Characterization of an Indian isolate of Carnation mottle virus infecting carnations†

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Carnation (*Dianthus caryophyllus* L.) is an important cut-flower crop. It is susceptible to infection by several viruses, which cause significant losses to all types of carnations. Carnation mottle virus (CarMV) is one of the most important viruses among them. Ninety-three carnation cultivars collected from different parts of India were screened serologically with DAS-ELISA using polyclonal IgG. About 90% of the cultivars tested were found positive for CarMV, indicating the widespread nature of CarMV in India. CarMV was separated from other carnation viruses by host-range studies and maintained on *Saponaria vaccaria* and *Catharanthus roseus*. The virus was purified from infected *S. vaccaria* leaves and characterized by SDS–PAGE. Morphological studies of CarMV were conducted by electron microscopy and immune electron microscopy. The electron micrograph showed isometric particles of about 30 nm in diameter, typical of CarMV. Complete coat protein (CP) and movement protein (p7 and p9) genes of CarMV were amplified by RT–PCR with virus-specific primers. IC–RT–PCR was also used for sensitive detection of CarMV. Sequence alignment of the CP gene of Indian isolate of CarMV with other established isolates further confirmed the virus as CarMV. Though the amino acid sequence of CP was highly homologous, there are distinct differences. The Indian isolate is different from the already available classification of CarMV isolates. Isolates from the world belong to either the PK ($P^{164}K^{331}$) or AN ($A^{164}N^{331}$) group, while the Indian isolate belongs to a new group PN ($P^{164}N^{331}$). The p7 protein showed 85–98% amino acid similarity with the available protein sequences. The p9 protein showed 91–96% amino acid similarity with the available protein sequences of CarMV.

CARNATION (*Dianthus caryophyllus* L.) is one of the most important cut-flower crops grown worldwide on commercial scale, and it ranks among the top five cut-flowers. The main production areas are in western Europe, Latin America, Japan, eastern Europe and Australia. Other important areas are South East Asia, the US, Central America and Israel[1]. Cut-flower cultivation is also on the rise in India.

Carnation is susceptible to infection by several viruses that cause significant losses[2]. These are Carnation mottle virus (CarMV)[3], Carnation vein mottle virus (CVMV), Carnation etched ring virus (CERV), Carnation necrotic fleck virus (CNFV), Carnation latent virus (CLV) and Carnation ringspot virus (CRSV)[1]. Among them, CarMV is the most important and widespread virus[4]. CarMV is the type member of the genus *Carmovirus* and belongs to the family Tombusviridae. CarMV causes significant economic losses to farmers. Although its infection leads to mild symptoms, it causes severe infection in all types of carnations. This virus is responsible for the poor quality of cut-flower in terms of size, split calyces and reduced vigour, in addition to lesser yields in terms of lateral shoots, total number of flowers and fresh weight[2]. Virus infection not only affects the flower quality and shelf life, it also weakens the plant making it susceptible to infection by other pathogens present in nature. It can be easily transmitted by contact and cropping operations[1]. CarMV is considered relatively specific to carnation, but natural infection on other ornamental plants like *Begonia elatior* from Denmark[5] and *Daphne* sp. from New Zealand[6] has been reported. A survey in California demonstrated that the incidence was as high as 78% in flower-production greenhouses[7], while other studies have reported[8,9] incidence as high as 100%. Symptoms on carnation are generally indistinct, consisting of a mild mottling of the leaves, apparent only in soft growth. The wax on mature leaves of the plants masks the symptoms, so that the flowering plant may appear symptomless[4].

In the present study, CarMV occurring in carnations growing in different parts of India was screened using bioassay and ELISA. The virus was purified and characterized by SDS–PAGE. Morphological studies were carried out using Electron Microscopy (EM) and Immune Electron Microscopy (IEM). Viral coat protein (CP) and movement protein (MP) genes (*p7* and *p9*) were amplified using RT–PCR. Immunocapture-Reverse Transcription Polymerase Chain Reaction (IC–RT–PCR) was used for sensitive detection of CarMV. The amplified fragments were sequenced and analysed by alignment with other established strains of CarMV.

†The nucleotide sequence data here have been submitted in the EMBL nucleotide database under the accession numbers AJ549954 (CP), AJ584842 (P7) and AJ584843 (P9). Sequences of designed primers AOSCarMVCPu and AOSCarMVCPd have been submitted to the EMBL database under accession number AJ566191 and AJ566192 respectively.

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Experimental procedure

Host range and symptomatology

The leaf tissue of carnation was homogenized in 0.025 M sodium phosphate buffer, pH 7.6 and inoculated onto the leaves of *Catharanthus roseous*, *Chenopodium amaranticolor*, *Chenopodium quinoa*, *Cucumis sativus*, *Gomphrena globosa*, *Lycopersicon esculentum*, *Medicago sativa*, *Nicotiana clevelandii* and *Saponaria vaccaria*. The plants (maintained at 25–30°C) were observed for symptoms during 2–3 weeks post-inoculation.

Virus purification

Inoculated *S. vaccaria* plants harvested 8–10 days after inoculation were used for virus purification[10]. The partially pure virus preparation was further purified by sucrose density gradient.

SDS–PAGE

SDS–PAGE was performed as described earlier[11] with 12% resolving gel and 5% stacking gel. The gel was stained with Coomassie Brilliant Blue and then destained. The gel was photographed in the Gel Documentation System (Alpha Innotech Corp.).

DAS–ELISA

Ninety-three carnation varieties from different locations in India were screened by ELISA[12] using polyclonal antisera (Agdia, USA).

EM

The mid-vein portion of symptomatic leaves of *S. vaccaria* was chopped into ~1 mm pieces and placed in 3% glutaraldehyde for 2–3 h. They were then crushed with uranyl acetate and kept at 4°C overnight. EM grids were prepared with this extract.

IEM

Clarified crude virus extract (CVE) and clarified virus concentrate (CVC) were prepared from symptomatic *S. vaccaria* leaves and the grid was analysed as described earlier[13].

Primer design

The primers for CP and MP genes (*p7* and *p9*) were designed on the basis of conserved sequences obtained after multiple alignments of these sequences present in the NCBI database. The consensus sequences obtained at both the 5′ and 3′ regions were used as upstream and downstream primers respectively. The pair of primers, CPu-5′-ggggATCCgTATggAAAATAAAggAg-3′ and CPd 5′-AACTgCAgTCACATCCTATAAACAACCCATT-3′, was synthesized from Biobasic, Canada.

RNA isolation and RT–PCR

Total RNA was extracted from infected leaf tissue of *S. vaccaria* using Tri-reagent (Sigma, USA). For reverse transcription reaction (50 µl), 10 µl total RNA (~1–2 µg) was converted to cDNA using Mu–MLV RT as described by the manufacturer (Amersham Pharmacia Biotech).

Further amplification (in 37 cycles) of the cDNA was carried out in Mastercycler (Eppendorf) with Advantage HF2 PCR System (Clontech, USA) according to the manufacturer's instructions, annealing the template and primers at 57°C.

IC–RT–PCR was performed as described earlier[14]. The amplified product was electrophoresed in 1% (w/v) TAE agarose gel and stained with ethidium bromide (0.5 µg/ml). The DNA was visualized and photographed using a transilluminator. A 100 bp DNA ladder was used as size standard.

Cloning and sequencing of PCR-amplified CP and MP gene

The PCR-amplified DNA fragment was cut from the gel and eluted using gel extraction kit (QBIOGENE). The amplified PCR product was then cloned into pGEM-T easy vector (Promega, USA) using *Escherichia coli* JM101 by the standard molecular biology procedure[11]. Recombinant clones were identified and three clones of each CP and MP gene were sequenced using Sanger's dideoxy chain termination method[15] in an automated sequencer (ABI Prism 310, Applied Biosystems, USA) using Big Dye Terminator Sequencing v3 sequencing kit (Applied Biosystems, USA).

Sequence analysis

The nucleotide sequence was aligned with CP gene of CarMV from the database using BLAST[16]. The program BLASTP was used to search the amino acid sequence database. Pairwise comparisons were performed by the ALIGN-2 program utilizing the DOTHELEX algorithm[17]. Multiple alignments were generated by the MULTALIN program[18].

Results

Bioassay

The inoculated host plants *C. roseous*, *C. sativus*, *G. globosa*, *L. esculentum*, *N. clevelandii* and *S. vaccaria*, showed mosaic symptoms after 15 days of inoculation. *C. amaranti-*

color and *C. quinoa* showed chlorotic local lesions after 4 days of inoculation. *S. vaccaria* and *C. roseus* were used as maintenance and propagation host respectively for CarMV. *C. amaranticolor* proved to be the best bioassay host. The results of host-range experiments were finally confirmed by ELISA.

ELISA

Ninety-three carnation cultivars from different locations in India were screened serologically through DAS–ELISA technique. Among them, 87 cultivars (~93%) were found to be positive for the presence of CarMV (Table 1).

Table 1. Carnation cultivars used for ELISA test

Carnation cultivar	ELISA	Carnation cultivar	ELISA
Acca Pola	+	Liberty	+
Aicardi	+	Loris	+
Alima	+	M. Collette	+
Aliso	+	Malaga	+
Antille	+	Mastu	+
Ariane	+	Mercia	+
Arthur Sim	+	Michelle	+
Astrid	+	Middle Sand	+
Awanti No. 3	+	Mistral	+
Blade Pink	+	Monalisa	+
Bottom Nibera	+	New Espana	+
Bottom Norman	+	Nordika	–
Bright Levdevory	+	Oasis	–
Cabret	+	Orange Triumph	+
Candy	+	Paraoo Fancy	+
Castellero	+	Peachy Mambo	+
Charmint	+	Petra Alekptera	+
Charmour	+	Pink Aicardi	+
Cherrio	+	Pinkdona	+
Cherry Solar	–	Pintoo	+
Dalila	+	Pleasure	–
Delphe White	+	Prinidello	+
Dessio	+	Purple Chopin	+
Dona Brecas	+	Red Arhradite	+
Dusty Pink	+	Red Carso	+
Eastern Light	+	Roggio-de-sole	+
Equinoxe	+	Saleya	+
Espana	+	Salmanca	+
Etore	+	Scania	+
Firato	–	Shocking Pink	+
Flair	–	Solar	–
Forka	+	Sorriso	+
Garbo	+	Sunrise	+
Indalo	+	Super Green	+
Irma	+	Talima	+
Jack	+	Tasman	+
Josh	+	Tikal	+
Laspama	+	Top	+
Lavender Lace	+	Top Hidalgo	+
Leon	+	Top Solar	+
Liberty	+	Trendy	+
Loris	+	White Candy	+
M. Collette	+	White King	+
Malaga	+	White Pink	+
Mastu	+	White Wedding	+
Mercia	+	Yellow Candy	+
Michelle	+		

Virus purification

The virus was purified using an already established protocol[10] with infected *S. vaccaria* tissue. The purified virus was found to be infectious when inoculated on *C. amaranticolor* and *S. vaccaria*. When electrophoresed through SDS–PAGE, a single distinct band of 34 kDa was seen (Figure 1), which corresponds to the expected value reported for CarMV[19].

EM and IEM

EM examination of negatively stained preparations of CVC and CVE prepared from *S. vaccaria* leaves showed the presence of isometric virus particles 30 nm in diameter (Figure 2 *a* and *b*). IEM studies showed enhanced trapping with CarMV-specific antiserum (Figure 2 *b*)[20].

RT–PCR and IC–RT–PCR

RT–PCR was performed for the amplification of complete CP and MP genes (*p7* and *p9*), while IC–RT–PCR was also performed for CP gene amplification. Gel electrophoresis showed a single amplification product of ~1050 bp in *S. vaccaria* and carnation cultivar tested (Figure 3 *a*) for CP, while amplifications of ~200 and ~250 bp (Figure 3 *b*) were observed for the *p7* and *p9* genes respectively, as expected. Primers were specific and could amplify the complete CP gene and MP genes of the virus.

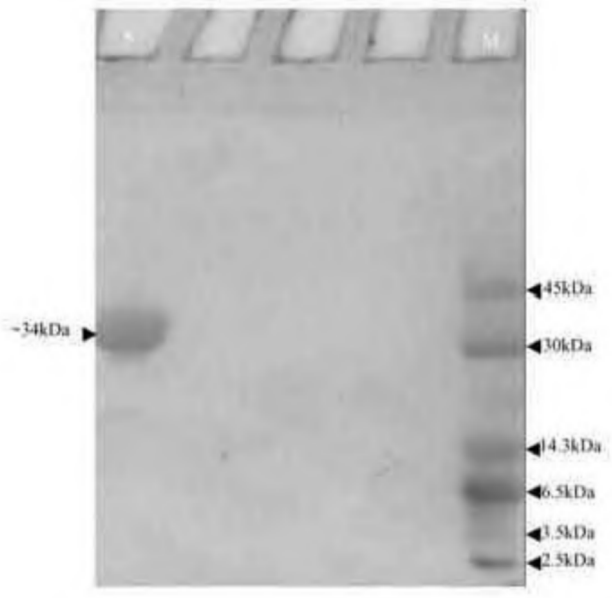

Figure 1. SDS–PAGE analysis of purified virus preparation revealing the presence of a single band of ~34 kDa corresponding to the coat protein of CarMV.

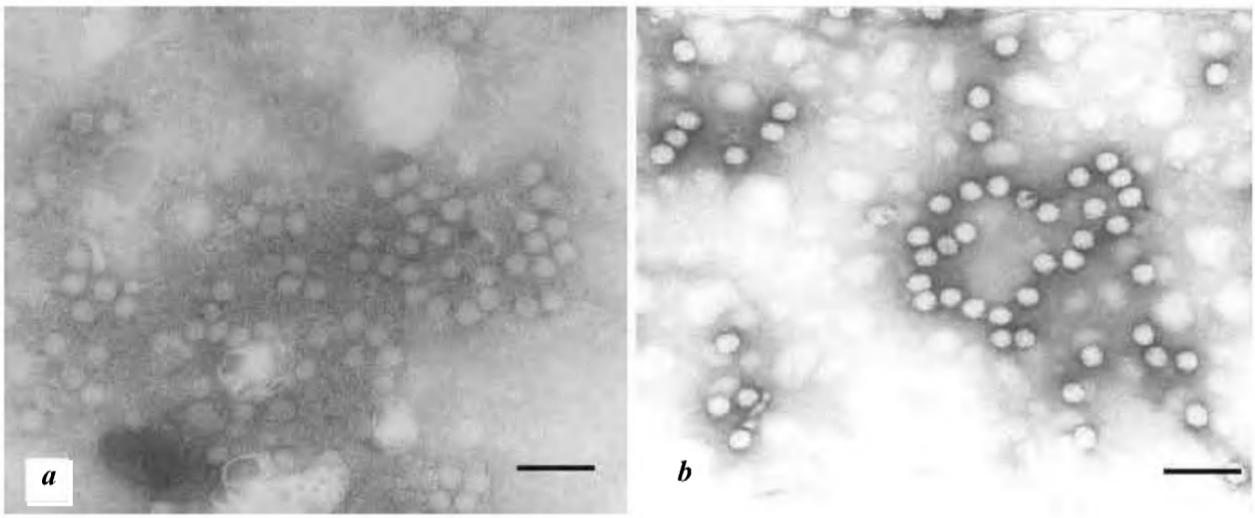

Figure 2. ***a***, Electron microscopic examination of negatively stained preparations from clarified virus extracts and clarified virus concentrates prepared from *C. roseous* leaves showing the presence of isometric virus particles 30 nm in diameter. Bar = 250 nm. ***b***, IEM showing enhanced trapping with CarMV-specific antiserum. Bar = 250 nm.

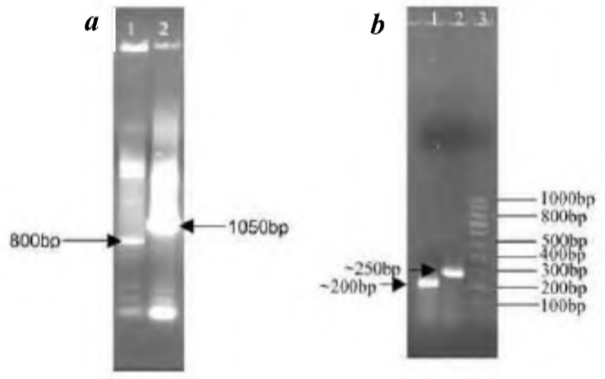

Figure 3. ***a***, Amplification of CarMV coat protein gene. Amplification is ~ 1050 bp in size. Lane 1, 100 bp marker; lane 2, ~ 1050 bp amplified fragment. ***b***, Amplification of *p7* and *p9* movement protein genes of CarMV. Lane 1, Amplification of *p7* gene (~ 200 bp); lane 2, Amplification of *p9* gene (~ 250 pb); lane 3, 100 bp marker.

Sequencing of amplified PCR product and sequence analysis

On sequencing, the cloned PCR product for the CP gene was found to be 1047 bp long, while the *p7* and *p9* genes were found to be 184 and 254 bp in length respectively. The sequences obtained were submitted to EMBL database (accession numbers AJ549954 (CP), AJ584842 (*p7*) and AJ584843 (*p9*)). The nucleotide sequences were translated to amino acid sequences and were aligned with other available sequences in the database. Figure 4 shows the alignment of CarMV CP (present study) with several other CarMV isolates. The sequence was 97% similar to other established isolates. However, there were distinct differences at 164 and 331 positions in the amino acid sequence. Figures 5 and 6 show the alignment of translated sequence of *p7* and *p9* with different strains of CarMV available in the database.

Discussion

In India CarMV occurrence was suspected since more than the last two decades, but no conclusive report was published. Bansal and Singh[21] reported that *C. amaranticolor* and *C. quinoa* were the local lesion hosts of CarMV. Furthermore, Singh and Singh[22] reported *Aphis gossypii* to be the vector of CarMV. *C. amaranticolor* and *C. quinoa* are local lesion hosts for many of the carnation viruses, including CVMV, CRSV and CLV. In general, Carmoviruses (type member CarMV) have not been shown to be transmitted by aphids, although other carnation viruses have *Chenopodium* sp. as the local lesion host. Further, the earlier data were based on host range and aphid transmission, and not supported by EM and serology. In the present study CarMV occurrence was established using EM, serological and nucleic acid-based techniques that are more reliable.

CarMV is present in more than 90% of the carnation cultivars screened through ELISA (Table 1). Due to the high incidence of infection of CarMV (though it may cause mild symptoms), accompanied by its various effects on carnation and flower production, CarMV needs to be considered as an important carnation virus. Its high incidence has also been recorded worldwide[1,9,23]. CarMV is therefore highly infectious. The high incidence of CarMV infection obtained by DAS–ELISA indicates its widespread nature and also the poor maintenance state of carnations in this part of the world. DAS–ELISA is the most commonly used indexing method for CarMV[2,24].

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                1                                                   50
Indian          MENKGEKIAM NPTVQALAQK GDKLAVKLVT RGWASLSTNQ KRRAEMLAGY
Spain           MENKGEKIAM NPTVQTLAQK GDKLAVKLVT RGWASLSTNQ KRRAEMLAGY
Italy           MENKGEKIAM NPTVQTLAQK GDKLAVKLVT RGWASLSTNQ KRRAEMLAGY
Netherlands     MENKGEKIAM NPTVQTLAQK GDKLAVKLVT RGWASLSTNQ KRRAEMLAGY
Japan           MENKGEKIAM NPTVQTLAQK GDKLAVKLVT RGWASLSTNQ KRRAEMLAGY
Australia       MENKGEKIAM NPTVQTLAQK GDKLAVKLVT RGWASLSTNQ KRRAEMLAGY
France          MENKGEKIAM NPTVQTLAQK GDKLAVKLVT RGWASLSTNQ KRRAEMLAGY
Colombia        MENKGEKIAM NPTVQTLAQK GDKLAVKLVT RGWASLSTNQ KRRAEMLAGY
USA             MENKGEKIAM NPTVQTLAQK GDKLAVKLVT RGWASLSTNQ KRRVEMLAGY
Israel          MENKGEKIAM NSTVQTLAQK GDKLAVKLVT RGWASLSTNQ KRRAEMLAGY
China           MENKGEKIAM NPTVQTLAQK GDKLAVKLVT RGWASLSTNQ KRRAEMLAGY
Consensus       MENKGEKIAM NpTVQtLAQK GDKLAVKLVT RGWASLSTNQ KRRaEMLAGY

               51                                                  100
Indian          TPAVLAFTPR RPRMTNPPPR TSRNSPGQAG KSMTMSKTEL LCTVKGTTGV
Spain           TPAILAFTPR RPRMTNPPPR TSRNSPGQAG KSMTMSKTEL LCTVKGTTGV
Italy           TPAILAFTPR RPRMTNPPPR TSRNSPGQAG KSMTMSKTEL LSTVKGTTGV
Netherlands     TPAILAFTPR RPRMTNPPPR TSRNSPGQAG KSMTMSKTEL LSTVKGTTGV
Japan           TPAILAFTPR RPRMTNPPPR TSRNSPGQAG KSMTMSKTEL LSTVKGTTGV
Australia       TPAILAFTPR RPRMTNPPPR TSRNSPGQAG KSMTMSKTEL LSTVKGTTGV
France          TPAILAFTPR RPRMTNPPPR TSRNSPGQAG KSMTMSKTEL LSTVKGTTGV
Colombia        TPAILAFTPR RPRMTNPPPR TSRNSPGQAG KSMTMSKTEL LSTVKGTTGV
USA             TPAILAFTPR RPRMTNPPPR TSRNSPGQAG KSMTVSKTEL LSTVKGTTGV
Israel          TPAVLAFTPR RPRMTNPPPR TSRNSPGQAG KSMTMSKTEL LSTVKGTTGV
China           TPAILAFTPR RPRMTNSPPR TSRNSPGQAG KSMTMSKTEL LCTVKGTTGV
Consensus       TPA!LAFTPR RPRMTNpPPR TSRNSPGQAG KSMTmSKTEL LsTVKGTTGV

               101                                                 150
Indian          IPSFEDWVVS PRNVAVFPQL SLLAMNFNKY RITALTVKYS PACSFETNGR
Spain           IPSFEDWVVS PRNVAVFPQL SLLATNFNKY RITALTVKYS PACSFETNGR
Italy           IPSSEDWVVS PRNVAVFPQL SLLATNFNKY RITALTVKYS PACSFETNGR
Netherlands     IPSFEDWVVS PRNVAVFPQL SLLATNFNKY RITALTVKYS PACSFETNGR
Japan           IPSFEDWVVS PRNVAVFPQL SLLATNFNKY RITALTVKYS PACSFETNGR
Australia       IPSFEDWVVS PRNVAVFPQL SLLATNFNKY RITALTVKYS PACSFETNGR
France          IPSFEDWVVS PRNVAVFPQL SLLATNFNKY RITALTVKYS PACSFETNGR
Colombia        IPSFEDWVVS PRNVAVFPQL SLLATNFNKY RITALTVKYS PACSFETNGR
USA             IPSFGDWVVS PRNVAVFPQL SLLATNFNKY RITALTVKYS PACSFETNGR
Israel          TPSFEDWVVS PRNVAVFPQL SLLATNFNKY RITALTVKYS PACSFETNGR
China           IPSFEDWVVS PRNVAVFPQL SLLATNFNKY RITALTVKYS PACSFETNGR
Consensus       iPSFeDWVVS PRNVAVFPQL SLLAtNFNKY RITALTVKYS PACSFETNGR

               151                                                 200
Indian          VALGFNDDAS DTPPTTKVGF YDLGKHVETA AQTAKDLVIP VDGKTRFIRD
Spain           VALGFNDDAS DTPPTTKVGF YDLGKHVETA AQTAKDLVIP VDGKTRFIRD
Italy           VALGFNDDAS DTPPTTKVGF YDLGKHVETA AQTAKDLVIP VDGKTRFIRD
Netherlands     VALGFNDDAS DTPPTTKVGF YDLGKHVETA AQTAKDLVIP VDGKTRFIRD
Japan           VALGFNDDAS DTPATTKVGF YDLGKHVETA AQTGKDLVIP VDGKTRFIRD
Australia       VALGFNDDAS DTPATTKVGF YDLGKHVETA AQTGKDLVIP VDGKTRFIRD
France          VALGFNDDAS DTPATTKVGF YDLGKHVETA AQTAKDLVIP VDGKTRFIRD
Colombia        VALGFNDDAS DTPATTKVGF YDLGKHVETA AQTAKDLVIP VDGKTRFIRD
USA             VALGFNDDAS DTPPTTKVGF YDLGKHVETA AQTAKDLVIP VDGKTRFIRD
Israel          VALGFNDDAS DTPPTTKVGF YDLGKHVETA AQTAKDLVIP VDGKTRFIRD
China           VALGFNDDAS DTPPTTKVGF YDLGKHVETA AQTAKDLVIP VDGKTRFIRD
Consensus       VALGFNDDAS DTPpTTKVGF YDLGKHVETA AQTAKDLVIP VDGKTRFIRD

               201                                                 250
Indian          SASDDAKLVD FGRIVLSTYG FDKANTVVGE LFIQYTIVLS DPTKTAKISQ
Spain           SASDDAKLVD FGRIVLSTYG FDKADTVVGE LFIQYTIVLS DPTKTAKISQ
Italy           SASDDAKLVD FGRIVLSTYG FDKADTVVGE LFIQYTIVLS DPTKTAKISQ
Netherlands     SASDDAKLVD FGRIVLSTYG FDKADTVVGE LFIQYTIVLS DPTKTAKISQ
Japan           SASDDAKLVD FGRIVLSTYG FDKADTVVGE LFIQYTIVLS DPTKTAKISQ
Australia       SASDDAKLVD FGRIVLSTYG FDKADTVVGE LFIQYTIVLS DPTKTAKISQ
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Figure 4. *Contd...*

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France      SASDDAKLVD FGRIVLSTYG FDKADTVVGE LFIQYTIVLS DPTKTAKISQ
Colombia    SASDDAKLVD FGRIVLSTYG FDKADTVVGE LFIQYTIVLS DPTKTAKISQ
USA         SASDDAKLVD FGRIVLSTYG FDKANTVVGE LFIQYTIVLS DPTKTAKISQ
Israel      SASDDAKLVD FGRIVLSTYG FDKADTVVGG LFIQYTIVLS DPTKTAKISQ
China       SASDDAKLVD FGRLVLSTYG FDKADTVVGE LFIQYTIVLS DPTKTAKISQ
Consensus   SASDDAKLVD FGRiVLSTYG FDKA#TVVGe LFIQYTIVLS DPTKTAKISQ

              251                                                300
Indian       ASNDKVSDGP TYVVPSVNGN ELQLRVVAAG RWCIIVRGTV EGGFTKPTLI
Spain        ASNDKVSDGP TYVVPSVNGN ELRLRVVAAG KWCIIVRGTV EGGFTKPTLV
Italy        ASNDKVSDGP TYVVPSVNGN ELQLRVVAAG KWCIIVRGTV EGGFTKPTLI
Netherlands ASNDKVSDGP TYVVPSVNGN ELQLRVVAAG KWCIIVRGTV EGGFTKPTLI
Japan        ASNDKVSDGP TYVVPSVNGN ELQLRVVAAG KWCIIVRGTV EGGFTKPTLI
Australia    ASNDKVSDGP TYVVPSVNGN ELQLRVVAAG KWCIIVRGTV EGGFTKPTLI
France       ASNDKVSDGP TYVVPSVNGN ELQLRVVAAG KWCIIVRGTV EGGFTKPTLI
Colombia     ASNDKVSDGP TYVVPSVNGN ELQLRVVAAG KWCIIVRGTV EGGFTKPTLI
USA          ASNDKVSDGP TYVVPSVNGN ELQLRVVAAG KWCIIVRGTV EGGFTKPTLI
Israel       ASNDKVSDGP TYVVPSVNGN ELQLRVVAAG KWCIIVRGTV EGGFTKPTLI
China        ASNDKVSDGP TYVVPSVNGN ELQLRVVAAG KWCIIVRGTV EGGFTKPTLI
Consensus    ASNDKVSDGP TYVVPSVNGN ELqLRVVAAG kWCIIVRGTV EGGFTKPTL!

             301                                                348
Indian       GPGISGDVDY ESARPIAICE LVTQMEGQIL NITKTSAEQP LQWVVYRM
Spain        GPGISGNVDY ESARPIAICE LVTQMEGQML HITKTSAEQP LQWVVYRM
Italy        GPGISGDVDY ESARPIAICE LVTQMEGQIL KITKTSAEQP LQWVVYRM
Netherlands GPGISGDVDY ESARPIAICE LVTQMEGQIL KITNTSAEQP LQWVVYRM
Japan        GPGISGDVDY ESARPIAVCE LVTQMEGQIL NITKTSAEQP LQWVVYRM
Australia    GPGISGDVDY ESARPIAVCE LVTQMEGQIL NITKTSAEQP LQWVVYRM
France       GPGISGDVDY ESARPIAVCE LVTQMEGQIL NITKTSAEQP LQWVVYRM
Colombia     GPGISGDVDY ESARPIAVCE LVTQMEGQIL NITKTSAEQP LQWVVYRM
USA          GPGISGDVDY ESARPIAICE LVTQMEGQIL KITKTSAEQP LQWVVYRM
Israel       GPGISGDVDY ESARPIAVCE LVTQMEGQIL KITKTSAEQP LQWVVYRM
China        GPGISGDVDY ESARPIAICE LVTQMEGQML KITKTSAEQP LKVVVYRM
Consensus    GPGISG#VDY ESARPIA!CE LVTQMEGQiL kITKTSAEQP LqwVVYRM
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Figure 4. Multiple sequence alignment of coat protein of different CarMV isolates inclusive of the Indian isolate of CarMV. Amino acids in bold italics show variable positions, whereas amino acids in bold are those on the basis of which a new group for CarMV was proposed, i.e. $P^{164} \rightarrow A^{164}$ correlating with $K^{331} \rightarrow N^{331}$.

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             1                                                  50
 Indian      MDIESEVPVI EKQMLAGNRG KQKTRRSVAK DAIRKPASDS TNGGNWVNVA
 USA         MDIESEVPVV EKQMLAGNRG KQKTRRSVAK DAIRKPASDS TNGGNWVNVA
 Spain       MDIESEVPVV EKQMLAGNRG KQKTRRSVAK DAIRKPASDS TNGGNWVNVA
 Spain       MDIESEVPVV GKQMLAGNRG KQKTRRSVAK DAIRKPASDS TNGGNWVNVA
 USA         MDIEPEVPVV EKQMLAGNSG KQKTRRSVAK DAIRKPASDS TNGGNWVNVA
 Spain       MDIEPEVPVV EKQMLAGNSG KQKTRRSVAK DAIRKPASDS TNGGNWVNVA
 Netherlands MDIEPEVPVV EKQMLAGNRG KQKTRRSVAK DAIRKPASDS TNGGNWVNVA
 Spain       MDIEPEVPVA EKQALAGNRG KQKTRRSVAK DAIRKPASDS TNGGNWVNVA
 Spain       MDIEPEVPVV GKQTLAGNRG KQKTRRSVAK DAIRKPASDS TNGGNWVNVA
 Italy       MDIEPEVPVV GKQTLAGNRG KQKTRRSVAK DAIRKPASDS TNGGNWVNVA
 Israel      MDIEPEVPVV GKQTLAGNRG KQKTRRSVAK DAIRKPASDS TNGGNWVNVA
 Australia   MDIEPEVPVV GKQTLAGNRG KQKTRRSVAK DAIRKPASDS TNGGNWVNVA
 Colombia    MDIEPEVPVV GKQTLAGNRG KQKTRRSVAK DAIRKPASDS TNGGNWVNVA
 Italy       MDIEPEVPVV GKQTLAGNRG KQKTRRSVAK DAIRKPASDS TNGGNWVNVA
 Netherlands MDIEPEVPVV GKQTLAGNRG KQKTRRSVAK DAIRKPASDS TNGGNWVNVA
 Spain       MDIEPEVPVV GKQMLAGNRG KQKTRRSVAK DAIRKPASDS TNGGNWVNVA
 Japan       MDIEPEVPVV GKQMLAGNRG KQKTRRSVAK DAIRKPASDS TNGGNWVNVA
 Spain       MDIEPEVSVV GKQTLAGNRG KQKTRRSVAK DAIRKPASDS TNGGNWVNVA
 France      MDIEPEVSVV GKQTLAGNRG KQKTRRSVAK DAIRKPASDS TNGGNWVNVA
 Spain       MDIEPEVPVV EKQTLVGNRG KQKTRRSVAK DAIRKPASDS TNGGNWVNVA
 Spain       MDIEPEVSVV GKQTLAGNRG KQKTRXSVAK DAIRKPASDS TNGGIWVNVA
 Israel      MDIEPEVPVV GKQMLTGNRG KQKTRRSGGQ DAIRKPASDN ANGGNWVNVA
Consensus    MDIEpEVpV! gKQmLaGNRG KQKTRrSvak DAIRKPASDs tNGGnWVNVA

             51        61
 Indian      DKIEVHIHFN F
 USA         DKIEVHIHFN F
 Spain       DKIEVHIHFN F
 Spain       DKIEVHIHFN F
 USA         DKIEVHIHFN F
 Spain       DKIEVHIHFN F
 Netherlands DKIEVHIHFN F
 Spain       DKIEVHIHFN F
 Spain       DKIEVHIHFN F
 Italy       DKIEVHIHFN F
 Israel      DKIEVHIHFN F
 Australia   DKIEVHIHFN F
 Colombia    DKIEVHIHFN F
 Italy       DKIEVHIHFN F
 Netherlands DKIEVHIHFN F
 Spain       DKIEVHIHFN F
 Japan       DKIEVHIHFN F
 Spain       DKIEVHIHFN F
 France      DKIEVHIHFN F
 Spain       DKIEVHIHFN F
 Spain       DKIEVHIHFN F
 Israel      DKIEVHIHFN F
Consensus    DKIEVHIHFN F
```

Figure 5. Multiple sequence alignment of translated sequences of *p7* gene of CarMV isolates.

```
                1                                                   50
Indian          MPSANLHLIV LTGEIGLMLL IRINCTFTST FSLPPLVTLN QIIALSFCGL
USA             MPSVNLHLIV LTGVIGLMLL IRLRCTFTST FSLPPLVTLN QIIALSFCGL
Spain           MPSVNLHLIV LTGVIGLMLL IRLRCTFTST FSLPPLVTLN QIIALSFCGL
Spain           MPSVNLHLIV LTGVIGLMLL IRLRCTFTST FSLPPLVTLN QIIALSFCGL
Spain           MPSVNLHLIV LTGVIGLMLL IRLRCTFTST FSLPPLVTLN QIIALSFCGL
USA             MPSVNLHLIV LTGVIGLMLL IRLRCTFTST FSLPPLVTLN QIIALSFCGL
Spain           MPSVNLHLIV LTGVIGLMLL IRLRCTFTST FSLPPLVTLN QIIALSFCGL
Spain           MPSANLHLIV LTGVIGLMLL IRLRCTFTST FSLPPLVTLN QIIALSFCGL
Spain           MPSANLHLIV LTGVIGLMLL IRLRCTFTST FSLPPLVTLN QIIALSFCGL
Australia       MPSANLHLIV LTGVIGLMLL IRLRCTFTST FSLPPLVTLN QIIALSFCGL
Colombia        MPSANLHLIV LTGVIGLMLL IRLRCTFTST FSLPPLVTLN QIIALSFCGL
France          MPSANLHLIV LTGVIGLMLL IRLRCTFTST FSLPPLVTLN QIIALSFCGL
Netherlands     MPSANLHLIV LTGVIGLMLL IRLRCTFTST FSLPPLVTLN QIIALSFCGL
Netherlands     MPSANLHLIV LTGVIGLMLL IRLRCTFTST FSLPPLVTLN QIIALSFCGL
Spain           MPSANLHLIV LTGVIGLMLL IRLRCTFTST FSLPPLVTLN QIIALSFCGL
Italy           MPSANLHLIV LTGVIGLMLL IRLRCTFTST FSLPPLVTLN QIIALSFCGL
Israel          MPSANLHLIV LTGVIGLMLL IRLRCTFTST FSLPPLVTLN QIIALSFCGL
Spain           MPSANLHLIV LTGVFGLMLL IRLRCTFTST FSLPPLVTLN QIIALSFCGL
Spain           MPSVNLHLIV LTGVIGLMLL TRLRCTFTST FSLPPLVTLN QIIALSFCGL
Italy           MPSANLHLIV LTGVIGLMLL TRLRCTFTST FSLPPLVTLN QIIALSFCGL
Japan           MPSVNLHPIV LTGVIGLMLL IRLRCTFTST FSLPPLVTLN QIIALSFCGL
Israel          MPSVNLHLIM LTGVIGLMLL IRLRCTFTST FSLPPLVTLN QIIALPFCGL
Consensus       MPSaNLHLIV LTGvIGLMLL iRlrCTFTST FSLPPLVTLN QIIALSFCGL

                51                                  84
Indian          LLNSISRAER ACYYNYSVDS SKQQHISVST PNGK
USA             LLNSISRAER ACYYNYSVDS SKQQHISIST PNGK
Spain           LLNSISRAER ACYYNYSVDS SKQQHISIST PNGK
Spain           LLNSISRAER ACYYNYSVDS SKQQHISIST PNGK
Spain           LLNSISRAER ACYYNYSVDS SKQQHISIST PNGK
USA             LLNSISRAER ACYYNYSVDS SKQQHISIST PNGR
Spain           LLNSISRADR ACYYNYSVDS SKQQHISIST PNGK
Spain           LLNSISRAER ACYYNYSVDS SKQQHISIST PNGK
Spain           LLNSISRAER ACYYNYSVDS SKQQHISIST PNGK
Australia       LLNSISRAER ACYYNYSVDS SKQQHISIST PNGK
Colombia        LLNSISRAER ACYYNYSVDS SKQQHISIST PNGK
France          LLNSISRAER ACYYNYSVDS SKQQHISIST PNGK
Netherlands     LLNSISRAER ACYYNYSVDS SKQQHISIST PNGK
Netherlands     LLNSISRAER ACYYNYSVDS SKQQHISIST PNGK
Spain           LLNSISRAER ACYYNYSVDS SKQQHISIST PNGK
Italy           LLNSISRAER ACYYNYSVDS SKQQHISIST PNGK
Israel          LLNSISRAER ACYYNYSVDS SKQQHISIST PNGK
Spain           LLNSISRAER ACYYNYSVDS SKQQHISIST PNGK
Spain           LLNSISRAER ACYYNYSVDS SKQQHISIST PNGK
Italy           LLNSISRAER ACYYNYSVDS SKQQHISIST PNGK
Japan           LLNSISRAER ACYYNYSVDS SKQQHISIST PNGK
Israel          LLNSISRAER ACYYNYSVDS SKQQHISIST PNGK
Consensus       LLNSISRAER ACYYNYSVDS SKQQHIS!ST PNGK
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Figure 6. Multiple sequence alignment of translated sequences of *p9* gene of CarMV isolates.

Host-range results were in agreement with the earlier results[1,2,8]. CarMV was purified using the method described earlier[10] and used for morphological studies through EM. Isometric particle could be seen typical of CarMV[25]. Presence of CarMV was further strengthened by ISM studies that showed enhanced trapping with CarMV-specific antiserum and by SDS–PAGE analysis of the purified virus that revealed a single band of ~ 34 kDa (Figure 1), as has been reported[21].

RT–PCR and IC–RT–PCR (for CP only) using designed primers were also carried out for detection of this virus through amplification of CP, *p7* and *p9* genes. RNA isolated from different sources (purified preparation and field samples) gave expected results in RT–PCR and IC–RT–PCR.

The sizes of MP genes were identical to earlier reports. The p7 protein showed 85–98% amino acid similarity with the available protein sequences, while the nucleotide similarity was 94–98%. Similarly, the p9 protein showed 91–96% amino acid similarity with the available protein sequences. The amino acid sequences of p7 showed only one difference at the 10th position in the Indian isolate. Here the small aliphatic valine is replaced by the larger aliphatic isoleucine (Figure 5).

In case of the translated sequence for the *p9* gene, the Indian isolate differs at two positions, i.e. 14th and 24th. At the 14th position aliphatic, hydrophobic valine was replaced by charged, polar glutamic acid, while at the 24th position polar, charged arginine was replaced by un-

charged, polar asparagine (Figure 6). The effect of mutations at positions 164 and 331 could not be seen on symptomatology, host range or biological characteristics of the virus. The functional effects of these changes need to be investigated.

The size of CP is in accordance with that in an isolate characterized earlier[26]. Further, the sequence was highly conserved and found to be >95% similar at nucleotide and >97% similar at the amino acid level. The high level of sequence conservation has been reported earlier as well[26], where the authors studied 23 different CarMV isolates and obtained highly similar sequences. However, in the 23 isolates that were characterized, there was a remarkable co-variation in the amino acid sequence for CP between position Pro^{164} (located in the S-domain) and Lys^{331} (located in the P-domain). Proline at position 164 correlated with lysine at 331 in all the cases except an isolate 'SP-S', where histidine[26], another basic residue, was found at 331. The change in Pro^{164} (P) → Ala (A) correlated with the change in Lys^{331} (K) → Asn (N). Brunt has suggested the existence of tertiary interactions between these regions of the molecule and the isolates were classified as groups PK and AN[26]. However, our results are an exception and unique to India, as they do not fall in either of the two. The isolate characterized in the present study has P at 164 and N at position at 331. It has not been the case in any of the earlier characterized isolates. It should therefore be placed in a new group, i.e. PN. It must be emphasized, however, that the functional relevance of a change in $P^{164} \rightarrow A^{164}$ correlating with $K^{331} \rightarrow N^{331}$ needs to be established. Further, there are a number of residues where the amino acid sequence of the CP of the present isolate differs from established isolates. At position 16 there is alanine, while in other isolates this position is conserved for threonine. Similarly, positions M^{125}, N^{225} and R^{231} also differ and have T, D and K respectively, in other isolates (Figure 4).

Molecular studies carried out by us will help in the complete characterization of CarMV isolates from India. This will also help in developing diagnostics and hence raising virus-free/resistant plants.

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