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ORIGINAL ARTICLE

Molecular evidence of *Apple stem grooving virus* infecting *Ficus palmata*

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Abstract

Key message **The present study identified *Ficus palmata* as a new natural host of *Apple stem grooving virus*.**

Abstract *Ficus palmata*, growing in the campus of CSIR-IHBT, was found to exhibit virus-like symptoms which include necrotic and chlorotic spots, chlorosis, leaf deformation and marginal chlorosis. Surveys were conducted in three different areas of district Kangra; leaf samples from symptomatic and asymptomatic plants were collected and subjected to DAS-ELISA, NASH and RT-PCR. Combined results of the three detection techniques revealed the presence of ASGV in 15/30 tested samples, thereby confirming the presence of ASGV in 50% of the samples. Out of the 15 positive samples, 5 randomly selected samples were confirmed at the molecular level through sequencing of the partial replicase gene of ASGV and complete CP gene sequences were used for characterization and phylogenetic analysis. Further, all the five positive samples were also analyzed for any probable mixed infection of major apple viruses, viz., *Apple stem grooving virus* (ASGV), *Apple chlorotic leafspot virus* (ACLSV), *Apple stem pitting virus* (ASPV) and *Apple mosaic virus* (ApMV) by multiplex RT-PCR. The results revealed that the samples were positive only for ASGV. The phylogenetic analysis based upon the CP gene revealed that the five characterized isolates (Fi-15, Fi-16, Fi-19, Fi-20 and Fi-29) were grouped into two separate clusters and shared 88.5–100% (nt) and 95.3–100% (aa) sequence identity among themselves. Out of the five, four isolates (Fi-15, Fi-19, Fi-20 and Fi-29) were 100% identical to each other, while they shared 88.5 and 95.3% sequence identity with the fifth isolate (Fi-16) at nucleotide (nt) and amino acid (aa) levels, respectively. Further among the five isolates, the variant Fi-16 isolate was used for mechanical inoculation on herbaceous hosts. Mechanical inoculation studies of the variant Fi-16 isolate revealed that this isolate could be successfully transmitted mechanically to *Chenopodium amaranticolor, Cucumis sativus, Chenopodium quinoa, Phaseolus vulgaris, Nicotiana benthamiana and Nicotiana glutinosa*. To the best of our knowledge, this is the first report of *F. palmata* as a new natural host of ASGV.

Keywords ASGV · *Ficus palmata* · Host range · DAS-ELISA · NASH · RT-PCR

Introduction

Ficus commonly known as 'Fig' is one of the largest genera of angiosperms with about 800 species and 2000 varieties of woody trees, shrubs and vines in the family *Moraceae* and is considered one of the most diversified genera with regard to its habitats and life forms (Hamed 2011; Chaudhary et al. 2012). *Ficus palmata* is a deciduous, moderate-sized tree, 6–10 m in height, having a worldwide distribution from northeast Africa—Egypt, Eritrea, Ethiopia, Somalia, and Sudan through Arabia and temperate Asia to northern India, Nepal and Pakistan. In India, it is found in the states of Andhra Pradesh, Bihar, Delhi, Gujarat, Madhya Pradesh, Maharashtra, Rajasthan, Tamil Nadu, Uttar Pradesh, Punjab, Himachal Pradesh, and Jammu and Kashmir thereby

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extending from plains to ~1700 m in the Himalayas and is often cultivated for its fruit (Parmar and Kaushal 1982). *Ficus palmata* also known as 'bedu' (Kumaun, Uttrakhand), 'pheru', 'khemri' (Dehradun, Uttrakhand), 'phegra', 'anjir' (Himachal Pradesh), 'fagu' (Punjab), etc. is the wild relative of Fig-producing tree, whose species *carica* is known for the commercially important Fig (Parmar and Kaushal 1982; Patil and Patil 2011). The overall production of fig in world stands at 1.0×10^6 MT; the country with the largest produce is Turkey followed by Egypt, Algeria, Morocco, Iran, Syria, United States, Brazil, Albania and Tunisia (FAO 2011).

Ficus trees are propagated by grafting or self-rooted cuttings; the agropractices used for grafting and to obtain cuttings for rooting favor the spread of pathogens such as viruses and phytoplasmas. In recent years, several viruses from different families (*Closteroviridae, Bunyaviridae, Flexiviridae, Partitiviridae, Tymoviridae and Caulimoviridae*) have been reported to infect *ficus* (Martelli 2011). *Fig leaf mottle-associated virus*-1 (FLMaV-1) belonging to the family *Closteroviridae* was the first virus found to be infecting the Fig trees (Elbeaino et al. 2006).

Apple stem grooving virus (ASGV), family *Betaflexiviridae*, genus *Capillovirus* is a plus-sense ssRNA, flexuous, filamentous virus, about 600–700 nm in length and 12 nm wide (Lister 1970). ASGV is known to be transmitted by grafting and mechanical inoculations. However, in some cases like *Lilium longiflorum, Malus platycarpa* and *Chenopodium quinoa*, seed transmission is also reported (Van der Meer 1976; Inouye et al. 1979). It is one of the most important latent viruses of apple crop and has the potential to cause serious economic losses (Nemeth 1986; Welsh and van der Meer 1989). Apart from infecting apple, it also infects several other important crops such as *Actinidia, Citrus, Lilium*, Japanese apricot (*Prunus mume*), cherry, pear, and *Nandina domestica* (Lovisolo et al. 2003; Takahashi et al. 1990; Clover et al. 2003; Inouye et al. 1979; Shim et al. 2004; Tang et al. 2010; Bhardwaj et al. 2014).

In sensitive cultivars of apple and pear, ASGV causes stem grooving, necrosis and deformation on top-grafting unions (Motoshima et al. 1983; Nemeth 1986; Yoshikawa 2000), while on other hosts, it causes chlorotic leaf spot, brown line above the graft union, fruit rind intumescence in mandarin, interveinal mottling, leaf distortion, foliar ringspots and chlorosis (Welsh and van der Meer 1989; Yoshikawa 2000; Lovisolo et al. 2003; Clover et al. 2003; Bhardwaj et al. 2014).

Ficus palmata trees were found to be widely distributed in the campus of Council of Scientific and Industrial Research-Institute of Himalayan Bioresource Technology (CSIR-IHBT), Palampur, H.P, India, and a significant number of these trees displayed virus-like symptoms. Based on the symptoms and owing to the widespread presence of *F. palmata* trees in and around the apple germplasm field of this institute [where previously apple cultivars tested positive for ASGV (Accession numbers: FM204881 (Negi et al. 2010); LN627002, LN627003 and LN627004], one of the *Ficus* plants was tested for the presence of ASGV through RT-PCR and was found positive for ASGV infection. Further, to elucidate whether the presence of this virus in *F. palmata* was due to close proximity to infected apple plants or *F. palmata* being a natural host of ASGV, leaf samples were collected from three different areas of district Kangra, Himachal Pradesh (located away from the apple germplasm field) and were tested for ASGV infection. In this communication, data on ASGV incidence in *F. palmata*, host range studies and sequence information for five isolates of the virus are being presented and to the best of our knowledge, this is the first report of *F. palmata* as a new natural host of ASGV.

Materials and methods

Plant material

In 2013, we noticed that *F. palmata* trees growing in and around the apple germplasm field of CSIR IHBT were displaying severe virus-like symptoms and similar symptoms were also observed on the *F. palmata* trees growing away from the apple germplasm field. To find out the prevalence of ASGV on the *F. palmata* trees, a total of thirty symptomatic and asymptomatic leaf samples were collected from three different areas, viz. CSIR-IHBT (samples from near and away from apple germplasm field), CSKHPKV Palampur and Dharamshala of district Kangra, Himachal Pradesh, and tested for the presence of ASGV.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from the *F. Palmata* tree growing near the apple germplasm field of CSIR-IHBT using CTAB method (Zeng and Yang 2002) and subjected to RT-PCR using detection primers ASGV4F and ASGV4R (Kummert et al. 1998), which amplify the partial replicase gene of ASGV. First-strand cDNA synthesis was carried out in a reaction mixture of 25 µl, containing 1 µg RNA, 5 µl Mu-MLV reverse transcription buffer (5×) (USB, Cleveland, Ohio, USA), 1 µl (10 pmol/µl) of downstream primer (Kummert et al. 1998), 1.5 µl dNTP mix (40 mM), 0.5 µl (200 U/µl) of Mu-MLV reverse transcriptase (USB) and 0.5 µl (40 U/µl) RNase inhibitor (USB, USA). RT reaction was incubated at 37 °C for 75 min followed by 70 °C for 5 min for enzyme inactivation.

PCR was carried out in thin-walled 0.2-ml tubes in 9700 Thermal Cycler (Applied Biosystems, USA). The reaction

mixture (50 µl) consisted of 5 µl Taq DNA polymerase buffer (10×) (Merck Biosciences, India), 1.5 µl dNTP mix (10 mM), 10 pmol each of upstream and downstream primers (Kummert et al. 1998), 0.5 µl (3 u/µl) Taq DNA polymerase (Merck Biosciences, India) and 5 µl cDNA. Denaturation was performed at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 40 s, primer annealing at 62 °C for 40 s, extension at 72 °C for 1 min, and a final elongation at 72 °C for 8 min. The amplified PCR products were electrophoresed in 1% agarose gel at 80 V, stained with ethidium bromide and visualized under UV transilluminator.

The positive amplicon (obtained from the *F. Palmata* tree growing near the apple germplasm field of CSIR-IHBT) was eluted from the gel using GeneJET Gel Extraction kit (Thermo Scientific, Waltham, Massachusetts, USA) and cloned into pGEM-T easy vector (Promega, Madison, Wisconsin, USA). Recombinant plasmid was purified using GenElute Plasmid Miniprep kit (Sigma Aldrich, USA) and sequenced in an automated DNA sequencer (ABI PRISM®3130xl Genetic Analyzer) using ABI prism Big Dye™ Terminator v3.1 Ready Reaction Cycle Sequencing kit (Applied Biosystems, USA), sequencing both strands. Sequence was analyzed with the help of BLAST (http://blast.ncbi.nlm.nih.gov/) and was found to match with ASGV sequence. The sequence was submitted to GenBank database (accession number LN849916).

Double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) and nucleic acid spot hybridization (NASH)

To assess the occurrence of ASGV in *F. Palmata*, 30 leaf samples were collected from the trees growing at three different areas, viz. CSIR-IHBT, CSKHPKV Palampur and Dharamshala of district Kangra, Himachal Pradesh, and screened for the presence of ASGV infection by DAS-ELISA (Clark and Adams 1977) using commercially available reagents (Adgen Phytodiagnostics, UK). To further confirm the results obtained from DAS-ELISA, the samples were tested by NASH. Total RNA was extracted from all the symptomatic and asymptomatic leaf samples using CTAB method (Zeng and Yang 2002) and blotted on to nylon membrane (Ambion, USA) in a vacuum blot system (Hoefer, USA) according to the standard protocol (Sambrook et al. 1989) and crosslinked by UV irradiation. A complete coat protein (CP) gene of ASGV characterized from the apple cultivar Starkrimson, FM204881 (Negi et al. 2010) (cloned in pGEM®-T easy vector) was used for the preparation of antisense DIG-labeled riboprobe using DIG RNA labeling mix [Roche Diagnostics, Germany (http://stanxterm.aecom.yu.edu/wiki/data/Product_manuals_attach/DIGRNALabel.pdf)] as per the manufacturer's instructions. The membrane was hybridized at 65 °C with the riboprobe generated from cloned ASGV CP. Chemiluminescent detection was done using CDP star (Ambion, Life technologies, USA) as per the manufacturer's instructions.

The results obtained by ELISA and NASH were also validated by RT-PCR using detection primers (Kummert et al. 1998). Out of the positive samples, four samples giving amplification for the partial replicase gene of ASGV were randomly selected, cloned and sequenced as mentioned elsewhere in this manuscript and submitted to GenBank database (accession numbers LN849917, LN849918, LN849919 and LN849920). Thus, a total of five samples were confirmed through sequencing and found positive for ASGV. Among the five samples, one was collected from the *F. Palmata* tree growing near the apple germplasm field of CSIR-IHBT and rest four from the trees growing away from the field (i.e., one from the campus of IHBT, one from CSKHPKV Palampur and two from Dharamshala).

Molecular characterization of coat protein (CP) gene

To characterize the complete coat protein gene of ASGV from *F. palmata*, all the five RT-PCR-positive samples (confirmed with detection primers of Kummert et al. 1998) were chosen and analyzed using the CP primers ASGV5641/ASGV6396, which gives an amplification of ~750 bp (Nickel et al. 2001). RT-PCR was performed as described above (in RT-PCR section). Amplicons obtained were eluted, cloned and sequenced as mentioned for replicase gene.

The above-characterized five samples were also tested for the presence of other major apple viruses (ASGV, ACLSV, ApMV and ASPV) through multiplex RT-PCR (Kumar et al. 2014).

Phylogenetic analysis

Sequences were analyzed by BLAST and compared with already-characterized ASGV isolates. Multiple alignments were carried out with the help of MultAlin software available online at http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html.

The phylogenetic tree for partial replicase was constructed using maximum likelihood method [implemented in MEGA 5.2, best fit model: T92 + G (Tamura-3-parameter using discrete Gamma distribution), model of nucleotide substitutions] and 1000 bootstrap replicates. ASGV sequences used for the phylogenetic analysis of the partial replicase gene are as follows: apple (India; HE978837, FN565167, Germany; JX080201), kiwi (China; AF522459), and Citrus (Taiwan; AY646511, China; KC588947, JQ765412).

While the phylogenetic tree for complete CP was constructed using maximum likelihood method [implemented in MEGA 5.2, best fit model: JTT (Jones Taylor Thornton),

model of amino acid substitutions], choice for the best fit models were made in MEGA 5.2 software (Tamura et al. 2011) and support was assessed using bootstrap analysis (1000 replicates). ASGV sequences used for the phylogenetic analysis of the CP gene are as follows: apple (Brazil; AF438409, Japan; NC001749, India; FM204881, LN627002, LN627003, LN627004, LN627005, South Korea; AY596172, China; JX885580, Korea; AF465354), pear (India; FN393044, China; AY886760), lily (Japan; D16681), kumquat (Taiwan; AY646511), Meyer lemon (USA; EU553489) and kiwi (China; AF522459, India Ki-1; HG796196). *Potato virus* X (PVX) Acc. no. NC011620 was taken as an outgroup.

Mechanical inoculation on herbaceous plants

An apple isolate of ASGV LN627003 [characterized from Red Chief cultivar (Ap RC), growing at the apple germplasm field of CSIR-IHBT] (unpublished data) was taken as a positive control for mechanical inoculation experiments.

The extract from one of the positive samples (Fi-16, which gave bright signal in NASH) was mechanically inoculated with 0.1 M sodium phosphate buffer (pH 8.0) using carborundum (in our earlier studies on ASGV, we standardized this buffer for rub inoculation) on *Chenopodium amaranticolor, Cucumis sativus, Chenopodium quinoa, Phaseolus vulgaris, Nicotiana benthamiana* and *Nicotiana glutinosa*. The experiment was conducted in two replicates with three plants, inoculated for each experiment. The plants were grown up to 6 weeks in the greenhouse for symptom development. All the symptomatic or non-symptomatic plants were checked and confirmed through RT-PCR using coat protein primers (Nickel et al. 2001).

Results

Sample collection, virus detection and characterization

Leaf samples were collected from *F. palmata* trees growing in the vicinity of the apple germplasm field along with additional samples, from three different places of district Kangra, viz. CSKHPKV, CSIR-IHBT campus Palampur and Dharamshala. It was observed that 80% of the trees displayed virus-like symptoms, viz necrotic spots, chlorosis, chlorotic spots, and marginal chlorosis, while 20% were symptomless (Fig. 1).

All the samples were tested for ASGV infection. Twelve out of thirty samples were found positive (Sr. no 2, 3, 8, 11, 15, 16, 19, 20, 21, 26, 27 and 29) in DAS-ELISA with OD_{405} values 2.5 to 3x the negative control. All these samples along with additional three samples (Sr. no 7, 10 and 12) were found positive by NASH. The results of DAS-ELISA and NASH were further validated by RT-PCR, which confirmed the presence of ASGV in 15 samples (Sr. no 2, 3, 7, 8, 10, 11, 12, 15, 16, 19, 20, 21, 26, 27 and 29) (Supplementary Table 1).The combined results of all the three detection techniques confirmed the presence of ASGV in 50% of *F. palmata* samples.

About 63% of the symptomatic trees were found to be infected with ASGV while none of the asymptomatic trees carried the virus. Amongst the ASGV-positive samples, 58% had chlorotic spots, 45.8% necrotic spots, 20.8% had chlorosis along the margin. Chlorosis was also observed on 46.6% negative plants and all the samples which displayed leaf deformation were also found negative for the virus (Supplementary Table 1).

Five positive samples (including one, growing in the vicinity of apple germplasm field) showing distinct signals in NASH were randomly selected and subjected to RT-PCR using detection primer (which amplifies the partial replicase gene of the virus). An amplification of ~ 574 bp was obtained and all the five amplicons were cloned, sequenced and submitted to GenBank database. The accession numbers LN849916, LN849917, LN849918, LN849919 and LN849920 were obtained. The samples were named as Fi-15, Fi-19, Fi-20, Fi-29 and Fi-16, respectively. Further, the complete CP gene of ASGV was amplified from the five isolates described above using complete CP primers. An amplification of ~ 750 bp which includes 714 bp of complete CP was obtained. The amplicons were cloned, sequenced and submitted to GenBank database with accession numbers LN559078 (Fi-15), LN559079 (Fi-16), LN559080 (Fi-19), LN559081 (Fi-20) and LN559082 (Fi-29). The five positive samples (Sr. no.15, 16, 19, 20 and 29) when tested through multiplex RT-PCR, for the presence of four major apple viruses, viz. ACLSV, ASGV, ASPV and ApMV, showed positive results for ASGV only, with an amplification of ~ 200 bp while none of the other viruses (i.e., ACLSV, ASPV and ApMV) were detected.

Phylogenetic analysis

Phylogenetic analysis based on the partial replicase gene at nucleotide level revealed that the five isolates of ASGV from *F. palmata* grouped themselves into two separate clades (Fig. 2). The four isolates (Fi-15, Fi-19, Fi-20 and Fi-29) shared 99–100% sequence identity among themselves, whereas the fifth isolate (Fi-16) shared 97.5–98.4% sequence identity with them. All these *F. palmata* isolates of ASGV were found to be most closely related to the apple isolates from India (HE978837, FN565167) and Germany (JX080201) and shared 97.3–99.6% sequence identity at nucleotide level. However, the sequence identity of the present isolates with the kiwi (AF522459) isolate was 83–84%

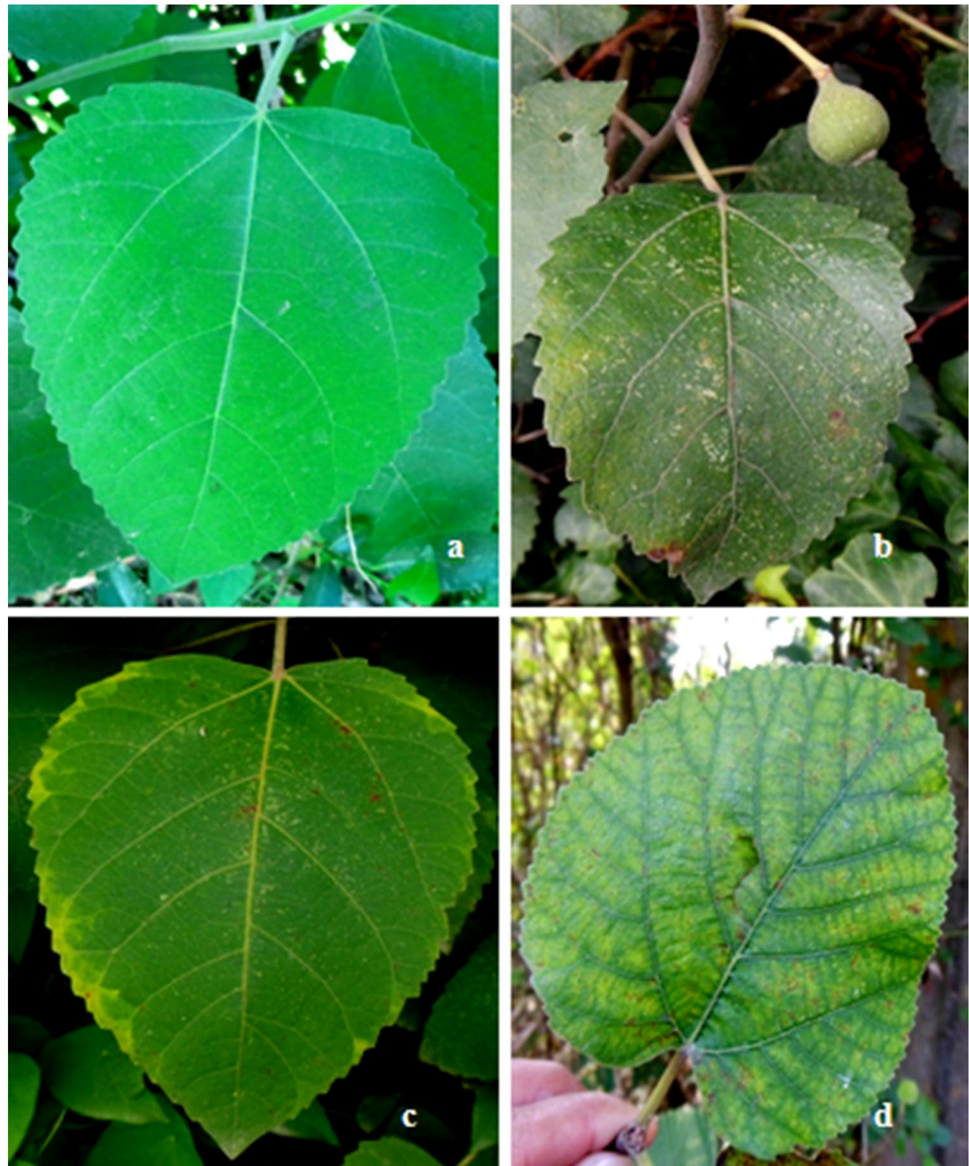

Fig. 1 Virus-like symptoms on *Ficus palmata* as in the study area. **a** Healthy leaf, **b** Chlorotic and necrotic spots along with leaf deformation, **c** Chlorotic spots along with the chlorosis along the margin of the leaf, **d** Chlorosis along with necrotic spots

Fig. 2 Phylogenetic tree constructed using maximum likelihood method [implemented in MEGA 5.2, best fit model: T92+G (Tamura-3-parameter using discrete Gamma distribution), model of nucleotide substitutions] and 1000 bootstrap replicates, showing genetic relationship of the present ASGV (*Ficus palmata*) isolates with other ASGV isolates from various hosts and countries. *Fi* fig, *Ap* apple, *Qt* kumquat, *Ki* kiwi, *CTLV Citrus tatter leaf virus*

while the sequence identity with the Citrus isolates [*Citrus tatter leaf virus* (CTLV): AY646511, KC588947, JQ765412] was found to be 75–76%.

Phylogenetic analysis done for the complete CP gene of ASGV revealed that the five isolates (Fi-15, Fi-16, Fi-19, Fi-20 and Fi-29) clustered in two separate groups. Sequence identity matrix computed for the isolates of ASGV showed that CP sequences of four out of the five isolates (Fi-15, Fi-19, Fi-20 and Fi-29) were 100% identical to each other and shared 88.5 and 95.3% sequence identity with Fi-16 at nucleotide (nt) and amino acid (aa) levels, respectively. Phylogenetically, as expected the four isolates clustered into one group while Fi-16 grouped separately from the four (Fi-15, Fi-19, Fi-20 and Fi-29) (Fig. 3).

The four isolates were most closely related to the apple isolates from India (FM204881, LN627002, LN627003, LN627004); Brazil (AF438409) and China (JX885580) sharing a sequence identity of 97.6–99% (at nt) and 97.8–100% (at aa) level and clustered together. The four isolates from *F. palmata* grouped with the apple isolates (LN627002, LN627003 and LN627004) of ASGV characterized from Starkrimson (Ap-SK), Red Chief (Ap-RC) and Red Fuji (Ap-RF) cultivars growing at the apple germplasm field of CSIR-IHBT. Further, these four isolates shared only 90.3% (at nt) and 97.4% (at aa) level sequence identity with isolate from Vance Delicious (Ap-VD) cultivar (LN627005) characterized from the same apple germplasm. This indicates that there is natural variance of isolates present under the same field conditions.

Whereas the fifth isolate Fi-16, different from rest of the four isolates, was found to be closely related to the kiwi (Ki-1) and pear isolates from India sharing 98.7 and 99.1% (aa) sequence identity. This isolate shared a sequence identity of 87.6–88.6% (at nt) and 93.2–94.9% (at aa) level with the apple isolates (LN627002, LN627003, LN627004 and LN627005) of ASGV characterized from the apple cultivars growing at CSIR-IHBT.

When compared to other isolates from different geographical locations and hosts, the four isolates (Fi-15, Fi-19, Fi-20 and Fi-29) shared 88–98 and 94–100% sequence identity while Fi-16 shared 87–99 and 92–99% identity at nucleotide and amino acid levels, respectively. The phylogenetic clustering shows that these five isolates from *F. palmata* group themselves irrespective of the host and geographic locations.

Mechanical inoculation on herbaceous plants

Fi-16 isolate (showing bright signal in NASH and grouping separately in the phylogenetic tree) was chosen for the mechanical inoculation studies. The other *F. palmata* isolates clustered with the apple isolate LN627003 [characterized from Red Chief cultivar (Ap RC), growing at the apple germplasm field of CSIR-IHBT]. In our own earlier mechanical inoculation studies (unpublished data), we transferred this isolate to various herbaceous hosts and observed the following symptoms: chlorotic spots on *C. quinoa, C. amaranticolor* and *N. benthamiana* (Fig. 4a, b, d) and mild chlorotic spots along with chlorosis on *N. glutinosa* (Fig. 4c). Because of this information in hand we used this isolate as a positive control for mechanical inoculation experiments. This isolate of apple shared 97.8% sequence identity with the four (Fi-15, Fi-19, Fi-20 and Fi-29) isolates characterized from *F. palmata* but shared a sequence identity of 87.6% at nucleotide and 93.2% at amino acid level with the fifth isolate (Fi-16).

Being comparatively a distinct isolate the extract from Fi-16 was mechanically inoculated into six different

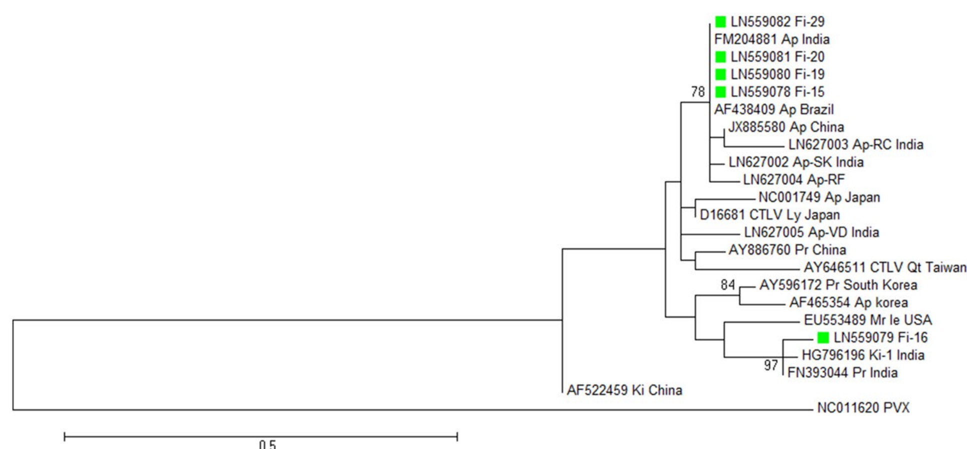

Fig. 3 Phylogenetic tree constructed using maximum likelihood method [implemented in MEGA 5.2, best fit model: JTT (Jones Taylor Thornton), model of amino acid substitutions and 1000 bootstrap replicates], showing genetic relationship of the present ASGV (*Ficus palmata*) isolates with other ASGV isolates from various host and countries. *Fi* fig; *Ap* apple; *RC* Red Chief; *RF* Red Fuji; *SK* Starkrimson; *VD* Vance Delicious; *Pr* pear; *Ki* kiwi; *Ly* lily; *Qt* kumquat; *Mr le* Meyer lemon; *CTLV Citrus tatter leaf virus; PVX, Potato virus* X

Fig. 4 Host range studies and symptoms induced by apple isolate (cultivar Red Chief Ap-RC) on various herbaceous hosts; **a** *C. quinoa* and **b** *C. amaranticolor* showing chlorotic spots; **c** *N. glutinosa* with mild chlorotic spots; *N. benthamiana* with chlorotic spots. Corresponding healthy controls for each of the plants inoculated were symptomless and were negative when tested through RT-PCR

herbaceous hosts, namely *C. amaranticolor, C. sativus, C. quinoa, P. vulgaris, N. benthamiana* and *N. glutinosa*. Chlorotic spots were observed on *C. quinoa* and *C. amaranticolor* (Fig. 5a, b), while leaf distortion with mild chlorotic spots were observed on *N. benthamiana* (Fig. 5c). Chlorotic spots along with chlorotic mosaic were seen on *N. glutinosa* (Fig. 5d) whereas leaf distortion with chlorotic streaks were observed on *C. sativus* and *P. vulgaris* but the size of chlorotic streaks was comparatively smaller in case of *C. sativus* (Fig. 5e, f). All the symptomatic host plants gave positive amplification for complete CP of ASGV (data not shown). The corresponding mock inoculated controls for all the hosts were symptomless and negative in RT-PCR.

Discussion

The results of this study provides an insight into prevalence of ASGV infection in its new host *F. palmata*, which is widely distributed in the northwestern region of Himachal Pradesh (district Kangra, viz. Dharamshala and Palampur) located 32.12°N 76.53°E at an altitude of around 1300 masl. In the current study, the presence of ASGV on *F. palmata* was found to be associated with symptoms of chlorotic and necrotic spots and chlorosis along the margins.

Phylogenetically, out of the five isolates characterized from *F. palmata*, four (Fi-15, Fi-19, Fi-20 and Fi-29) clustered with the apple isolates characterized from the cultivars growing at the apple germplasm field of CSIR-IHBT, Palampur. The close clustering of these isolates indicates that there might be some alternate mode of transmission besides grafting (possibly an insect vector). However, the presence of ASGV-positive *F. palmata* trees located away from the apple germplasm field of CSIR-IHBT indicates towards the natural infection of ASGV on *F. palmata*.

On comparison of the symptoms induced by *F. palmata* (Fi-16) and control apple (Ap-RC) isolates of ASGV on various herbaceous hosts, it was observed that these isolates induced similar symptoms of chlorotic spots on *C. quinoa* and *C. amaranticolor*, whereas the symptoms observed on *N. benthamiana* and *N. glutinosa* were distinct. Fi-16 induced mild chlorotic spots along with leaf distortion on *N.*

Fig. 5 Host range studies and symptoms induced by *Ficus palmata* Fi-16 isolate on various hosts; **a** *C. quinoa* and **b** *C. amaranticolor* showed chlorotic spots; **c** *N. benthamiana* showed leaf distortion along with mild chlorotic spots; **d** *N. glutinosa* displayed chlorotic spots and chlorotic mosaic; **e** *Cucumis sativus* and **f** *Phaseolus vulgaris* showed symptoms of leaf distortion along with chlorotic streaks. Corresponding healthy controls for each of the plants inoculated were symptomless and were negative when tested through RT-PCR

benthamiana while apple isolate (Ap-RC) showed only chlorotic spots and no distortion was observed. Likewise, symptoms of chlorotic spots along with chlorotic mosaic were induced by Fi-16 on *N. glutinosa* whereas only mild chlorotic spots were observed in case of apple isolate (Ap-RC).

Further, the variable Fi-16 isolate clustered closely with Ki-1 isolate from kiwi (Bhardwaj et al. 2014) and were compared based upon the symptoms induced on herbaceous hosts. Results revealed that both the isolates induced similar chlorotic spots on *C. quinoa* and *C. amaranticolor*. However, the symptoms induced by Fi-16 on *N. glutinosa, P. vulgaris* and *C. sativus* were very distinct from those induced by kiwi (Ki-1) isolate (Bhardwaj et al. 2014). Previously, in 2003, Clover et al. (kiwi isolate) reported necrotic lesions with chlorotic margins on *C. quinoa* and necrotic lesions, veinal necrosis and foliar chlorotic mottling on *P. vulgaris* whereas the isolate Fi-16 behaved differently and induced chlorotic spots on *C. quinoa* and leaf distortion along with chlorotic streaks on *P. vulgaris*. Hence, symptomatically the Fi-16 isolate is distinct from other isolates reported so far. The difference in symptoms observed on different hosts may be attributed to some extent to the difference in amino acid sequence of coat protein gene. Since symptom development is a complex phenomenon involving interactions between viral genes or their products and host plant factors, alteration of the amino acid sequence of the CP or Movement Protein (MP) genes may alter the symptom development (Shintaku and Palukaitis 1992; Banerjee et al. 1995; Rao and Grantham 1995; Fujita et al. 1996; Moreno et al. 1997; Andersen and Johansen 1998; Szilassy et al. 1999; Takeshita et al. 2001; De Assis Filho et al. 2002). However, establishment of complete genome information will be much more important in this regard.

The expanding host range of ASGV to tree species such as *Ficus* indicates that there must be some other mode of transmission (possibly a vector or through root bridges) which is so far unexplored and needs to be worked out. To the best our knowledge, the current study is the first report of molecular characterization, diversity analysis, incidence and host range studies of ASGV infecting *F. palmata*.

Author contribution statement PB: experimental work and manuscript preparation; VH: overall guidance and manuscript editing.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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