Molecular variability analyses of *Apple chlorotic leaf spot virus* capsid protein

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The complete sequences of the coat protein (CP) gene of 26 isolates of *Apple chlorotic leaf spot virus* (ACLSV) from India were determined. The isolates were obtained from various pome (apple, pear and quince) and stone (plum, peach, apricot, almond and wild Himalayan cherry) fruit trees. Other previously characterized ACLSV isolates and *Trichoviruses* were used for comparative analysis. Indian ACLSV isolates among themselves and with isolates from elsewhere in the world shared 91–100% and 70–98% sequence identities at the amino acid and nucleotide levels, respectively. The highest degree of variability was observed in the middle portion with 9 amino acid substitutions in contrast to the N-terminal and C-terminal ends, which were maximally conserved with only 4 amino acid substitutions. In phylogenetic analysis no reasonable correlation between host species and/or geographic origin of the isolates was observed. Alignment with capsid protein genes of other *Trichoviruses* revealed the TaTao ACLSV peach isolate to be phylogenetically closest to *Peach mosaic virus*, *Apricot pseudo chlorotic leaf spot virus* and *Cherry mottle leaf virus*. Recombination analysis (RDP3 ver.2.6) done for all the available ACLSV complete CP sequences of the world and Indian isolates indicate no significant evidence of recombination. However, one recombination event among Indian ACLSV-CP isolates was detected. To the best of our knowledge, this is the first report of complete CP sequence variability study from India and also the first evidence of homologous recombination in ACLSV.

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1. Introduction

ACLSV was first reported in *Malus* spp. from the US by Mink and Shay in 1959 (Burnt *et al.* 1996). ACLSV is the type species of the genus *Trichovirus*, family *Betaflexiviridae* (Carstens 2010). It has filamentous particles approximately 600–700 nm in length that contain a polyadenylated, single-stranded, plus-sense RNA and multiple copies of a single coat protein (CP) of 21 kDa (Yoshikawa and Takahashi 1988). The importance of ACLSV is also due to its worldwide occurrence and its large host range on pome and stone fruit crops, which are of great economic value. The complete nucleotide sequences of ACLSV isolates from apple (Jelkmann 1996), cherry (German *et al.* 1997), peach (Marini *et al.* 2008) and plum (German *et al.* 1990; Sato *et al.* 1993) have been determined.

ACLSV is one of the important latent viruses infecting apple. ACLSV infection rates of up to 80–100% in many commercial apple cultivars with yield losses of the order of 30–40% have been reported (Nemchinov *et al.* 1995; Wu *et al*. 1998; Cembali *et al*. 2003). ACLSV is generally asymptomatic in most apple cultivars, but in sensitive cultivars, malformation and reduction in leaf size and chlorotic rings or line patterns are common. The severity of symptoms elicited by ACLSV depends largely on the plant species and virus strains (Németh 1986). Infections

Keywords. ACLSV; coat protein; India; phylogenetic analysis; recombination; variability

Abbreviations used: ACLSV, *Apple chlorotic leaf spot virus;* CP coat protein, DAS, double antibody sandwich; PREs, potential recombination events; RDP, Recombination Detection Program; RT-PCR, reverse transcription-polymerase chain reaction

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in stone fruits are also normally latent, but severe graft incompatibilities in some *Prunus* combinations in nurseries have been reported (Ulubas and Ertunc 2005). Some virulent strains cause symptoms ("butteratura" or "viruela") in fruits of apricot (Liberti *et al.* 2005). The virus is reported to causes dark green sunken mottle, severe leaf and fruit deformation, known as "butteratura", in peach (Sutic *et al.* 1999), bark split and pseudopox in some plum cultivars (Dunez *et al*. 1972) and graft incompatibility in apricot (Desvignes and Boye 1989). Incidence of diseases reduces the quality and quantity of these fruits. Viral diseases cause economic losses through lower yields and reduced quality of plant products. Damage is more profound in perennial crops than in annuals. The virus is reported to be transmitted by mechanical inoculations, grafting and unclean horticultural practices. Spread of ACLSV in the field has been detected, but the natural mode of spread is still unknown.

In the present study various pome and stone fruits grown in the hill state of Himachal Pradesh (HP), India, were investigated for the presence of ACLSV by double antibody sandwich (DAS)-ELISA and reverse transcription-polymerase chain reaction (RT-PCR). Restricted surveys were also conducted in Jammu and Kashmir (J&K) and some parts of Uttrakhand. We report here the characterization of 27 ACLSV CP isolates (26 complete and 1 partial) from different host species and locations in India. The phylogenetic relationships, biological properties of some isolates and variability of Indian ACLSV-CP isolates with all the available complete and partial ACLSV pome and stone fruit isolates from the world (table 1) were examined to detect possible heterogeneity and evolution.

2. Materials and methods

2.1 *Sample collection*

Surveys were undertaken in the major pome- and stone-fruit-growing states in India to identify the incidence of ACLSV over a period of 3 years (2007–2009). Typical symptoms of virus infection such as leaf deformation, curling, shot holes, necrotic spots, mosaic and mild chlorotic ring-like symptoms were observed on the leaves of some plants. However, most of the plants were apparently healthy. Wild apricot and wild Himalayan cherry were also sampled to check the incidence of virus on them as these are the wild relatives of *Prunus* spp. and are commonly used as rootstock for the cultivated ones.

2.2 *DAS-ELISA, host range and RT-PCR*

Preliminary detection of the virus was done by DAS-ELISA (Clarks and Adams 1977) by using commercially available ELISA reagents for ACLSV (Agdia, USA) as per the manufacturer's instructions. The tests were carried out in triplicate, and absorbance was measured at 405 nm. Samples that gave maximum ELISA readings were used for mechanical inoculations on herbaceous hosts – *Chenopodium quinoa, C. amranticolor, Phaseoulus vulgaris* and *Vigna sinensis* (var. Chitlidana). To prepare cDNA, total RNA was isolated from the positive pome and stone fruit samples using RNeasy Plant mini kit (Qiagen, Germany) as per the manufacturer's instructions. The degenerate primers for amplification of complete CP and part of 3UTR region of ACLSV were designed (accession numbers: AM490253 and AM490254) for molecular detection. RT-PCR was carried out in thin-walled 0.2 ml tubes in Thermocycler 9700 (Applied Biosystems, USA). The reaction mix (50 μl) containing 1× Taq DNA polymerase buffer (Invitrogen, USA), 3 mM dNTP mix (Fermentas, Lithuania), 4 ng of each downstream and upstream primers, 0.75 mM magnesium chloride, 1.5 U Taq DNA polymerase (Invitrogen, USA) and ~50 ng cDNA. Denaturation was performed at 94°C (the annealing temperature for the primers was standardized for each isolate) followed by extension at 72°C for 1 min for 30 cycles. A final elongation step at 72°C for 10 min was also performed. The PCR product was analysed on 1% agarose gels and visualized in UV transilluminator by ethidium bromide (1 μl /ml) staining.

2.3 *Cloning and sequencing*

Amplified DNA was eluted from gel by AuPrep gel extraction kit (Life technology Ltd., India) and ligated in pGEM-Teasy vector (Promega, USA). The ligated product was transformed in *Escherichia coli* DH5α. Routine molecular biology techniques (boiling prep plasmid isolation and restriction digestion) were performed as detailed by Sambrook *et al.* (1989) to identify recombinant plasmid. AuPrep mini plasmid kit (Life technology Ltd., India) was used to purify recombinant plasmid. Sequencing was performed using ABI prism Big Dye Terminator ver.3Ready reaction Cycle sequencing Kit (Applied Biosystems) in an automated sequencer (ABI Prism 310) with T7 and SP6 primers using the Sanger's Dideoxy chain termination method (Sanger *et al*. 1977). Three independent clones from a particular transformation experiment were sequenced.

ACLSV-CP sequences obtained from the present study were aligned with earlier deposited sequences (partial and complete) from the NCBI database (table 1). Percentage sequence identities among the isolates were obtained using Clustal W software (Higgins *et al*. 1994). Multiple sequence alginment for the available complete and partial ACLSV-CP isolates was done by MultAlign programme (Corpet 1998) for determining the sequence variability. The phylogenetic trees were constructed using MEGA version

Table 1. List of all the ACLSV-CP isolates characterized from the world and other *Trichovirus* isolates used as out-group in the study

S. no	Accession no.	Isolate name	CP	Country	Source
1.	ABL63752	BR-1	Full (F)	Brazil (Brz)	Apple (Ap)
2.	P54890	–	F	Japan (Jap)	Apple (Ap)
3.	BAA03643	P-205	F	Japan (Jap)	Apple (Ap)
4.	AB326230	GC10j	F	Japan (Jap)	Apple (Ap)
5.	AB326229	GC10h	F	Japan (Jap)	Apple (Ap)
6.	AB326228	GC10f	F	Japan (Jap)	Apple (Ap)
7.	AB326227	GC10c	F	Japan (Jap)	Apple (Ap)
8.	AB326226	GC10a	F	Japan (Jap)	Apple (Ap)
9.	AB326225	MO-5	F	Japan (Jap)	Apple (Ap)
10.	AB326224	B6	F	Japan (Jap)	Apple (Ap)
11.	AB326223	A4	F	Japan (Jap)	Apple (Ap)
12.	AAT80319	AT-43	Partial (P)	Hungary (Hun)	Apple (Ap)
13.	AAT80320	AT-49	P	Hungary (Hun)	Apple (Ap)
14.	ABG75614	SKIL	P	Isarel (Isr)	Apple (Ap)
15.	CAE52470	M93	P	Albania (Alb)	Apple (Ap)
16.	CAE52469	M76	P	Albania (Alb)	Apple (Ap)
17.	CAE52468	M54	P	Italy (Ita)	Apple (Ap)
18.	ABC59575	P10R1D3	P	Bulgaria (Bul)	Apple (Ap)
19.	CAE52485	MP-Tur	P	Turkey (Tur)	Apple (Ap)
20.	CAE52486	MP02	P	Italy (Ita)	Apple (Ap)
21.	CAE52481	M119	P	Albania (Alb)	Apple (Ap)
22.	CAE52482	M139	P	Albania (Alb)	Apple (Ap)
23.	CAE52483	M62	P	Albania (Alb)	Apple (Ap)
24.	CAE52484	MP-CI	P	China (Chi)	Apple (Ap)
25.	ABK62735	ACLSV-C	F	China (Chi)	Apple(Ap), Peach (Pe)
26.	AJ586650	PE154	P	Hungary (Hun)	Peach (Pe)
27.	EU223295	TaTao	F	USA	Peach (Pe)
28.	AJ586651 (APCLSV)	PE297	P	Jordan (Jor)	Peach (Pe)
29.	AAU93348	HBP	F	China (Chi)	Peach (Pe)
30.	AJ586646	PE 118D	P	Hungary (Hun)	Peach (Pe)
31.	AJ586644	PE- FC	P	Italy (Ita)	Peach (Pe)
32.	AAU06132	AP 10	P	Turkey (Tur)	Peach (Pe)
33.	ABC59574	R1D2P-L	P	Bulgaria (Bul)	Peach (Pe)
34.	AJ586652	PE 56	P	Italy (Ita)	Peach (Pe)
35.	AJ586649	PE153	P	Lebanon (Leb)	Peach (Pe)
36.	AJ586647	PE151	P	Lebanon (Leb)	Peach (Pe)
37.	AJ586648	PE152	P	Lebanon (Leb)	Peach (Pe)
38.	AJ586650 (APCLSV)	PE154	P	Hungary (Hun)	Peach (Pe)
39.	AJ586645 (APCLSV)	PE150	P	Italy (Ita)	Peach (Pe)
40.	AAU06131	KP2	P	Turkey (Tur)	Peach (Pe)
41.	ABC59572	R2D43	P	Bulgaria (Bul)	Peach (Pe)

Table 1. (*Continued*)

42.	AAT80323	P-1	P	Hungary (Hun)	Peach (Pe)
43.	AAT75238	Kuerel	F	China (Chi)	Pear (Pr)
44.	AM292923	–	P	Greece (Gre)	Quince (Qu)
45.	AE52472	AlF5	P	Italy (Ita)	Almond (Ald)
46.	AJ586621	Al-19	P	Italy (It)	Almond (Ald)
47.	DQ329160	P1R9D9	P	Bulgaria (Bul)	Sweet cherry (Che)
48.	X99752	Balton-1	F	France (Fra)	Sweet cherry (Che)
49.	AY730560	ASwC43	P	Turkey (Tur)	Sweet cherry (Che)
50.	AY677105	C-1	P	Hungary (Hun)	Wild cherry (Che)
51.	AY677106	C-2	P	Hungary (Hun)	Wild cherry (Che)
52.	AAF67188	SX/2	F	Poland (Pol)	Plum (Pl)
53.	AAA42589	P863	F	France (Fra)	Plum (Pl)
54.	NP_040553	–	F	France (Fra)	Plum (Pl)
55.	AJ243438	PBM1	F	Germany (Ger)	Plum (Pl)
56.	AJ586623	Apr-109	P	Spain (Spa)	Apricot (Apr)
57.	AJ586629	Apr-110	P	Spain (Spa)	Apricot (Apr)
58.	AJ586622	Apr-103	P	Spain (Spa)	Apricot (Apr)
59.	AJ586630	Apr-20	P	Italy (Ita)	Apricot (Apr)
60.	AJ586632	Apr-60	P	Spain (Spa)	Apricot (Apr)
61.	AJ586633	Apr-61	P	Spain (Spa)	Apricot (Apr)
62.	AJ586634	Apr-62	P	Spain (Spa)	Apricot (Apr)
63.	AJ586635	Apr-63	P	Italy (Ita)	Apricot (Apr)
64.	AJ586631	Apr-3	P	Jordan (Jor)	Apricot (Apr)
65.	AJ586636 (APCLSV)	APR-EA5	P	Turkey (Tur)	Apricot (Apr)
66.	ABA18642 (PeMV)	CL-2	F	USA	Peach (Pe)
67.	NP_062430 (CMLV)	SA1162-21	F	USA	Sweet cherry (Che)

4 (Tamura *et al*. 2007) by neighbour joining method and 1000 bootstrap replicates with a cut-off value of 65% to determine the relationship of Indian ACLSV-CP isolates with other ACLSV-CP isolates available from the world. Other *Trichoviruses – Peach mosaic virus* (PeMV) and *Cherry mottle leaf virus* (CMLV) CP isolates along with newly classified four *Apricot pseudo chlorotic leaf spot virus* (APCLSV) partial CP isolates (Liberti *et al*. 2005) were used as the out-group (table 1) to have a better understanding of phylogeny of ACLSV-CP isolates. Earlier the four APCLSV partial CP isolates sharing amino acid sequences identity of 88–97% were classified as ACLSV isolates (Al-Rwahnih *et al*. 2004). Analysis was done in groups defined as follows: isolates from a particular host, all isolates from pomes, all isolates from stones, only Indian isolates, all isolates from India with other *Trichoviruses* as the out-group and one set of ACLSV complete CP sequences (ACLSV pome and stone fruit isolates together) with the out-group. Radiated tree was constructed to identify clusters, divergence and define evolutionary relationship of the isolates concerned.

All the complete ACLSV-CP sequences were also analysed for putative recombination by Recombination Detection Program (RDP) ver. 3.26 (Martin *et al.* 2005) in order to identify any recombination and subsequent evolution of isolates.

3. Results

3.1 *DAS-ELISA, RT-PCR, cloning and sequencing*

ACLSV antibodies reacted positively with flower and young leaf samples of pome and stone fruits collected during spring. Samples that had ELISA readings atl east two times or greater than the negative/healthy control's readings (0.255) were considered to be ACLSV-positive. ACLSV detection was more reliable in spring season with the use

of flower petals and buds. ACLSV came across as a major virus on apple with disease incidence of 85–90% in HP (data not shown). The infection was very widespread as ACLSV was confirmed in about 14 commonly grown apple cultivars from the 18 tested (data not shown). From survey in HP, it was evident that a significant percentage of other pome and stone fruits were also ACLSV-positive. Initial surveys in J&K and Uttrakhand pointed towards ~40% ACLSV incidence on apple. PCR using primers specific for complete ACLSV-CP gave amplicons of expected size (~800 bp). The positive clones were sequenced and submitted to GenBank (table 2).

3.2 *Host range studies*

Host range studies were done using a few isolates that gave the highest ELISA readings. Mechanical inoculation with ACLSV India11 (apple, Kinnaur) isolate showed severe leaf deformation, vein clearing and chlorosis in *C. amranticolor*; severe chlorosis in *C. quinoa*; severe mottling, chlorosis and necrotic spots in *P. vulgaris* and severe necrotic lesions in *V. sinensis* (var. Chitlidana). Mild chlorosis in *C. amranticolor* and *V. sinensis* (var. Chitlidana) was obtained by inoculating ACLSV isolate India13 (apple, Palampur). Severe chlorosis and curling in *C. amranticolor*, necrotic spots in *V. sinensis* (var. Chitlidana), severe chlorosis in *C. quinoa* and mild chlorotic spots in *P. vulgaris* was observed with India15 (apple, Kashmir) isolate. India16 (apple, Uttrakhand) isolate showed severe chlorosis and vein clearing in *V. sinensis* (var. Chitlidana), whereas India20 (peach, Bajaura) showed chlorosis only in *C. amranticolor*. India27 (wild Himalayan cherry, Palampur) ACLSV isolate showed severe leaf curling and chlorosis in *V. sinensis* (var. Chitlidana) and severe chlorotic spotting in *C. amranticolor*. However, only

Table 2. All the Indian ACLSV-CP isolates characterized from India

S. no.	Accession no.	Place	Isolate	CP	Source	Variety
1.	AM494505	Nagri	India1	Full (F)	Apple (Ap)	Royal delicious
2.	AM494506	Dobi	India2	F	Apple (Ap)	Royal delicious
3.	AM494507	Solan	India3	F	Apple (Ap)	Royal delicious
4.	AM494508	Nihari	India4	F	Apple (Ap)	Royal delicious
5.	AM494509	Kalpa	India5	F	Apple (Ap)	Royal delicious
6.	AM494510	Bajaura	India6	F	Apple (Ap)	Golden delicious
7.	AM494511	Tissa	India7	F	Apple (Ap)	Royal delicious
8.	AM494512	Sangla	India8	F	Apple (Ap)	Royal delicious
9.	AM494513	Salooni	India9	F	Apple (Ap)	Royal delicious
10.	AM494514	Palampur	India10	F	Apple (Ap)	Vance delicious
11.	AM408891	Kinnaur	India11	F	Apple (Ap)	Royal delicious
12.	AM409322	Kotgarh	India12	F	Apple (Ap)	Red gold
13.	AM709776	Palampur	India13	F	Apple (Ap)	Bright n Early (BE)
14.	AM709777	Palampur	India14	F	Apple (Ap)	Scarlet Gala(SG)
15.	FN550875	Kashmir	India15	F	Apple (Ap)	Gala Mast
16.	FN550876	Uttrakhand	India16	P	Apple (Ap)	–
17.	AM882705	Palampur	India17	F	Plum (Pl)	–
18.	AM931534	Palampur	India18	F	Plum (Pl)	Kala amritsari
19.	AM882704	Palampur	India19	F	Pear (Pr)	–
20.	AM498047	Kullu	India20	F	Peach (Pe)	Elberta
21.	AM931533	Palampur	India21	F	Peach (Pe)	Shane Punjab
22.	AM498050	Solan	India22	F	Peach (Pe)	–
23.	AM498049	Salooni	India23	F	Quince (Qu)	–
24.	AM498046	Solan	India24	F	Almond (Ald)	–
25.	AM498045	Solan	India25	F	Apricot (Apr)	–
26.	AM498048	Kullu	India26	F	Wild apricot (Apr)	Chuli (rootstock)
27.	AM498044	Palampur	India27	F	Wild Himalayan Cherry (Che)	rootstock

–, no symptoms were obtained.

mild chlorosis was obtained in *C. quinoa* and *P. vulgaris* developed mild chlorotic spots and mottling (table 3).

3.3 *Phylogenetic and recombination analysis*

All the Indian ACLSV-CP isolates showed sequence identity at an amino acid level of 91–100% with each other and 87–100% with isolates from elsewhere. The recently obtained ACLSV TaTao peach isolate was the most variable, sharing sequence identity of 72–73% and 71–77% at amino acid levels with ACLSV-CP isolates from India and elsewhere, respectively. Multiple sequence alignment of Indian isolates indicates differences in amino acids (aa) towards the middle and C-terminal of the CP. Maximum variability was evident in the middle portion (37–100aa) (Supplementary figure 1). The Indian isolates may be divided into two groups *viz*. group I and group II, on the basis of co-variation and differences of these amino acids (table 4). However, more elaborate host range studies and confirmation by mutational analysis is necessary. ACLSV TaTao peach isolate had the most variable amino acid sequence.

The phylogenetic analysis using radiated tree with CP sequences from ACLSV isolates clearly indicates differences in phylogeny. However, in the rectangular-type phylogenetic tree, there was sufficient variation among the isolates of ACLSV-CP for them to be arranged on several different branches. All ACLSV isolates fell in one cluster of the tree with a few branches showing relatively significant bootstrap values (Supplementary figure 2a). No region-wise and host-specific clustering was observed among Indian isolates.

In recombination analysis of all available complete ACLSV-CPs from India and elsewhere, five unique recombination signals were detected. However, only event 1 seemed significant (table 5) as they were detected by five of the recombination detection programs.

4. Discussion

The virus capsid protein has a role in replication, symptom modulation, cell-to-cell movement, systemic spread and suppression of RNA silencing, in addition to virion formation (Callaway *et al*. 2001; Thomas *et al*. 2003; Lu *et al*. 2004). Earlier variability analysis of ACLSV partial CP sequences showed only slight variation in the N-terminal portion while the C-terminal was maximally conserved. Partial CP sequences were clustered into two groups *viz.* A and B (Al Rawahnih *et al.* 2004). Group B comprised only four isolates (APR-EA5, PE154, PE150 and PE297; table 1). However, later, Liberti *et al.* (2005) confirmed that members in group B were infact APCLSV isolates, with partial CP amino acid sequences 88–97% identical to other APCLSV isolates.

Recently, classification for ACLSV-CP based on covariation of the five amino acids at positions 40, 59, 75, 130 and 184, which were highly conserved within two

Table 3. Details of symptoms obtained on various herbaceous plants after mechanical inoculation

S. no.	Isolate	*C. amranticolor*	*C. quinoa*	*V. Sinensis* (var. Chitlidana)	*P. Vulgaris*	Group
1	India11 (apple, Kinnaur)	Severe leaf deformation, vein clearing and chlorosis	Severe chlorosis	Necrotic lesions	Severe mottling, chlorosis and necrotic spots	P-205
2	India27 (wild Himalayan cherry, Palampur)	Severe chlorotic spotting	Mild chlorosis	Severe leaf curling and chlorosis	Mild chlorotic spots and mottling	P-205
3	India13 (apple, Palampur)	Mild chlorosis	–	Mild chlorosis	–	B-6
4	India15 (apple, Kashmir)	Severe curling and mild chlorosis	Severe chlorosis	Necrotic spots	Mild chlorosis	P-205
5	India16 (apple, Uttrakhand)	Mild chlorosis	–	Severe chlorosis and vein clearing	–	P-205
6	India20 (peach, Bajaura)	Chlorosis	–	–	–	P-205

–, no symptoms were obtained.

Table 4. Variation of 17 amino acids in ACLSV-CP in Indian isolates

Amino acid positions	37	*40*	*59*	60	72	*75*	82	83	86	88	94	97	98	*130*	137	*184*	192
Gp1	T	*A*	*L*	L/T	V	*F*	N	L	Ile	R	P	S	N	*S*	G	*M*	V
Gp2	M/I	*S*	*V*	A/V	I	*Y*	G	M	A	K	T/S	N	S	*T*	S	*L/Ile*	I

The five amino acids conserved in the P-205 group and B-6 group classified by Yaegashi *et al.* (2007) have been italicized. The amino acids underlined show covariation.

Table 5. Recombination analysis results for all the available complete ACLSV-CP sequences used in the study (all events show possible misidentification of daughter)

Event no.	Major parent	Minor parent	Daughter	Breakpoints	Detected by	Average *P*- value
1	India7 (apple)	India12 (apple)	India20 (peach)	186–498	BOOTSCAN	2.877×10^{03}
					MAXCHI	3.399×10^{04}
					CHIMAERA	1.823×10^{02}
					LARD	1.166×10^{07}
					3SEQ	4.481×10^{06}
2	AAA42589 (plum)	India3 (apple)	India24 (almond)	26–211	SiScan	2.344×10^{02}
	AAA42589 (plum)	India3 (apple)	India25 (apricot)	26–211	SiScan	2.344×10^{02}
3	AAA42589 (plum)	India15 (apple)	India9 (apple)	26–211	SiScan	9.724×10^{04}
			ABL63752 (apple)	104–533	SiScan	9.724×10^{04}
			India26 (apricot)	104–533	SiScan	9.724×10^{04}
4	AB326224 (apple)	India7 (apple)	AAA42589 (plum)	23–104	GENECOV	4.413×10^{04}
	AB326224 (apple)	India7 (apple)	NP_040553	23–104	GENECOV	4.413×10^{04}
5	India20 (peach)	India7 (apple)	AAA42589 (plum)	333–538	SiScan	6.223×10^{04}

clusters, was proposed by Yaegashi *et al*. (2007). The clusters were designated as "P205 type" for isolates containing the combination alanine 40, valine 59, phenylalanine 75, serine 130 and methionine 184, whereas the isolates containing serine 40, leucine 59, tyrosine 75, threonine 130 and leucine 184 combination were designated as "B6 type". Moreover, mutational studies concluded that the substitution of a single amino acid (Ala40 to Ser40 or Phe75 to Tyr75) resulted in extreme reduction in the accumulation of viral genomic RNA, double-stranded RNAs and viral proteins (movement protein and CP) in infiltrated tissues, suggesting that the combinations of the two amino acids at positions 40 and 75 are important for effective replication in host plant cells (Yaegashi *et al*. 2007).

4.1 *ELISA, host range and phylogenetic analysis*

The importance of spring season and flower samples for ACLSV detection from various pome and stone fruits by ELISA has been confirmed time and again (Polák and Svoboda 2006; Llacer *et al*. 1985). Most of the samples giving higher ELISA readings except india 13 isolate, when mechanically inoculated on herbaceous plant produced comparatively severe symptoms pointing towards greater infectivity of the isolate and/or better virus titre. All the six isolates used for host range studies share 92–100% sequence identity at the amino acid level. The isolates India13 and India20 (100% sequence identity at the amino acid level) had mild symptoms, whereas isolates India11 and 27 (96% sequence identity at the amino acid level) had the most severe symptoms. In accordance to latest classification based on co-variation of five amino acids proposed by Yaegashi *et al*. (2007), the former two isolates classify as B6 type and the later two as P205 type. However, phylogenetic tree shows isolate India27 to be relatively far from P205 type and nearer to B6 type (figure 2b) and thus have amino acid sequence intermediate to both types (Supplementary figure 1). The severity of symptoms due to inoculation of India27 isolate in *V. sinensis* and mild symptoms in *C. amranticolor* (figure 1) could be attributed to this separation from P205 type (Supplementary figure 2b) and variability in amino acid sequence. Similarly, the changes in amino acids, apart from the five amino acid co-variation, in India11 isolate could be the reason for pronounced symptom of this P205 type isolate on herbaceous hosts, and this needs to be further ascertained by mutational studies. The role of co-varying amino acids at positions 59, 130, 150, 184 and 192 also needs to be ascertained by mutational study and host range study for more isolates.

On analysing ACLSV stone fruit isolates from world, we found that with the exception of ACLSV partial CP cherry isolates P1R9D9 (ABC59573) and C-2 (AAT80322), all clustered as B6 type, suggesting co-evolution of stone fruit isolates. However, the Indian stone fruit isolates did not show such co-evolution (data not shown). The Indian almond isolate (India24) was unique as it had asparatic acid (D) residue at 151 position instead of asparagines (N) as in the remaining isolates (Rana *et al.* 2008a), with the exception of an Indian apple isolate, India3 (Supplementary figure 1). It is worth mentioning that these isolates were from the same orchard and in close proximity to each other. However, the isolates share different phylogeny (India3 – P205 type, India24 – B6 type), sharing 93% sequence identity at the amino acid level.

Wild and cultivated apricot isolates (India26, 25) were highly similar, showing up to 94% sequence identities at the

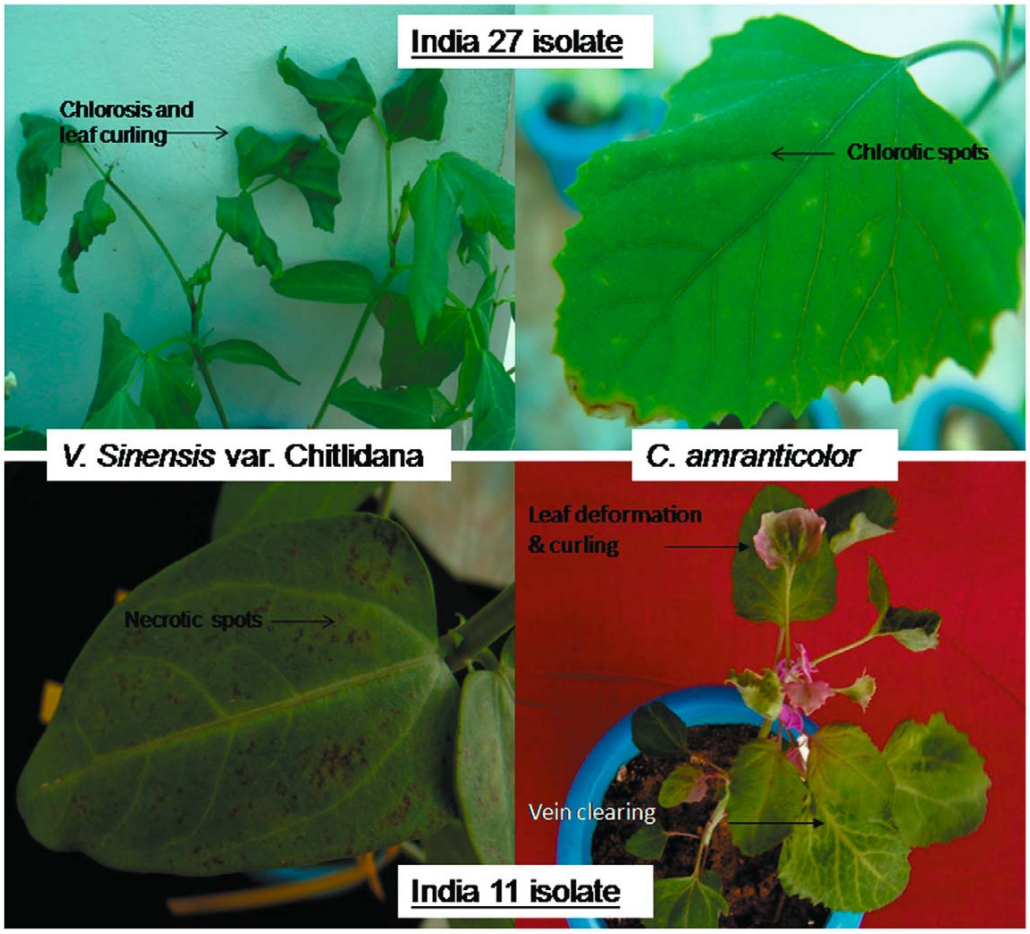

Figure 1. Symptoms on *V. sinensis* and *C. amranticolor* after mechanical inoculation of India 11 and India27 isolate.

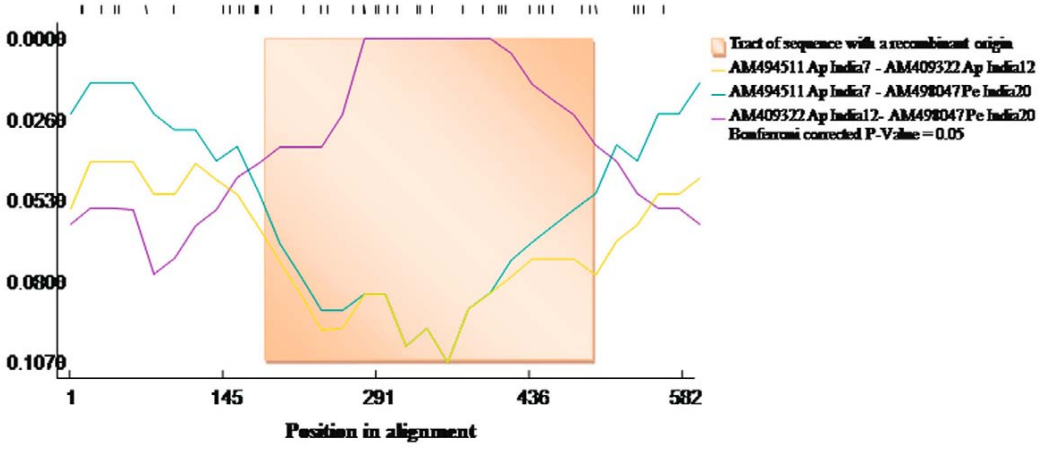

Figure 2. Distance plot showing recombination between AM494511 (India7, major parent) and AM409322 (India12, minor parent) and formation of daughter isolate (India20, AM498047) when all the complete ACLSV isolates were analysed by RDP3.

nucleotide level (Rana *et al*. 2008c). Sequence alignments of ACLSV apricot isolates at the amino acid level showed that most of the variability was present in the N-terminal part of the CP cistron (overlapping with the movement protein,

i.e. from 60 to 100 amino acids), whereas the C-terminus was significantly less divergent. The Indian wild apricot ACLSV isolate clusters with B6 type, which is a less infective cluster of ACLSV isolates. While cultivated apricot is falls in P205 type. The percentage sequence identity of Himalayan wild cherry ACLSV isolate (India27) with different cherry isolates at the nucleotide level ranges from 78% to 90% (Rana *et al.* 2007). The Indian cherry isolate clustered as P205 type but shows closeness to B6 type in the phylogenetic tree. The host range study supports this relatedness to B6 type with differences in infectivity as compared with other P205 type India11 (apple) isolates used in the study. The substitution of amino acid at positions 60, 94 and 98 could be responsible for variation in symptoms produced in the host range study for isolates sharing 96% sequence identity at the amino acid level and similar phylogeny (both are P205 type).

Indian peach sequence India21 and India22 clustered together, sharing 98% amino acid identity, whereas India20 isolate falls in separate group, showing differences in phylogeny when all partial and full CP peach isolates of the world were compared. The India20 isolate shared 92% sequence identity with India21 and India22 isolates at the amino acid level and is the only P205 type peach isolate reported until now from the world. Multiple sequence alignment shows that maximum variability occurs between amino acid positions 33 to 98 among all ACLSV peach isolates. Among all ACLSV-CP peach sequences, India20 sequence was the only sequence having valine and phenylalanine at positions 59 and 75, respectively, similar to apple (India1, 3, 4), plum (India17, 18) and quince (India23) isolates. It also indicated more variability at the amino acid level with sequence identity of less than 87.1%, supporting the criteria for difference in the species (Adams *et al.* 2004). The India21 and India19 (pear) ACLSV isolates (100% identity) were obtained from different corners of the same orchard, indicating spread by unclean horticultural practices of pruning.

Both the Indian isolates of ACLSV from plum (India17, 18) were 100% identical at the nucleotide and amino acid levels, and the only plum isolates to cluster with P205 type. The isolates were obtained from trees growing in different orchards. Phylogenetic analysis of all the available ACLSV plum isolates show that the Indian isolates are relatively closer to the SX/2 plum pseudopox isolate (B6 type) of ACLSV from Poland than any other ACLSV plum isolate although the sequence identity is 91% at the amino acid level, well within the criteria for difference in the species (Adams *et al.* 2004). Among plum isolates, the Indian sequences were unique in having valine and phenylalanine at positions 59 and 75, respectively.

The percentage sequence identity of 41 ACLSV apple isolates (16 Indian, characterized in this study, and 25 others) at the amino acid level ranged from 89% to100 % (data not shown). These isolates also fall into two distinct clusters, viz. P205 type and B6 type, as described earlier. The isolates also show variability at certain amino acid positions (table 3). The host range study of both India11 and India13 ACLSV isolate clearly points to the effect of difference in phylogeny and sequence variability (91% sequence identity at the amino acid level). The Indian ACLSV-quince isolate exhibited nucleotide and amino acid sequence identities of 84% and 87%, respectively, with the partial ACLSV-CP from Greece (Rana *et al*. 2008b). The Indian isolate clusters in P205 type, whereas the Greek isolates clusters with B6 type. The Indian pear isolate shares 91% sequence identity to the Kuerel isolate at the amino acid level (AAT75238), but they fall in different clusters (data not shown).

Earlier studies (Al Rawahnih *et al.* 2004) have reported quite good separation between ACLSV-CP isolates infecting *Prunus* on one hand and *Malus/Pyrus* on the other. However, it is quite interesting that in several cases our result showed very closely related ACLSV-CP isolates obtained from pome and stone fruits. This is evident from clustering of ACLSV plum (India17, 18), quince (India23) and peach (India22) with various ACLSV apple isolates from different locations in HP (figure 2a).

4.2 *Recombination analysis*

Recombination data is useful for defining evolutionary relationships of the isolates concerned. Recombination analysis of all available complete ACLSV-CP sequences from India and elsewhere gave five potential recombination events (PREs). However, only event 1 seemed significant (table 4) as it was detected by five of the recombination detection programs, viz. MAXCHI (average *P*-value=3.399×10^{04}), LARD (average *P*-value=1.166×10^{07}), CHIMAERA (average *P*-value=1.823×10^{02}), 3SEQ (average *P*-value=4.481×10^{06}) and BOOTSCAN (average *P*-value=2.877×10^{03}), with identical breakpoints between nucleotides from 186 to 498 in India7 (major parent) and India12 (minor parent) isolates. The presence of this recombination event has been depicted with the help of a graphical representation (figure 2), which clearly shows breakpoints and location of recombination sites. The breakpoints were also confirmed by drawing the phylogenetic tree of 1–185,186–498 and 499–582 nt of all the Indian ACLSV-CP isolates. All the sequences involved including the daughter isolate (India20, peach) fall in the P205 group, which has Ser40 and Tyr75, indicative of higher infectivity.

In event 3, recombination in plums between French (AAA42589, major parent) and India15 (AM882705, minor parent) isolates gave India9 apple, India24 apricot and Brazilian apple isolates with different breakpoints. However, this and other recombination events were detected by only one of the recombination program and with insignificant average *P*-values (table 4).

Individual recombination analysis for all the pome and stone fruit isolates was also carried out. Plum isolates indicated possible minor parenting by Indian isolates (India17, 18) for P863 (AAA42589), PBM1 (CAB46654) and major parenting for SX/2 (AAF67188), French (NP_ 040553) isolates with SX/2 and P863 isolates, respectively. All the ACLSV-peach sequences analysed by RDP indicated one recombination event in the Siscan programme, between the isolate HBP and India20 but with very low probability. Recombination events with insignificant average *P*-values were detected by single RDP for most of the Indian apple isolates among themselves and with Japanese (AB326230) ACLSV apple isolate. Recombination analysis performed for the ACLSV cherry, almond, apricot, plum, apple isolates individually and for all stone fruit isolates indicated no possible recombination events. Although a number of PREs were detected in analysis of ACLSV-CP isolates from peach and pome fruit isolates, none was found to be significant.

The pattern of closeness of *Maloidae*/*Prunus* ACLSV isolates (in phylogenetic and recombination analysis) might reflect transmission routes that may not be prevalent in other countries but have an effect in India. The interesting clustering of pome fruit isolates having different geographic origins needs to be further substantiated by further studies. The observation indicates the spread through infected planting materials like rootstocks and scions from state nurseries to farmers. One other important factor responsible for high sequence identities among pome and stone fruit isolates in India could be the mixed cultivation of these fruits. Vector (nematodes, bees), natural reasons (root graftings, pollen transmission) and unclean horticultural practices may also be the factors responsible.

The present analysis thus confirms the existence of differences in phylogeny among Indian ACLSV-CP isolates although they share high sequence identity (91–100% at the amino acid level). The variability among Indian isolates was supported by host range studies and molecular data analysis (indicating variation in amino acids). The lack of any significant recombination among isolates (Indian and world) points toward ACLSV- CP being a non-target sequence for virus recombination and subsequent evolution of the virus. Further studies to identify interacting domains and/or amino acids of CP in ACLSV are needed for the development of management strategies.

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