Diagnosis of a new variant of soybean yellow mottle mosaic virus with extended host-range in India

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Abstract Soybean yellow mottle mosaic virus (SYMMV, genus *Carmovirus*) was previously known to occur in South Korea and USA causing bright yellow mosaic in soybean. In this study, SYMMV (Car-Mb14 isolate) was isolated from mungbean (*Vigna radiata*) exhibiting mild mottling and puckering symptoms in the experimental field at Indian Agricultural Research Institute, New Delhi during 2012. The virus isolate, Car-Mb14 induced veinal mottling, mild mottling, chlorotic blotching, local and systemic necrosis in soybean, mungbean, blackgram, French bean and guar bean, respectively. The symptomatology of the present isolate of SYMMV was different from the previously reported South Korean isolate, as the later did not induce symptoms in any of the above legumes other than soybean. The present isolate was phylogenetically distinct and shared 90–93 % sequence identity in coat protein (CP) of 52 SYMMV isolates reported from Korea and USA. In order to know the serological relationships, the CP gene of the present isolate was over expressed as a 39 kDa protein in *E. coli* and an antiserum of 1:16,000 titer against the recombinant CP was produced. Serological cross reactivity analysis revealed that SYMMV was serologically related to blackgram mottle virus but not to cowpea mottle virus, the other legume infecting carmoviruses. The antiserum was used to detect prevalence of SYMMV in legume crops by

ELISA. Out of 145 field samples of legumes (mungbean, blackgram, French bean and soybean) collected from different places in India, SYMMV was detected only in 16 samples of mungbean and one sample of blackgram. The natural infection of SYMMV in mungbean and blackgram was further confirmed based on CP gene sequence. This study provides evidence of occurrence of a new variant of SYMMV with distinct symptom phenotype and extended host-range in India.

Keywords Carmovirus · Blackgram mottle virus · Soybean yellow mottle mosaic virus · Host reaction · ELISA · Recombinant coat protein · Western blot

Introduction

Soybean yellow mottle mosaic virus (SYMMV), a member of the genus *Carmovirus* (Family: *Tombusviridae*) has been reported only from North America and South Korea [7, 11]. SYMMV has isometric virion of 28–30 nm, which contains a single copy of positive sense ssRNA of 4009 nucleotides (nt) lacking the 3' poly (A) tail and 5' cap structure. The viral genome encodes six ORF's: ORF1 and ORF2 encode replicase associated proteins; ORF 3 and ORF 4 encode the double gene block proteins required for *in planta* movement of the virus, ORF5 encodes a coat protein (CP) and ORF6 is of unknown function. Host range studies of SYMMV revealed that SYMMV produced symptoms only on soybean (*Glycine max*) and it was experimentally transmitted to only mungbean, which although did not produce any symptoms [11].

Mungbean (*Vigna radiata*) is one of the most important legume crop in India. Several viruses, mungbean yellow mosaic virus [8], groundnut bud necrosis virus (GBNV)

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[18], cowpea mild mottle virus [10], bean common mosaic virus [5] and blackgram mottle virus (BMoV) [12] are known to cause yellow mosaic, bud necrosis, mottle mosaic, mosaic and mottle diseases, respectively in mungbean in India. However, so far, natural infection of mungbean by SYMMV is neither known in India nor in any other country.

During 2012 cropping season, mungbean (*V. radiata*) plants were observed to develop systemic mild mottling and puckering in the experimental farm of Indian Agriculture Research Institute (IARI), New Delhi. The symptomatic mungbean leaves contained isometric particles (28–30 nm) and moderately reacted with the antiserum to BMoV (Ac Diagnostics, Fayetteville, USA) in DAC-ELISA suggesting that the virus could be a member of the genus *Carmovirus*. The further investigations based on transmission, host reactions, purification, serology and CP sequence analysis provide evidence of natural infection of mungbean by a novel strain of SYMMV in India.

Materials and methods

Virus isolate

Surveys were undertaken to detect carmovirus in blackgram and mungbean crops in the experimental fields of IARI during 2012–2013. The leaf samples showing mild mottling symptoms were tested by ELISA with the polyclonal antiserum to BMoV. The leaf dip transmission electron microscopy (JEM-1011 model) was conducted by staining the grids with 2 % uranyl acetate. For sap inoculation, the inoculum was prepared (1:2 w/v) in 0.1 M sodium phosphate buffer, pH 7.2, containing 0.1 % β -mercapto ethanol and the leaves of French bean (*Phaseolus vulgaris*) cv. Pusa Parvati predusted with Carborundum (600 mesh) was inoculated. An isolate (Car-Mb14) of the virus was established and maintained on French bean cv. Pusa Parvati by periodic sap inoculation.

Sap transmission to different plant species

In order to study the host reactions, French bean (cv. Pusa Parvati) samples containing isometric particles or testing positive by DAC-ELISA were homogenized in 0.1 M sodium phosphate buffer and the sap was used to inoculate leaves of different plant species listed in the Table 1. Ten seedlings of each plant species were inoculated in an environment controlled greenhouse at 26–28 °C at the National Phytotron Facility (NPF), IARI. The inoculation experiment was repeated twice. Inoculated plants were regularly observed for symptom expression and infection was confirmed by electron microscopy, ELISA and RT-

PCR. The progeny virions derived from these alternate hosts were used to back inoculate mungbean plants and infection was confirmed by DAC-ELISA and RT-PCR.

Virus purification

Virus was purified from 100 g of frozen leaf tissues of French bean cv. Pusa Parvati collected at 20–25 days post inoculation (dpi). The extract was clarified with 10 % chloroform for 15 min and the supernatant was centrifuged at 100,000g for 90 min at 4 °C on 10 % sucrose cushion. The pellets were resuspended in 0.01 M phosphate buffer, pH 7.0 and examined in electron microscope. The molecular weight (MW) of the viral CP subunit was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using 12 % resolving gel and 5 % stacking gels. The gel was stained with Coomassie Brilliant Blue solution (Sigma-Aldrich Corp., St. Louis, MO, USA). The Western blot was conducted using antiserum to BMoV.

RT-PCR

RNA extraction was carried out from symptomatic mungbean leaves as well as from purified virus preparation (1000 μ g) using RNeasy Plant Mini Kit (Qiagen, Chatsworth, CA). Virion RNA was separated on 1 % denaturing agarose gel containing formaldehyde and ethidium bromide [14]. Two pairs of degenerate primers were designed based on available sequences of the legume infecting carmoviruses viz., *Cowpea mottle virus* (CPMoV) (NC_003535, U20976) and SYMMV (FJ457015, FJ707484, NC_011643) (Table 2). Based on the sequenced information generated from the Car-Mb14 isolate using the degenerate primers, another pair of primers was designed to amplify the complete CP gene (Table 2). The first strand cDNA was synthesized in a 20.0 μ l reaction mixture containing 4.0 μ l of 5 $\times$ first strand buffer, 4.0 μ l of 2.5 mM dNTP mix, 1.0 μ l of RNase inhibitor, 1.0 μ l of 10 μ M reverse primer, 1.0 μ l of SuperScriptTM III Reverse Transcriptase enzyme (200 U/ μ l, Invitrogen, Carlsbad, CA), 5.0 μ l of RNA template (400–500 ng) and the final volume was adjusted with nuclease free water. The mixture was subjected to 25 °C for 5 min followed by 50 °C for 60 min and was inactivated at 70 °C for 15 min using a thermal cycler (Biometra T personal, Germany). The PCR was conducted in 50 μ l reaction mixture containing 2.0 μ l of cDNA, 5.0 μ l of 10 $\times$ *ExTaq* Buffer, 4.0 μ l of 2.5 mM dNTP mix, 2.0 μ l of 10 μ M of each primer, 0.25 μ l of (1.25 U) of *TaKaRa Ex Taq[®]* DNA polymerase (TaKaRa, Japan) and nuclease-free water to make up the volume. The reaction mixture was subsequently subjected to 35 cycles, each consisting of denaturing at 94 °C for 45 s, primer

Table 1 Responses of different plant species following sap inoculation with Car-Mb14 isolate

Plant species	No of symptomatic plants out of ten inoculated			Symptoms ^b			Confirmation	
	Rep I	Rep II	Local	Dpi ^a	Systemic	Dpi	ELISA ^c	RT-PCR ^d
Amaranthaceae								
<i>Spinacia oleracea</i>	0	0	Ns	0	Ns	0	—	Nt
Cucurbitaceae								
<i>Benincasa hispida</i>	0	0	Ns	0	Ns	0	Nt	Nt
<i>Citrullus lanatus</i> cv. Sugar Baby	0	0	Ns	0	Ns	0	—	Nt
<i>Cucumis sativus</i>	0	0	Ns	0	Ns	0	Nt	Nt
<i>Lagenaria siceraria</i>	0	0	Ns	0	Ns	0	Nt	Nt
<i>Luffa aegyptiaca</i>	0	0	Ns	0	Ns	0	Nt	Nt
Leguminaceae								
<i>Arachis hypogaea</i>	3	2	Mcl	30	Ns	0	—	Nt
<i>Cyamopsis tetragonoloba</i>	10	10	Nls	7–8	Vn	15–20	+	+
<i>Glycine max</i> cv. Pusa-22	10	10	Mo	7–10	Vmm	15–18	+	+
Pusa-9712	10	10	Mo	7–10	Vmm	15–18	+	+
<i>Phaseolus vulgaris</i> cv. Anupama	4	5	Mo	7–9	Mo, P	17–19	+	Nt
Chitra	7	6	Nls	3–4	Mmo	13–14	+	Nt
Contender	8	9	Mo	5–7	Clb	20–23	+	Nt
Kentucky Wonder	10	10	Nls	6–8	Clb	20–25	+	Nt
Pusa Parvati	10	10	Mo	5–7	Clb, Mo &P	20–25	+	+
Roshini	0	0	Ns	0	Ns	0	—	Nt
Selection-8	10	10	Nls	5–8	Clb	15–17	+	Nt
<i>Vigna mungo</i> cv. Co-5	10	10	Uc, Ve	6–7	Mm	15–17	+	Nt
Bhalabu Urd	10	10	Vn	6–7	Clb	17–19	+	+
Bharabanki local	9	8	Mo	8–9	Mo	18–20	+	Nt
<i>Vigna radiata</i> cv. Pusa Vishal	10	10	Nls, Vn	6–7	Mm	15–20	+	+
SML-668	10	10	Nls	7–9	Mm	18–20	+	+
<i>Vigna unguiculata</i> cv. Pusa Komal	0	0	Ns	NS	Ns	0	—	+
Solanaceae								
<i>Nicotiana benthamiana</i>	0	0	Ns	0	Ns	0	—	Nt
<i>Solanum lycopersicum</i>	0	0	Ns	0	Ns	0	—	Nt

^a Days after post inoculation^b Key to symptoms: *Clb* chlorotic blotches, *LR* leaf rolling, *Mcl* marginal chlorosis, *Mo* mottling, *Mm* mild mottle, *Mmo* mild mosaic, *Ns* no symptoms, *Nls* necrotic lesions, *P* puckering, *Uc* upward cupping, *Ve* veinal enation, *Vn* veinal necrosis, *Vmm* veinal mild mottle^c Absorbance value at 405 nm (1 h reading): 0.134–3.00; Healthy plant sample value: 0.051–0.271; +, positive results; —, negative results; Nt, not tested^d RT-PCR was carried out with primers RKJ224F and RKJ225R which amplifies coat protein region

annealing at 63 °C for 45 s, and extension at 72 °C for 1 min 20 s with the final extension at 72 °C for 10 min. The PCR product was analyzed in 1.5 % agarose gel. The specific band was excised and eluted using the SV Wizard PCR clean up system (Promega, Madison, WI, USA).

Molecular cloning and sequence analysis of coat protein

The PCR product was ligated in pGEM-T easy vector (Promega, Madison, WI, USA). The selected recombinant

clones were sequenced using Sanger's dideoxy method at Department of Biochemistry, University of Delhi, South Campus, New Delhi, India. The sequence of cloned fragment was assembled using BioEdit [3], and the amino acid sequence was deduced using Expasy translate tool [2]. For comparison of the sequence with the other carmoviruses, multiple alignments were conducted and a sequence identity matrix was prepared using BioEdit software. For the comparison of the isolates color graph was constructed based on pair wise percent sequence identity of the CP using Microsoft Excel (Version 2007). The phylogenetic

Table 2 Details of the primers designed for detecting *Carmovirus* in legume samples

Name	Primer sequence (5' to 3') ^a	Annealing temperature (°C)	Expected size (bp)	Region of the genome ^b
RKJ199F	tgagytatggggwactcttatg	57	460 bp	Partial CP region
RKJ200R	gggwyraksagytggagmtgta			
RKJ207F	tggcwtgtgcyatyacgtgg	61	2.3 kbp	Middle of RdRp to end of CP region
RKJ206R	cacccccacccgaaggatttctg			
RKJ224F	atgaattcatgaatgaacaatgctcaccgt	67	1065 bp	Complete CP region
RKJ225R	caaagcttagtgtgtgggttttaactggcgttgta			

CP coat protein, RdRP RNA dependent RNA polymerase

^a Restriction sites used for cloning purpose were underlined

^b Region of the genome amplified with the degenerate primers designed from *Cowpea mottle virus* (CPMoV; NC_003535, U20976) and *Soybean yellow mottle mosaic virus* (SYMMV; FJ457015, FJ707484, NC_011643)

Fig. 1 Symptoms in leguminous plant species followed by mechanical sap inoculation of SYMMV isolate Mb-14. Pinpoint necrotic spots on the inoculated leaf and systemic veinal necrosis on guarbean (a, b). Veinal mild mottling on systemic leaves of soybean (c). Systemic chlorotic blotches, puckering and leaf deformation symptoms on frenchbean cv. Pusa Parvati (d, e). Systemic mild mottling in mungbean cv. SML 668 (f). Chlorotic blotches on blackgram cv. Bhalabhu urd (g). Detection of the Car-Mb14 in different plant species by RT-PCR using coat protein specific primers (h, i) (RKJ224F and RKJ225R; *M1* GeneRuler 1 Kb DNA Ladder, *M2* GeneRuler 1 Kb plus DNA Ladder, *Lanes* B guar bean, C soybean, D, E French bean, F greengram, G blackgram)

analysis was carried out in MEGA 6.0 [17]. The evolutionary history of Car-Mb14 with the other carmoviruses was inferred using the neighbor-joining (NJ) method. The phylogenetic relationship of Car-Mb14 with other SYMMV isolates, based on the CP sequence available in the database was determined by maximum parsimony analysis in Mega 6.0.

Preparation of coat protein gene expression construct

The CP gene was further sub-cloned at *EcoRI* and *HindIII* restriction sites in pET28a (+) expression vector (Novagen, San Diego, USA) and transformed into *E. coli* BL21 (DE3) cells. A single clone containing the complete Car-

Mb14 CP in the correct open reading frame was selected for expression studies.

Expression of recombinant protein

Expression of recombinant CP was carried at 2, 3, 4, 5 and 6 h after induction with 1.0 mM of isopropyl- β -D-thiogalactopyranoside (IPTG) at 37 °C. Electrophoresis was carried out at 80 V for 2 h and the gel was stained with Coomassie brilliant Blue R 250 stain. The antigenicity of the recombinant protein was determined using antiserum to BMoV (1:200) in Western blots.

Production of polyclonal antibodies (PAb)

The expressed recombinant CP was purified from *E. coli* cells under denaturing conditions (6 M urea) using His-Bind purification Kit (Novagen, San Diego, USA). The purified protein was dialyzed using Small Wonder Lyser Kit (Promega, Madison, USA) to remove the salt impurities, lyophilized and quantified using NanoDropTM1000 spectrophotometer (Thermo Scientific, Willington, USA). Immunization was performed by injecting the purified recombinant protein (500 μ g) with an equal volume of Freund's incomplete adjuvant (Genei, Bangalore, India) intramuscularly in New Zealand white rabbit weekly for 5 weeks. 1 week after the last injection, the rabbits were bleed thrice at weekly interval. The serum was collected, mixed with 100 % glycerol (1:1, v/v) and stored at -80 °C.

Evaluation of PAb by ELISA

The antiserum was cross absorbed with the healthy leaf sap of French bean in PBS-TPO at 1:1 dilution (w/v) for 1 h at 37 °C. To determine the titer of the antiserum, indirect DAC ELISA with goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma, St. Louis, USA, 1:30,000) was performed using twofold dilution series of the antiserum [1]. The ELISA reaction was read at 405 nm, 1 h after the addition of substrate (p-nitro phenyl phosphate, 0.5 mg/ml; Sigma, St. Louis, USA) by using an ELISA reader (BIO-TEK instruments, Winooski, USA). The antiserum was used for the detection of the virus in the plant samples collected from the different locations. The selected ELISA positive samples were further confirmed by generating sequence information of CP gene. The cross reactivity of the antisera to Car-Mb14 and other carmoviruses namely, BMoV, Carnation mottle virus (CarMV), and CPMoV was analysed by ELISA using purified virus preparation of CarMb-14 isolate.

Fig. 2 Virion preparations resulting from typical carmovirus isolation protocols stained with 2 % (wt/vol) uranyl acetate and analyzed by electron microscopy (a). Separation of virion RNA prepared from purified virus on 1 % denaturing agarose gel containing formaldehyde and ethidium bromide. Lane M RNA MW marker, Lane 1 genomic RNA (b). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of isolated Car-Mb14 virus virions followed by staining with Coomassie brilliant blue (c). Lane M protein marker (blueye prestained protein marker)

Results

Field symptoms and virus detection

Out of 24 mungbean samples tested, 10 samples that showed mild mottle and puckering symptoms (cvs Pusa Vishal and SML-668) reacted with the antiserum to BMoV. However, none of the 30 blackgram samples tested by ELISA was positive. Isometric virus particles similar to carmovirus (~28–30 nm) were observed in ELISA positive samples of mungbean but not in blackgram samples. An isolate (Car-Mb14), that was positive by ELISA and showed presence of the isometric virions was readily sap transmitted to French bean cv. Pusa Parvati, which developed chlorotic blotches, mottling and puckering symptoms. RT-PCR with the degenerate primers to the conserved CP (RKJ199F and RKJ200R) resulted in amplification of a DNA band of 468 nt, which shared 89.0 and 94.0 % identity at nucleotide and amino acid level with the corresponding region of SYMMV (FJ494876) respectively, indicating the Car-Mb14 is an isolate of SYMMV.

Host responses

Out of 15 plant species inoculated, Car-Mb14 was transmitted to only six plant species of the family Leguminaceae (Table 1). The virus was highly sap transmissible to blackgram (*Vigna mungo*), guar bean (*Cyamopsis tetragonoloba*), French bean (*P. vulgaris*), mungbean (*V. radiata*) and soybean (*G. max*), where 100 % of the inoculated plants were infected by 5–10 dpi. The infected mungbean plants showed local necrotic specks at 6–7 dpi, and systemic symptoms such as mild mottle at 15–20 dpi. Symptoms on blackgram and French bean varied with the cultivars. The blackgram cultivar Co-5 showed leaf malformation with upward cupping and tip necrosis; cultivar Barabanki local developed systemic mottling; cultivar Bhalabu Urd developed necrotic specks, veinal necrosis on the inoculated leaves and systemic symptoms such as chlorotic blotches (Fig. 1g). The French bean cultivars Anupama, Contender and Pusa Parvati developed mottling symptoms in the inoculated leaves, whereas cvs Chitra, Kentucky Wonder, and Selection-8 developed small local necrotic spots and systemic mild chlorotic blotches, mild leaf rolling and puckering symptoms (Fig. 1d, e). The cv. Chitra however showed mild mosaic symptoms. The cv. Roshini did not develop any local or systemic symptoms. Guar bean (*C. tetragonoloba*) developed pin-pointed necrotic spots on the inoculated leaves at 7–8 dpi followed

by systemic veinal necrosis on subsequent leaves at 15–20 dpi (Fig. 1a, b). Soybean (*G. max*) cvs Pusa-22 and Pusa-9712 developed systemic veinal mild mottle symptoms (Fig. 1c). Cowpea cv. Pusa Komal produced no systemic symptoms, but was positive by RT-PCR. The progeny virion were stable and mechanical sap inoculation was highly efficient resulting in 100 % infection of mungbean after 1 week with similar symptoms. The plant species belonging to Amaranthaceae, Cucurbitaceae and Solanaceae neither produced any visible symptoms, nor they were positive by ELISA.

Virion properties

The purified virions were obtained from the mechanically inoculated French bean cv. Pusa Parvati. The yield of purified virus ranged from 70 to 80 mg/kg of infected leaf tissues. The purified virus preparation had an ultraviolet light absorption spectrum typical of nucleoprotein components with A₂₆₀/A₂₈₀ ratio of about 1.75. Electron microscopy revealed the presence of only isometric virus particles of 28 nm from the purified virus preparation (Fig. 2a). French bean cv. Pusa Parvati plants inoculated with the purified virus preparations showed symptoms similar to those described above. The purified virus preparation had one protein band of ~39 kDa in SDS PAGE (Fig. 2c). Similarly immunoblots of purified virus

Fig. 3 Two-dimensional pairwise colour chart of the Car-Mb14 with other carmovirus isolates based on coat protein gene amino acid sequence identity. A scale with the percent color code is presented (a). Phylogenetic relationship of Car-Mb14 isolate with other carmovirus species based on amino acid sequence of CP (b). The NJ tree was constructed in MEGA 6 programme. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to branches. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. *AnFBV* angelonia flower break virus (NC_007733), *CbMV* calibrachoa mottle virus isolate California (GQ244431), *CCIFV* cardamine chlorotic

fleck virus (NC_001600), *CarMV* carnation mottle virus (NC_001265), *CPMoV* cowpea mottle virus (NC_003535), *ELV* elderberry latent virus isolate (AY038066), *GaMV* galinsoga mosaic virus (Y13463), *HCRSV* hibiscus chlorotic ringspot virus (X86448), *HSRSV* honeysuckle ringspot virus (NC_014967), *JINRV* Japanese iris necrotic ring virus (NC_002187), *MNSV* melon necrotic spot virus (NC_001504), *NLVCV* nootka lupine vein-clearing virus (NC_009017), *PSNV* pea stem necrosis virus (NC_004995), *PFBV* pelargonium flower break carmovirus (AJ514833), *SgCV* saguaro cactus virus (NC_001780), *SYMMV* SYMMV (FJ457015), SYMMV-Mb14 isolate of the present study (KF619242), *TCV* turnip crinkle virus (M22445)

against BMoV polyclonal antiserum also showed the presence of ~39 kDa band (Fig. 6c).

Nucleic acid preparations from the purified virus had an ultraviolet spectrum with A260/A230 and A260/A280 ratios of about 2.15 and 2.24, respectively. The RNA isolated from the purified virus displayed a single major fraction of ~4 kb on 1 % denaturing agarose gel (Fig. 2b).

Coat protein sequence and phylogenetic relationships

RT-PCR of Car-Mb14 with the CP specific primers (RKJ224F and RKJ225R) resulted in the amplification of 1065 nt (GenBank: KF619242), which encoded a protein of 39 kDa. The pair wise sequence analysis of CP gene showed that Car-Mb14 had maximum identity both at nucleotide (82 %) and amino acid (93 %) level with SYMMV reported from South Korea (NC_011643). In contrast, only 29–63 and 16–64 % identities at nucleotide

and amino acid level, respectively were observed with the CP gene of other carmoviruses. Alignment of CP amino acid sequence of Car-Mb14 isolate with SYMMV Korean isolate (KI) and USA isolate (MS1) showed a difference of 27–28 aa (Fig. 4). The phylogenetic analysis based on CP of 18 recognised carmovirus species showed two major clusters, where Car-Mb14 formed a subgroup with CPMoV and SYMMV (Fig. 3b). The CP gene sequence of 52 isolates of SYMMV is available in the GenBank, of these, only one isolate originated from soybean in USA and rest of the isolates were from South Korea either infecting cultivated soybean (*G. max*) or wild species (*G. soja*). The comparison of the present isolate (Car-Mb14) with the 52 other isolates showed that the present isolate shared 90–93 % amino acid sequence identity. The phylogenetic analysis revealed that the isolates from South Korea and USA belonged to the three major clades, where as the Indian isolates showed a completely different phylogenetic divergence from all these isolates (Fig. 5).

Fig. 4 Multiple alignments of deduced amino acid sequences of the coat protein region of Indian isolates, Car-Mb14 (KF619242), Car-Mb18 (KR260902) and Car-BG 42 (KR260903) with the SYMMV

Korean isolate (FJ457015) and MS1 isolate (USA) (FJ707484). Asterisks denote identical amino acids

Fig. 5 Phylogenetic analysis of the SYMMV isolates from India (Car-Mb14, Mb18, BG42) with the other 52 SYMMV isolates from other parts of the world based on amino acid sequence (355 aa) of coat protein. Phylogenetic tree was inferred using maximum parsimony (MP) method conducted in MEGA 6. The consistency index is 0.89, the relation index is 0.93, and the composite index is 0.89 for all sites. The MP tree was obtained using the Subtree-Pruning-Regrafting algorithm (SPR) with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates) (SK-South Korea)

Expression of recombinant coat protein

The sequence analysis of the expression construct showed that the CP gene was inserted in frame with his-tag at both the ends. The preliminary SDS-PAGE analysis of soluble fraction and cell debris pellet from IPTG induced bacterial cultures showed that the protein of about 39 kDa was present in insoluble fraction. Following induction with IPTG, *E. coli* BL21 (DE3) cells harboring pET-Car-Mb14 efficiently produced recombinant protein where as uninduced bacterial culture did not show any over expression of CP (Fig. 6a Lanes 2–6; Lane 1). The test performed to optimize the period of IPTG induction for the production of recombinant CP showed that there was over expression at 2 h onward and increased with the duration of induction (Fig. 6a, Lanes 2–6). The yield of purified recombinant CP from 1.0 culture was 2.85 mg.

Evaluation of antiserum to recombinant protein

The antiserum developed against the expressed CP of Car-Mb14 detected the virus in purified preparation as well as in infected leaf tissues by DAC-ELISA. The polyclonal antiserum had a titer of about 1:16,000 when tested with

crude proteins of infected plants. The antiserum allowed the detection of virus in the infected samples in dilutions of antiserum ranging from 1:500 to 1:4000, where the mean absorbance at 405 nm of the infected sap decreased from 2.65 (1:500) to 0.277 (1:16,000) (Fig. 6d). The comparison of reactivity of different antisera to legume infecting carmoviruses showed that the antiserum to BMoV moderately reacted with SYMMV (Car-Mb14). The ELISA readings with BMoV antiserum were significantly low compared to that of SYMMV. The antiserum to CPMoV and CarMV however did not react with the Car-Mb14 (Fig. 6e).

Detection of the virus in legume samples

During 2014, a total of 145 legume samples were tested from the different locations to validate the Car-Mb14 antiserum by DAC-ELISA (Table 3). A total of 16 of the 82 mungbean samples were positive with the antiserum to Car-Mb14. Relatively higher number of samples from the mungbean cultivar SML668 were tested positive. Of the 31 blackgram samples tested, only one sample collected from IARI was found to be positive. French bean and soybean gave negative reaction with Car-Mb14 antiserum. Sequencing of one each ELISA positive mungbean (Car-Mb18; KR260902) and

Fig. 6 Electrophoretic analysis and titer determination of expressed SYMMV coat protein in *Escherichia coli*. **a** SDS-PAGE gel showing the expressed protein at 2, 3, 4, 5 and 6 h post induction with 1.0 mM IPTG. **b** Western blot of the samples in figure using BMoV polyclonal antisera (1:200), Lane UN total protein from uninduced cell culture. **c** 12 % SDS-PAGE showing purified recombinant CP. E1–E4 different elution fractions, Lane M pre-stained protein marker (BR

Biochem). **d** DAC-ELISA using different dilutions of SYMMV antiserum with the infected French bean leaf sap at dilution of 1:2. **e** DAC ELISA showing cross reactivity of commercial, inhouse generated SYMMV antisera, CPMoV and CarMoV antisera with purified virus (14 µg) at different dilutions. O.D. at 405 nm was recorded 1 h after adding substrate, p-nitrophenyl phosphate

blackgram (Car-BG42; KR260903) samples collected during 2014 from IARI field showed 99 and 100 % CP sequence identity, respectively with Car-Mb14 isolate.

Discussion

There are only three carmoviruses (BMoV, CPMoV and SYMMV) known to infect legume crops world over, of which only BMoV is known to occur in India [12, 19]. SYMMV is so far known to infect soybean in North America and South Korea [7, 11]. The present study for the first time provides evidence of occurrence of a distinct strain of SYMMV in mungbean in India.

BMoV has been reported as a distinct virus based on biological and serological properties [15]. Due to lack of genome sequence information, the molecular characterization of BMoV is pending and therefore, it is considered as a tentative member of the genus *Carmovirus*. In our

initial investigation the mungbean samples with mottling symptoms from IARI field showed positive reaction with the commercially available PAb to BMoV. However, the sequence information generated using the degenerate primer to the partial RdRp and CP genes of legume-infecting carmoviruses revealed close similarities with SYMMV. Thereafter, the complete CP sequence generated for the Car-Mb14 isolate showed 93 % identity with SYMMV and up to 63 % identity with the other carmoviruses. According to the ICTV guidelines for carmovirus species demarcation, <52 % amino acid sequence identity of the CP is considered for a distinct virus species [6]. Therefore, the complete sequence information of CP confirmed that the virus associated with the mild mottling symptoms of mungbean in the present study is an isolate of SYMMV.

Previously, SYMMV occurring in Korea was reported to produce bright yellow mosaic symptoms on soybean with no obvious visible disease symptoms on the other legumes including mungbean, French bean and cowpea [11].

Table 3 Detection of Car-Mb14 isolate in legume samples by DAC-ELISA using antiserum generated against the CP region of SYMMV

Source	Host plant	ELISA readings ^a	No. +ve samples/ samples tested ^b
Regional Agriculture Research Station, Lam, Guntur, Andhra Pradesh	<i>Vigna mungo</i>	0.12–0.15	0/7
	<i>Vigna radiata</i>	0.13–0.19	0/5
Sardarkrushinagar agricultural university, Gujarat	<i>Vigna radiata</i>	0.13–0.17	0/18
Indian Institute of Pulse Research, Kanpur, Uttar Pradesh	<i>Vigna mungo</i>	0.19–0.20	0/4
	<i>Vigna radiata</i>	0.12–0.66	1/4
University of Agricultural Sciences, Dharwad, Karnataka	<i>Glycine max</i>	0.13–0.19	0/8
	<i>Phaseolus vulgaris</i>	0.13–0.22	0/5
	<i>Vigna radiata</i>	0.15–0.20	0/5
Indian Agricultural Research Institute, New Delhi	<i>Glycine max</i>	0.26–0.32	0/19
	<i>Vigna mungo</i>	0.12–0.74	1/20
	<i>Vigna radiata</i>	0.32–2.51	15/50

^a Antiserum dilution 1:2000, Sap dilution 1:2, O.D. was recorded 1 h after adding substrate. Average healthy O.D. value ranged from 0.039 to 0.22

^b ELISA readings ≥ 2 of the healthy readings were considered positive

Contrastingly, SYMMV isolate from India (Car-Mb14) did not produce bright yellow mosaic symptoms in soybean; however it produced mild veinal mottling in soybean, mild mottling in mungbean, blackgram and French bean and local and systemic necrosis in guar bean. The Indian isolate of SYMMV shared the closest relationships with the Korean isolate of SYMMV with reference to the CP sequence, however they differed in symptomatology in several legume species. Therefore, the results suggest the present isolate from India is a distinct strain of SYMMV. Interestingly, the Car-Mb14 isolate shared similarities with BMoV in host-range and -reactions [12]. However, the Car-Mb14 isolate differed from BMoV by causing systemic veinal necrosis symptoms in guar bean. The genome sequence information of BMoV is not available and therefore it is not possible to compare the Car-Mb14 isolate with BMoV at molecular level. As the antiserum to BMoV is commercially available, we generated antiserum to Car-Mb14 isolate for serological comparison. Our results suggest that antisera to BMoV and the Indian isolate of SYMMV, although cross-react, are distinct. Further work is necessary to establish the identity of BMoV.

Under field conditions, SYMMV is often overlooked due to lack of prominent symptoms. Immunodiagnosis is popularly used for plant virus diagnosis, however serological study of SYMMV has not been reported so far. In this study, we produced bacterial expressed recombinant CP to generate SYMMV specific antiserum and utilized it for developing immune-diagnostic test. The CP gene of SYMMV was stably over expressed in *E. coli* and an antiserum with approximately 1:16,000 titer was produced against the recombinant CP. The recombinant approach is advantageous as it does not encounter the problems associated with the mixture of

closely related viruses, purification and maintenance of live virus culture. This strategy has been successfully used for a number of plant viruses from different genera however not for carmoviruses [4, 9, 13, 16]. To our knowledge, this is the first report of development of immunodiagnosis based on antiserum against bacterial expressed recombinant CP of any carmoviruses in general and SYMMV in particular. The antiserum to SYMMV efficiently detected the virus in the field samples at 1:2000 dilutions by ELISA. Testing of limited number of field samples of mungbean, blackgram and French bean from different places of India during 2014 showed low level incidence of the virus. The immunodiagnostic test of field samples was further confirmed by generating sequence information of CP gene for selected samples of mungbean and blackgram. SYMMV was rarely detected in blackgram. The positive results of ELISA and CP gene sequence of the only one field sample from blackgram confirmed natural occurrence of SYMMV in at least two legumes in India. The immunodiagnosis developed in this study will be useful to study epidemiology of SYMMV, and its impact alone or in mixed infection with other virus on the growth and yield of mungbean or other legume crops.

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