection in indicator cucumber (cv. Suyo) plant

Fig. 4 Molecular phylogenetic relationships of HSVd-mulberry isolates from India in this study, mulberry in Italy, Lebanon, Iran, and China, and major HSVd isolates from various crops reported so far. Mulberry isolates detected in this work are marked with a black star, and mulberry isolates in other countries are marked with a white star. Five representative groups [P-C, P-H/Cit3 (Amari et al. 2001), plum, citrus (Hadidi et al. 2017), grapevine-hop (Sahana et al. 2013) found in HSVd isolates are shown. The number of nucleotide differences and PWIS (pairwise identity scores) between mulberry isolates from India and those from Italy, Lebanon, Iran, China, and grapevines are shown in the right side of the figure
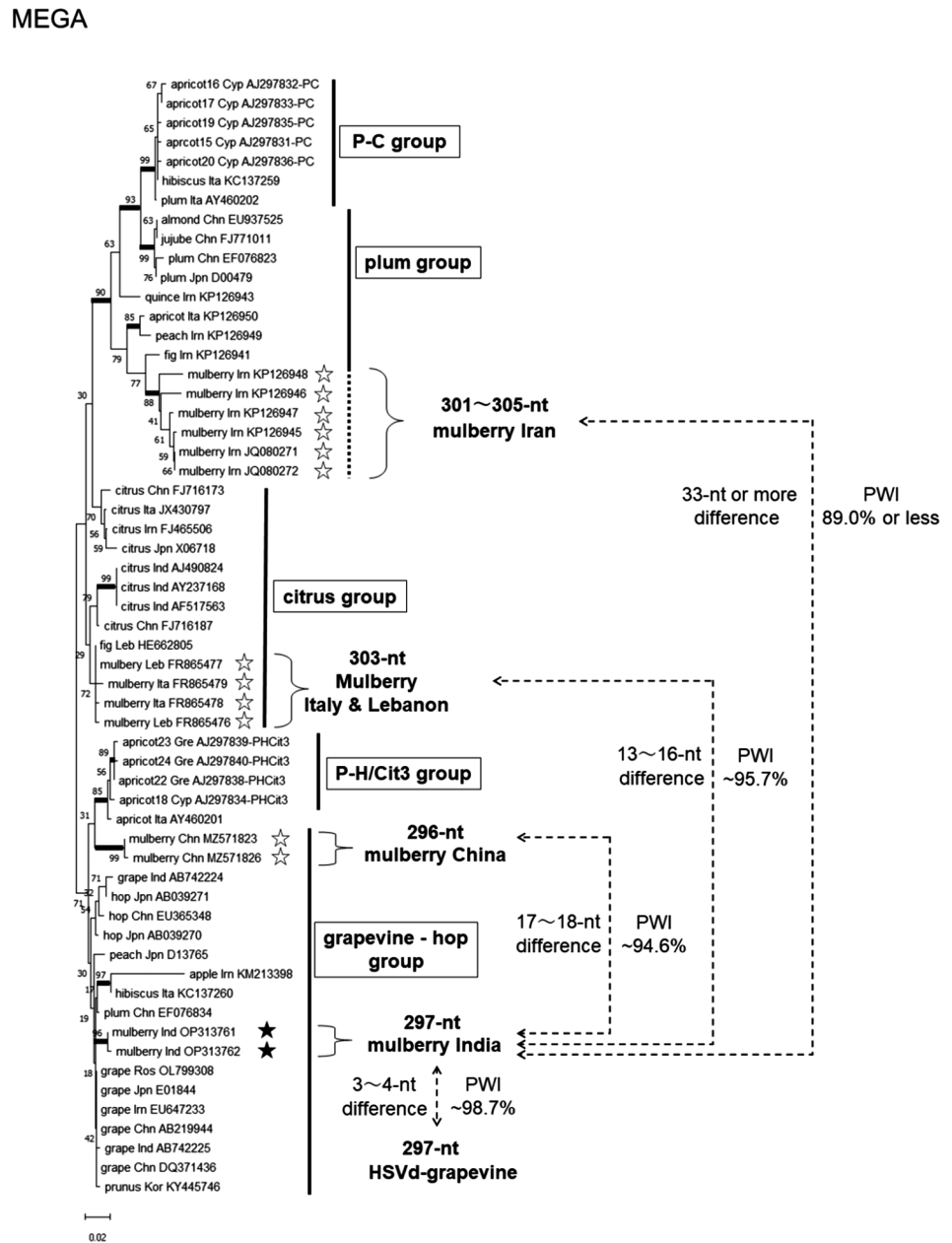
with the expression of typical HSVd-inducing symptoms. It is essential to analyze the relationship between HSVd and viral-like symptoms on mulberry trees in India by conducting infection experiments using mulberry seedlings. Studies also showed that agricultural tools could transmit HSVd during trimming, mechanical stripping, and grafting of infected scions (Hadidi et al. 2022). Since the outbreak, HSVd-associated crops have become widespread in recent years, they often remain symptomless but can act as potentially dangerous viroid-reservoir crop plants. Since mulberry is an important cash crop for feeding silkworms and now found as a new natural host for HSVd in India, hence,

further analysis of its origin and potential role as a source of infection for other horticultural plants is important. Further investigation is also required to find out the exact source of the HSVd infection in mulberry and also the mechanism of transmission from source plant(s) to mulberry or from mulberry to mulberry, as it was observed that nucleotide sequence divergence suggests HSVd infection in mulberry is not origined from single host but varies by the regions observed, in other words, it may be significantly influenced by the surrounding crop environment.

A. HSVd in mulberry plant from this study Karnataka, India (GenBank Acc No: OP313761.1)

B. HSVd in grapevine in India (GenBank Acc No: AB742225)

terminal left   pathogenicity   central   variable   terminal right

Fig. 5 Predicted secondary structures of the HSVd isolate of the present study along with reference sequences predicated through RNA structure 6.0. **A**: Secondary structure of HSVd isolates (Acc. No. OP313761) from this study; **B**: Secondary structure of previously reported Indian HSVd reference sequence (Acc. No. AB742225)

Declarations

Competing interests The authors declare no competing interests.

Conflict of interest The authors declare no conflict of interest.

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