Diversity of *Apple mosaic virus* Isolates in India Based on Coat Protein and Movement Protein Genes

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Abstract *Apple mosaic virus* (ApMV), an *Ilarvirus* is one of the most common pathogens of apple worldwide. During field surveys in commercial plantations of Himachal Pradesh and Jammu & Kashmir, observations of bright chlorotic mosaic like symptoms on apple trees indicated probable infection by the virus, which was later detected by double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA). An incidence of 24 and 28% (based on ELISA) was obtained as 6/25 and 15/53 samples from HP and J&K were positive, respectively. An amplification of approximately 700 and 850 bp was obtained for coat and movement protein genes (CP and MP), respectively. The CP was 223 amino acids in length and showed 87–99% identity when compared to 21 ApMV isolates. Whereas, MP (286 amino acids) showed 91–95% identity with other isolates. However, the gene sequences were quite conserved among Indian isolates and grouped together phylogenetically. CP of the Indian isolates showed maximum identity of 95% with Korean isolate (AY 125977) in apple and in other host these showed a maximum identity of 98% to Czech Republic pear isolate. MP showed maximum identity with Chinese isolate i.e., 95%. The diversity study

will also help in analyzing variability among the isolates and also to formulate diagnostic and resistance strategies.

Keywords ELISA · RT-PCR · ApMV · Coat protein and movement protein

Introduction

Apple (*Malus domestica* L; family Rosaceae) is a pomaceous perennial fruit crop cultivated commercially in Jammu and Kashmir (J&K), Himachal Pradesh (HP) and Uttarakhand (UK) hills in India. Delicious cultivars account for 85, 45 and 30% area under apple cultivation in HP, J&K and UK hills, respectively [8]. In India, mosaic symptoms were first noticed in Uttarakhand in 1957 [4]. Though India ranked seventh in apple fruit production, of late the productivity has been decreasing due to increase in disease incidence among other factors (biotic and abiotic). Many viruses have been reported in apples [12] viz. *Apple mosaic virus* (ApMV), *Apple chlorotic leaf spot virus* (ACLSV), *Apple stem grooving virus* (ASGV) and *Apple stem pitting virus* (ASPV) that cause significant yield losses [10]. The virus belongs to the genus *Ilarvirus* and family *Bromoviridae*, causes reduced shoot growth, fruit set, fruit weight, yield per tree and ascorbic acid content of the fruit [16]. More than 46% losses in Golden Delicious cultivar due to ApMV infection has been reported [5]. The virus causes line pattern symptoms in plum and is transmitted by root grafting [6] and most frequently through infected material and by mechanical inoculations onto herbaceous hosts [17]. Though related to *Prunus necrotic ring spot virus* (PNRSV) in particle characteristics, ApMV does not share antigenic similarity with it and spreads through thrips unlike PNRSV [2]. The genome of ApMV is tripartite having three RNAs

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viz. RNA1, RNA2 and RNA3. RNA1 and RNA 2 are monocistronic, encoding nonstructural proteins involved in viral replication whereas RNA 3 is bicistronic encoding 3a and 3b proteins. In RNA3, the proximal ORF 3a codes for the movement protein (MP) while the distal ORF 3b codes for coat protein (CP). The distal gene is expressed via a sub-genomic RNA, RNA 4. In the present study, the diversity of coat protein and movement protein gene of ApMV from Indian apple isolates was studied. For characterization of CP, already described primer sequences were utilized while for MP, primer pair was designed from available sequences in GenBank (NCBI) by multiple alignment.

Materials and Methods

Field Survey

Surveys were conducted in apple orchards of Himachal Pradesh and Kashmir for sample collection. In state of Jammu & Kashmir, four districts were monitored. Regions covered were Central Institute of Temperate Horticulture (CITH) at Srinagar, Shopian at Pulwama, Sopore at Baramulla and Gthlibag at Gandarbal. Kangra, Solan, Mandi, Chamba, Kinnaur, and Shimla districts were surveyed in HP. Samples were collected from different varieties including Red Delicious, Jonagold, Jonathan, Oregon Spur, Jonafree, Starkrimson, Golden Delicious, Lal Ambri, Sunehri, Gala Mast, Firdos, Red Chief and Red Fuji.

Enzyme Linked Immunosorbent Assay

Preliminary detection of the virus was carried out by ELISA using commercially available kit for ApMV (Bio-reba, Switzerland) analyzing samples in triplicate.

Reverse Transcription and Polymerase Chain Reaction (RT-PCR)

Infected leaf tissue was ground to fine powder in liquid nitrogen and RNA was isolated from 100 mg using NucleoSpin RNA Plant system (Machery-Nagel, Germany). The primers PPMCP-3 (5'-CTAATCGCTCCATCAT AATTC-3') and ApMVMPd (5'-TCATCCGCTTATA TTTCCAATG-3') for coat and movement protein genes respectively, were used for first strand synthesis. Reverse transcription was carried out in a 25 µl reaction mixture with 7 µl total RNA (1–2 µg), 1.5 µl of 200 nM PPMCP-3, 1.5 µl of 40 mM dNTP mix (Fermentas, Lithuania), 5 µl of 5× RT buffer and 100 U of M-MuLV Reverse Transcriptase (USB corporation, USA). The reaction mixture was incubated at 37°C for 75 min followed by incubation at 70°C for 5 min.

PCR was performed in a 50 µl reaction mixture containing 5 µl of 10× Taq Buffer A (Genei, India), 1.5 µl dNTP mix (10 mM), 0.5 µl Taq DNA polymerase (3U;Genei, India), 5 µl cDNA and 1 µl (200 nM) each of forward and reverse primers. In combination with above mentioned downstream primers, PPMCP-5 (5'-TC AACATGGTCTGCAAGTAC-3') and ApMVMPup (5'-A TGACAACACTGGGAGATAAAC-3') were used as forward primers respectively for CP and MP. PCR reaction was performed in G Storm thermal cycler (Genetechnologies, United Kingdom) and electrophoresed in 1% agarose gel with 1 µg/ml of ethidium bromide in 1× TAE buffer. Amplified products were cloned into pGEM®-T Easy vector (Promega, USA) and sequenced by dideoxy chain termination method [15] 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), sequencing both strands.

Sequence Analysis

The multiple alignment was done using Multalin interface. The evolutionary history was inferred using the Neighbor-Joining method [14]. The evolutionary distances were computed using the Poisson correction method [21] and are in the units of the number of amino acid substitutions per site. Evolutionary analyses were conducted in MEGA5 [19].

Results

Survey and ELISA

During surveys in HP and J&K, symptoms such as chlorosis, mosaic, leaf deformations, necrotic spots, shot holes and oak leaf pattern were noticed on leaves. In ELISA a sample was considered positive when the absorbance at 405 nm was $\geq 3\times$ than that of negative control. The readings were measured in triplicate in an ELISA reader (Bio-Rad, Model 680). The varieties Jonagold, Oregon Spur, Lal Ambri, Red Fuji, Gala Mast and Red Delicious were found positive. Disease incidence was 24 and 28% as 6/25 and 15/53 samples were found positive from Himachal Pradesh and Jammu & Kashmir, respectively.

RT-PCR

Out of 21 ELISA positive samples, 14 were analyzed by PCR. Amplification was obtained from six samples wherein both CP and MP were amplified. On sequencing the amplified products were of 689 and 861 bp for CP and

MP, respectively (EMBL accession numbers: FN435314, FN435315, FN435316, FN435317, FN547927, FN546183 for CP and FN597052, FN598483, FN568307, FN554697, FN551183, FN 568491 for MP).

Phylogenetic Analysis

Coat Protein

In BLAST search and phylogenetic analysis, CP of the isolates shared maximum identity with Czech Republic isolates (Fig. 1). Five of the Indian isolates clustered together with the exception of FN435316 which clustered with Pear isolates from Czech Republic (Fig. 1). However, with regard to MP all Indian isolates clustered together (Fig. 2). Multiple sequence alignment of CP of all the Indian isolates (FN435314, FN435315, FN435316, FN435317, FN547927

and FN546183) showed 87–99% identities with 21 other world isolates at amino acid level (Table 1). The CP accessions show 92–99% identity with other Indian isolates (FJ429309, FM178274, and FJ429311). The Indian isolates showed maximum identity with Korean isolate of apple (95%) accession no. AY125977. Comparative analysis of CP gene at amino acid level with Indian isolates FN435314, FN435315, FN435316, FN435317, FN547927 and FN546183 showed maximum identity of 96, 98, 96, 97, 97 and 97% with Czech Republic Pear isolates as other host. Among themselves they showed 92–99% identity (Table 1). Based on phylogenetic analysis of CP, all strains could be classified into five subgroups viz. subgroup I (apple and cherry isolates, India-this study), subgroup II (Pear strain, Czech Republic, FN435316-one of the Indian isolate-this study), subgroup III (apple isolate, Czech Republic), subgroup IV (apple isolates, USA and Korea) and subgroup V (ApMV isolates prunes, pear, hops and almond). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches [7]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis involved 27 amino acid sequences. There were a total of 208 positions in the final dataset.

Movement Protein

Phylogenetic tree based on MP gene (Fig. 2) revealed that the Indian isolates are closely clustered. Indian isolates were closely related (95%) to Chinese isolate (AM490197) and most distantly related to USA isolate U15608 (89%; Table 2). Figure 3 shows variability at amino acid level in MP. At position 24 instead of glutamine/arginine is present. At 25th position valine is present place of alanine. At 30th position glutamic acid is present instead of lysine. At 48th position valine is present in place of leucine. At 50th and 52nd position arginine is present instead of glutamine. Overall there was no charge difference due to amino acid changes. In U15608 78th to 89th positions show variability from other isolates as indicated in box (Fig. 3).

Discussion

Apple mosaic virus was found to have high level of incidence in India. In our studies ApMV infection was found in J&K and HP on apple and prunus trees. ApMV incidence in fruit crops like apple, plum, nectarine and apricot from HP and J&K was studied. In DAS-ELISA 13/198 samples from four districts of HP were come positive for ApMV indicating the disease incidence of approximately 6.5% [20]. The virus causes loss of 35.6% in hazelnut in Turkey [1]. The presence of ApMV in hazelnut

Fig. 1 Phylogenetic tree of all available coat protein sequences of *Apple mosaic virus* using neighbor-joining method with 1,000 bootstrap replicates. Branch lengths represent phylogenetic distances determined by distance matrices of nucleotide sequences. Numbers above critical branches are significant bootstrap values (>75%). For each sequence, corresponding isolate and country of origin are indicated

Fig. 2 Phylogenetic tree of complete nucleotide sequences of *Apple mosaic virus* movement protein gene constructed using neighbor-joining method with 1,000 bootstrap replicates. Branch lengths represent phylogenetic distances determined by distance matrices of nucleotide sequences. Numbers above critical branches are significant bootstrap values (>75%). For each sequence, corresponding isolate and country of origin are indicated

Table 1 Percent amino acid identity of coat protein gene of *Apple mosaic virus* with other isolates of the world from apple and other hosts

Accession no.	Source	Host	Country	% Identity with Indian isolates of ApMV					
				FN435314	FN435315	FN435316	FN435317	FN547927	FN546183
AY125977	ApMV	Apple	Korea	94	94	94	94	95	95
AY054385	ApMV	Apple	Czech Republic	93	93	93	93	95	95
AY542540	ApMV	Apple	Czech Republic	92	92	92	92	93	93
AY542541	ApMV	Apple	Belgium	94	95	94	94	93	93
S78319	ApMV	Apple	Germany	90	90	90	90	92	92
AM490197	ApMV	Apple	China	89	89	89	89	90	90
L03726U	ApMV	Apple	USA	93	93	93	93	90	90
AF548367	ApMV	Apple	Korea	93	92	93	93	83	83
U15608	ApMV	Apple	USA	93	92	93	93	83	83
U03857S	ApMV	Apple	Spain	60	60	60	60	51	51
AY542542	ApMV	Pear	Czech Republic	96	98	96	97	97	97
AY542545	ApMV	Pear	Czech Republic	96	98	96	96	97	97
AY542543	ApMV	Pear	Czech Republic	96	98	96	97	97	97
AY542546	ApMV	Pear	Czech Republic	96	97	96	96	96	96
AY542544	ApMV	Pear	Czech Republic	94	95	94	96	95	95
AY054389	ApMV	Pear	Czech Republic	90	90	90	90	92	92
AY054387	ApMV	Hop	Czech Republic	90	90	90	90	92	92
AY054386	ApMV	Prune	Czech Republic	90	91	90	90	92	91
AY054388	ApMV	Almond	Czech Republic	88	87	88	88	91	91
AM491772	PNRSV	Apple	India	60	60	60	60	51	51
FJ429311	ApMV	Apple	India	99	96	99	99	99	99
FJ429309	ApMV	Apple	India	95	92	95	95	92	92
FM178274	ApMV	Apple	India	99	96	99	99	99	99

Table 2 Percent sequence identity of movement protein gene of *Apple mosaic virus* at the amino acid level with other isolates of the world

Accession no.	Source	Host	Country	% Identity with Indian isolates of ApMV					
				FN597052	FN598483	FN568307	FN568491	FN554697	FN551183
U15608	ApMV	Apple	USA	91	91	91	91	89	92
AM490197	ApMV	Apple	China	95	95	95	95	93	95

Fig. 3 Multiple alignment of amino acid sequences of *Apple mosaic virus* movement protein from India showing the heterogeneity. Variable regions highlighted with bold letters. These letters represent

germplasm in Poland from plant material of foreign origin was verified [18]. The incidence of ApMV in north-east Spain in Negret hazel where virus-free trees were spread randomly with graft inoculated trees. When tested with ELISA after 10 years of planting it was noted that 15% of ungrafted trees contained this virus [3]. The multi-alignment with other sequences at the amino acid level showed maximum variability in the first 100 amino acids of the CP [9]. CP sequences characterized earlier (FM178274; FJ429311; FJ429309) from India were closely related (96, 95, and 89%) to the Korean isolate (AY125977) [20]. Whereas, CP sequences of isolates characterized in this study were related to isolates characterized from pear (Czech Republic). In the present study, the percent incidence of ApMV was 24 and 28% respectively, in HP and J&K. Analysis of more than 50 samples (from apple trees) by ELISA from Czech Republic revealed that 34 were positive (68%) for ApMV [13], on the higher side in comparison to India. In their studies, it was noticed that 4 out of 11 isolates had insertions of various lengths after nucleotide position 141 in CP [13]. However, our studies with regard to Indian isolates showed that nucleotide positions after 141 are conserved unlike reported earlier [13]. Although, with regard to MP, all the Indian isolates clustered together in phylogenetic tree (Fig. 2), there was some variability concentrated towards USA isolate (Fig. 2). Indian isolates could be classified into two subgroups mainly, these were I and II. Majority of isolates clustered into subgroup I while one of the isolate was in II, also occupied by pear isolate from Czech Republic (Fig. 1). Earlier studies on phylogenetic characterization of CP genes of ApMV isolates showed that these isolates were normally classified into three subgroups (I, II, and III) originating from apple, Hop/Pear and Almond, respectively [9]. Two pathotypes and three serotypes were found to be closely related to PNRSV isolates, which were antigenically related and clustered together with ApMV in the same subgroup [11].

It can be concluded that both CP and MP gene of ApMV are 91–100% conserved among them and show less variability at amino acid level with respect to different locations i.e. J&K and HP in India. Incidence of ApMV was higher in J&K in comparison to HP (visually and after

the change at amino acid level from other sequences given in the figure. Consensus sequence was shown in bottom row

testing). At amino acid level both CP and MP of Indian isolates showed maximum identity with Asian isolates in phylogenetic analysis. This is the first report of molecular characterization of movement protein gene of ApMV from Indian apple isolates. It was noted that recombination could be an important factor which is reflected in slightly different clustering of isolates with regard to CP and MP.

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