

Characterization of a new isolate of Bean yellow mosaic virus Group-IV Associated with Mosaic disease of Gladiolus in India

Charanjeet Kaur¹, Susheel Kumar¹, Raj SK^{1*}, Chauhan PS² and Neeta Sharma³

¹Plant Molecular Virology Laboratory, Council of Scientific and Industrial Research-National Botanical Research Institute, Lucknow-226001, U.P, India

²Department of Plant Microbe Interaction, Council of Scientific and Industrial Research-National Botanical Research Institute, Lucknow-226001, UP, India

³Department of Botany, University of Lucknow, Lucknow-226025, U.P, India

Abstract

A new isolate of *Bean yellow mosaic virus* of group-IV associated with leaf mosaic and flower color breaking symptoms of gladiolus has been characterized based on virus transmission, symptomatology, presence of flexuous rod-shaped particles of 720 nm × 11 nm, Western blot-immunoassay using BYMV antiserum and sequence analysis of full length viral genome (accession number: KM114059, CK-GL2 isolate). The CK-GL2 isolate shared 90-96% nucleotide identity with known BYMV isolates and showed close phylogenetic relationships with phylogenetic group IV isolates of BYMV. Recombination analysis showed eight recombination events in the genome of CK-GL2 isolate and suggested that 80% of the genome was recombinant of six different parental phylogenetic groups of BYMV.

Keywords: Gladiolus; Mosaic disease; Virus transmission; Particle morphology; Complete genome; Sequence analysis; *Bean yellow mosaic virus*

Introduction

Gladiolus (*Gladiolus* sp., family Iridaceae) is a vegetatively propagated ornamental plant of economic value which occupies a prominent position in global flower industry [1] and ranks amongst the top six flowers of export market. Gladiolus ranks second in area and production of cut flowers grown in India [2]. It is being grown in an area of 11,660 ha in the country with an estimated production of 1060 million cut flowers. The total floriculture export of gladiolus from India was 10,165.28 MT of value approximately 138 million rupees in the year 2012-13 [3]. Gladiolus is prone to viral, fungal, bacterial, pests and phytoplasmal diseases which affect its yield and production worldwide, causing losses to the floriculture industry [4,5]. These diseases reduce the quantity and quality of gladiolus flowers and in some cases may cause significant plant mortality [6].

Under natural conditions, various viruses infect gladiolus that include: *Arabis mosaic virus* (AMV), *Bean yellow mosaic virus* (BYMV), *Broad bean wilt virus* (BBWV), *Cucumber mosaic virus* (CMV), *Impatiens necrotic spot virus* (INSV), *Ornithogalum mosaic virus* (OrMV), *Strawberry latent ring spot virus* (SLRSV), *Tobacco mosaic virus* (TMV), *Tobacco rattle virus* (TRV), *Tobacco ring spot virus* (TRSV), *Tomato aspermy virus* (TAV) and *Tomato spotted wilt virus* (TSWV). Among them, BYMV is the most prevalent in gladiolus which causes mosaic symptoms on leaves, color-breaking in flowers and reduces plant vigor [7]. Limited work has been done in India on virus symptomatology, particle morphology and serological detection of BYMV [8]; CMV [9,10]. However, a detailed study on complete characterization of BYMV isolate has not been done so far in India.

Keeping in view of the high rate of mosaic disease incidence on gladiolus and deterioration in quality production in gladiolus grown in India, biological, serological, morphological and molecular characterization and identification of BYMV infecting gladiolus has been conducted with a long term goal to develop a disease management strategy in gladiolus.

Materials and Methods

Sample collection and estimation of disease impact

Two consecutive surveys were conducted in commercially cultivated fields near Lucknow and experimental plots at CSIR-National Botanical Research Institute, Lucknow, India during 2011-2012. Virus like symptoms of severe mosaic on leaves and sepals, and color breaking in floral petals were observed in all ten commercially grown gladiolus cultivars: Aldebaron, Decisso, Promise, Regency, Shagun, Snow Princess, Sylvia, Tiger Flame, True Love and Vink's Glory. Symptomatic as well as asymptomatic leaf samples of gladiolus from each cultivar were collected and stored at -80°C until further use.

Ten of the representative symptomatic and asymptomatic plants, from each cultivar, were also uprooted and used for comparison of morphological parameters. For estimation of yield losses, parameters like height of the plant, number of tillers per plant, number of leaves in each tiller, length of spikes, number of florets per spike, number of cormels per plant, and fresh weight of mother corms in each plant were measured and mean values of ten plants for each morphological parameter were computed in Table S1.

Virus transmission by mechanical (sap) inoculation and by aphids

The 1.0 g of symptomatic leaf tissue of gladiolus was macerated in 10 ml cold potassium phosphate buffer (0.1 M, pH 6.8, supplemented with

*Corresponding author: Raj SK, Plant Molecular Virology Laboratory, Council of Scientific and Industrial Research-National Botanical Research Institute, Lucknow-226001, UP, India, Tel: +91-522-2297950; Fax: +91-522-2205839; E-mail: skraj2@rediffmail.com

Received September 17, 2015; Accepted October 16, 2015; Published October 20, 2015

Citation: Kaur C, Kumar S, Raj SK, Chauhan PS, Sharma N (2015) Characterization of a new isolate of *Bean yellow mosaic virus* Group-IV Associated with Mosaic disease of Gladiolus in India. J Plant Pathol Microb 6: 309. doi:10.4172/2157-7471.1000309

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0.5% β -mercaptoethanol, 0.1% thioglycolic acid, 0.01 M EDTA, 0.1% Na_2SO_3 and 0.01 M DIECA additives) using a sterile pestle and mortar on ice. The homogenate obtained was inoculated onto carborandum dusted leaves of various recipient host species. Local and systemic symptoms on host plants were recorded 30 days post inoculation (dpi). For insect transmission, aphid (*Aphis craccivora* Koch) transmission studies were conducted as described earlier [8]. All inoculated plants were kept in insect-proof cages and observed for 30 days for symptom development, if any.

Partial purification of virus and transmission electron microscopy (TEM)

The virus was partially purified from infected gladiolus samples (after single lesion transfer) and formvar backed carbon coated copper grid (400 mesh, Canemco and Marivac Inc., Canada) were floated on 50 μl of virus preparation for 2 min. The excess buffer was washed with 10 mM phosphate buffer (pH 7.0) followed by three washes by sterile water. Virus preparations were negatively stained with 2% Uranyl acetate (pH 4.6), excess stain was soaked away with a piece of clean filter paper. The air-dried grids were observed in a TEM at 80,000 x magnification.

Cytological inclusion bodies and scrolls, formed due to potyvirus infection, were also observed in infected gladiolus leaf samples. The leaf was washed with 1X phosphate buffer saline (PBS, pH 7.2), fixed in 2.5% glutaraldehyde solution prepared in sodium cacodylate buffer (pH 7.2) for 2 h at 4°C. Samples were washed with 0.1 M sodium cacodylate buffer and post fixed in 1% osmium tetroxide for 2 h. Samples were again washed with sodium cacodylate, dehydrated in acetone series (15 to 100%) and embedded in araldite-DDSA mixture (Ladd Research Industries, USA). After baking at 60°C, blocks were cut to slices of 60-80 nm thickness by an ultra-microtome (Leica EM UC7) and sections were stained by 2% uranyl acetate and lead citrate. Analysis of sections was done under G2 spirit twin transmission electron microscope equipped with Gatan digital CCD camera (FEI TecnaiTM, Netherland) at 60 or 80 KV.

Western immuno blot analysis

To detect the potyvirus infection in naturally infected gladiolus and experimentally inoculated gladiolus and *V. faba* by sap, the Western blot immuno-assay was performed using antiserum of three potyviruses: BYMV, Narcissus potyvirus and *Sugarcane mosaic virus* (ScMV). Western blot immuno-assay was performed using crude sap of leaf samples [9]. After SDS-PAGE, proteins were transferred to a nitrocellulose membrane and processed separately with three antisera of BYMV (LOEWE, Sauerlach, Germany), narcissus potyvirus and ScMV, as described earlier [9]. The membrane was washed in blocking buffer TTBS (Tris 50 mM, NaCl 150 mM, Triton X-100, 0.1% and Tween-20, 0.05%) containing 5% non-fat dry milk powder for 2 h at room temperature and then transferred to a fresh blocking buffer containing 1:1000 diluted antisera and incubated for 3 h. After three subsequent washings with TTBS, the blot was transferred to anti-rabbit IgG alkaline phosphatase conjugate (1:10,000 dilutions) and incubated at 4°C overnight. Finally, the protein bands were elucidated by color-development reaction on adding BCIP/NBT (Sigma-Aldrich, Missouri, USA) in dark and the sizes of bands were assessed with PageRuler™ Prestained Protein Ladder marker (Thermo Fisher Scientific India Pvt. Ltd., India). The reaction was terminated by adding sterile water and the blot was air-dried.

Virus detection and full length genome amplification by reverse transcription-PCR and 5'-RACE

For virus detection, total genomic RNA was extracted from

naturally infected plant leaf (from field) and aphids (reared in insect house) using TRI reagent (Sigma-Aldrich, Missouri, USA) following the manufacturer's instructions except that the mixing of pellet was done by inverting or tapping of tube to avoid mechanical sheering of RNA. The extracted RNA was qualitatively analyzed and used as template for cDNA synthesis. Reverse transcription (RT) and polymerase chain reaction (PCR) were done separately.

For cDNA synthesis, 2 μg total RNA was denatured for 3 min at 70°C, quenched on ice and mixed with 100 pM reverse primer (Table S2). cDNA synthesis was carried out using MMuLV reverse transcriptase enzyme (Thermo Fisher Scientific India Pvt. Ltd., India) in a 20 μl reaction volume at 42°C for 45 min following the instruction manual. PCR was carried out using reverse and forward primers. For PCR, 5 μl of the cDNA mix was added to a 100 μl polymerase reaction mixture containing 200 mM of dNTPs, 2.5 mM MgCl_2 , 5U *Taq* polymerase (MBI, Fermentas, Inc., Hanover, MD, USA), 10X PCR buffer, 200 ng of each primer and RNase-free water. PCR was performed in Sure cyclor 8800 (Agilent Technologies Products (M) Sdn. Bhd., Penang, Malaysia). Then PCRs were performed with the conditions: initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 30 sec; annealing at 50°C, 54°C and 60°C for 30 sec for these primer respectively; and elongation at 72°C for 1 min; followed by final elongation at 72°C for 10 min.

The 5' untranslated region (UTR), P1 and partial HC-Pro regions of the potyvirus were amplified by 5'-RACE kit (FirstChoice™ RLM-RACE RNA Ligase Mediated RACE, Ambion, Life Technologies, USA) following manufacturer's instructions. For 5'-RACE, total RNA was processed with CIP and TAP enzymes; RNA adaptor was then ligated to 5'-end of this processed RNA. The adaptor ligated RNA was used for cDNA synthesis using HP-Rev primer. The cDNA was used as a template in PCR reactions with outer-5'-RACE-Forward and HP-Rev reverse primers. A Nested PCR was also performed using the above products as template with inner-5'-RACE-Forward and BYMV-Rev primers (which was designed from the 5'- HC-Pro sequence data) following manufacturer's instructions. The RT-PCR products were electrophoresed in 1% agarose gel and size of all amplified products was assessed with λ -DNA digested with *EcoRI/HindIII* as DNA marker (Thermo Fisher Scientific India Pvt. Ltd., India).

Cloning, sequencing and data analysis

All PCR amplified products were gel-purified using Wizard SV Gel and PCR CleanUp System kit (Promega Corp., Madison, USA). The products were cloned into pGEM-T easy vector system-I (Promega Corp., Madison, USA) and competent *Escherichia coli* DH5 α cells were transformed. Positive clones were confirmed by colony PCR. Three positive clones of each insert were sequenced using SP6 and T7 universal primers (Genei Pvt. Ltd., Bangalore, India). To obtain the complete viral genome, sequences from all PCR fragments were analyzed, assembled, consensus sequence was determined and submitted to the GenBank.

To the close homologues of the virus isolate, local alignment was done using full length viral genome in BLASTn (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and nucleotide identity was determined. The open reading frames (ORFs) located in the genome and their putative proteins were analyzed by ORF Finder (www.ncbi.nlm.nih.gov/projects/gorf/) and *Expasy* translation tools (<http://www.expasy.org/resources/search/keywords:translation>), respectively. The matrix for pairwise identity of BLASTn selected BYMV isolates and other closely related potyvirus isolates was obtained by Genomatix DiAlign2 program [11] by Clustal-W method. The evolutionary history of virus isolate

under study with selected potyviruses [12] and out-group members was inferred using full length nucleotide sequence in neighbor-joining method of MEGA v.6.1 program [13]. Trees were drawn to scale with branch lengths the same units as those of evolutionary distances used to infer the phylogenetic tree branch length in nucleotide substitutions per site between sequences. The tree was drawn using the close-Neighbor-Interchange algorithm with 1000 bootstrap replicates. Accessions used are given in Table 1-3.

Recombination analysis

Recombination analysis was carried out by Recombination Detection Program (RDP) version 4.0 [14]. Seven methods (RDP, GENECONV, BOOTSCAN, Maximum Chi Square, CHIMEARA,

SISCAN and 3Seq) in RDP were used for the detection of potential recombination event/s, identification of likely parents and localization of possible breakpoints. Only those events were considered for study which could be detected by more than one method. Details of recombination analysis are given in Table S4.

Results

Virus disease incidence in gladiolus cultivars

Natural infection of severe mosaic, leaf stripe and color breaking disease in various gladiolus cultivars was observed. Disease incidence was determined on the basis of symptoms. The severity of disease symptoms varied (severe, moderate and mild) among different

S. No.	Cultivar	Symptom	Observation of 2011		Observation of 2012	
			Infected/total plants	Disease incidence (%)	Infected/ total plants	Disease incidence (%)
1.	Tiger flame	Severe mosaic, color breaking	175/200	87.5	195/200	97.5
2.	Promise	Mosaic, color breaking	57/96	59.3	75/100	75.0
3.	Vink's glory	Mosaic	40/100	40.0	80/150	53.3
4.	Shagun	Mosaic, color breaking	36/90	40.0	90/100	90.0
5.	Regency	Mosaic	99/240	41.2	120/250	48.0
6.	Snow princess	Mild mosaic	62/200	31.0	100/250	40.0
7.	Aldebaron	Mild mosaic	34/100	34.0	70/150	46.6
8.	True love	Mosaic, color breaking	38/76	50.0	75/90	83.3
9.	Decisso	Mosaic	59/100	59.0	105/150	70.0
10.	Sylvia	Mosaic and color breaking	169/200	84.5	188/200	94.0

Table 1: Disease symptoms and disease incidences in various gladiolus cultivars growing at experimental plots of CSIR-NBRI in two consecutive years: 2011 and 2012.

Host species	Family	Local symptoms (7-10 dpi)		Systemic symptoms (25-30 dpi)	
		Type of symptoms	No. of plants showing symptoms*	Type of symptoms	No. of plants showing symptoms*
<i>Abelmoschus esculentus</i>	Malvaceae	-	0/10	-	0/10
<i>C. amaranticolor</i>	Amaranthaceae	NLL	7/10	-	0/10
<i>Capsicum annum</i>	Solanaceae	-	0/10	SM	6/10
<i>Chenopodium album</i>	Amaranthaceae	-	0/10	-	0/10
<i>Cicer arietinum</i>	Fabaceae	-	0/10	-	0/10
<i>Cucumis melo</i>	Cucurbitaceae	-	0/10	-	0/10
<i>Cucumis sativus</i>	Cucurbitaceae	-	0/10	-	0/10
<i>Cucurbita maxima</i>	Cucurbitaceae	-	0/10	-	0/10
<i>Datura innoxia</i>	Solanaceae	-	0/10	-	0/10
<i>Gladiolus</i> sp.	Iridaceae	-	0/10	SM	6/10
<i>Lagermaria sicemaria</i>	Cucurbitaceae	-	0/10	-	0/10
<i>Melilotus albus</i>	Fabaceae	-	0/10	MM	6/10
<i>Momordica charantia</i>	Cucurbitaceae	-	0/10	-	0/10
<i>N. glutinosa</i>	Solanaceae	-	0/10	-	0/10
<i>N. rustica</i>	Solanaceae	-	0/10	MM	5/10
<i>N. tabaccum</i> cv. White Burley	Solanaceae	-	0/10	-	0/10
<i>Petunia hybrida</i>	Solanaceae	-	0/10	SM	4/10
<i>Phaseolus vulgaris</i>	Fabaceae	-	0/10	-	0/10
<i>Physalis peruviana</i>	Solanaceae	-	0/10	SM	4/10
<i>Pisum sativum</i>	Fabaceae	-	0/10	MM	5/10
<i>Rhapanus sativus</i>	Brassicaceae	-	0/10	-	0/10
<i>Solanum lycopersicon</i>	Solanaceae	-	0/10	-	0/10
<i>Solanum melongena</i>	Solanaceae	-	0/10	-	0/10
<i>Spinacia oleracea</i>	Amaranthaceae	-	0/10	-	0/10
<i>V. faba</i>	Fabaceae	NLL	5/10	MM	8/10
<i>V. mungo</i>	Fabaceae	-	0/10	-	0/10
<i>Vigna radiata</i>	Fabaceae	NLL	4/10	-	0/10

*No. of plants showing symptoms out of 10 plants in each.

Abbreviations: NLL=Necrotic local lesions, MM=Mild mosaic, SM=Severe mosaic, and -=no symptoms developed.

Table 2: Local and systemic symptoms developed on various host species by mechanical inoculations of gladiolus virus.

Accession	Isolate	Phylogenetic group/ virus	Location	Host	% nt identity		Pairwise aa similarity of various genes of CK-GL2										
					BLASTn	Pairwise	PP	P1	HC Pro	P3	6K1	CI	6K2	VPg	Nla Pro	Nib	CP
D83749	MBGP	IV	Japan	-	96	94	94	98	98	96	96	97	100	98	96	78	93
NC_003492	MB4	IV	Japan	-	96	94	94	98	98	96	96	97	100	98	93	78	93
AB439730	G1	IV	Japan	<i>G. hybrida</i>	94	92	93	95	97	91	94	97	98	96	96	78	93
AY192568	GDD	IV	USA	<i>Gladiolus</i> sp.	94	92	93	93	97	92	90	98	96	97	96	79	91
AM884180	<i>Lisianthus</i>	IV	Taiwan	<i>E. russellianum</i>	93	90	92	94	96	91	94	97	98	97	96	78	87
AB079888	GB2	IV	Japan	-	93	90	92	93	96	95	96	98	94	98	96	76	89
JN692500	Vfaba2	IV	India	<i>V. faba</i>	93	90	90	94	96	91	94	97	98	97	96	82	76
AB439729	Gla	IV	Japan	<i>G. hybrida</i>	92	88	90	69	90	96	96	97	100	100	96	77	93
AB079886	M11	III	Japan	<i>G. hybrida</i>	90	85	89	70	95	92	94	97	100	97	93	79	87
AB079887	IbG	III	Japan	<i>G. hybrida</i>	90	85	89	69	95	92	94	96	100	97	96	79	86
JX156423	SW3.2	II	Australia	<i>Diuris</i> sp.	85	80	88	72	95	88	94	97	96	88	91	75	91
HG970850	MD7	II	Australia	<i>Lupinus cosentinii</i>	85	80	88	72	94	88	92	97	96	87	96	75	90
HG970863	AR87C	II	Australia	<i>L. angustifolius</i>	85	79	88	73	95	87	92	97	96	88	93	75	91
HG970855	LMBNN	II	Australia	<i>L. angustifolius</i>	85	80	88	71	95	87	94	97	96	88	93	75	91
HG970858	ES55C	II	Australia	<i>L. angustifolius</i>	85	80	88	72	95	88	94	97	96	88	93	75	91
HG970860	PN83A	I	Australia	<i>L. angustifolius</i>	85	80	88	71	94	89	96	97	88	90	93	76	92
HG970861	PN80A	I	Australia	<i>L. angustifolius</i>	85	79	88	71	94	89	96	96	86	90	92	76	92
HG970852	GB17A	I	Australia	<i>L. angustifolius</i>	85	79	88	72	94	87	90	97	88	91	91	76	91
FJ492961	Fr	I	S. Korea	<i>Freesia</i> sp.	85	79	88	71	94	87	96	96	88	90	92	75	91
HG970847	MD1	I	Australia	<i>L. cosentinii</i>	85	78	88	72	94	88	92	97	96	89	92	76	91
JX173278	KP2	I	Australia	<i>D. magnifica</i>	85	79	88	70	94	88	88	96	96	90	93	76	91
HG970851	SP1	I	Australia	<i>L. angustifolius</i>	85	79	88	69	94	88	94	97	94	91	92	76	91
HG970865	AR93C	I	Australia	<i>L. angustifolius</i>	85	79	88	71	94	87	94	96	96	90	92	76	91
HG970869	NG1	I	Australia	<i>L. angustifolius</i>	85	79	88	71	94	87	94	96	92	91	92	76	91
AB439732	92-1	V	Japan	<i>Trifolium pratense</i>	83	76	87	72	91	88	94	95	92	87	96	74	89
U47033	S	V	Australia	<i>V. faba</i>	83	78	86	68	89	90	96	95	94	86	93	75	88
HG970866	LP	VI	Australia	<i>L. pilosus</i>	81	73	86	72	91	87	92	92	92	88	92	75	88
HG970868	LPexFB	VI	Australia	<i>V. faba</i>	81	73	86	72	91	87	92	92	92	87	94	75	87
AB439731	90-2	VII	Japan	<i>V. faba</i>	80	72	85	69	94	82	88	92	90	88	92	74	87
HG970867	FB	VII	Australia	<i>V. faba</i>	80	72	85	69	95	83	88	92	92	89	91	74	87
DQ641248	WLMV	VIII	USA	<i>L. albus</i>	77	68	82	64	91	75	90	90	94	80	91	74	86
AB373203	CS	IX	Japan	<i>Pisum sativum</i>	75	63	80	58	86	75	94	91	88	83	86	70	84
NC_003536	CYVV	CYVV	Japan	<i>Phaseolus vulgaris</i>	-	45	64	44	78	46	73	76	60	66	69	59	71
HG970870	CYVV	CYVV	Australia	<i>T. repens</i>	-	44	65	44	78	46	73	76	58	66	69	59	71
NC_019409	KP	OrMV	Australia	-	-	24	39	13	41	21	34	52	35	48	44	46	63
NC_018455	CYNMV	<i>Macluravirus</i>	USA	<i>D. opposita</i>	-	7	11	-	-	-	-	-	-	-	-	-	-
NC_010521	SVYV	<i>Ipomovirus</i>	USA	Watermelon	-	06	13	-	-	-	-	-	-	-	-	-	-

Abbreviations: aa=amino acid, nt=nucleotide, PP=Polyprotein.

BYMV= *Bean yellow mosaic virus*, CYVV= *Clover yellow mosaic virus*, WLMV= *White lupin mosaic virus*, OrMV= *Ornithogalum mosaic virus*, SVYV= *Squash vein yellowing virus* and CYNMV= *Chinese yam necrotic mosaic virus*.

Table 3: Pairwise sequence similarity (%) of complete cds and various proteins of CK-GL2 with other potyvirus and closely related virus isolates reported worldwide.

cultivars of gladiolus and also varied in different years at NBRI. The disease incidence in ten cultivars of gladiolus varied from 31.0-87.5% in year 2011 (Table 1) which increased to 40-97.5% in the following year. Shagun, Sylvia and Tiger Flame cultivars were found to be the most susceptible cultivars for BYMV infection with $\geq 90.0\%$ disease incidence (Table 1). The most tolerant cultivars for BYMV infection with $\leq 48.0\%$ disease incidence were found to be Aldebaron, Regency and Snow Princess (Table 1).

Symptoms of viral disease in gladiolus and yield losses

The naturally infected gladiolus plants exhibited mild to severe mosaic symptoms on leaves, stem, and inflorescence; color breaking in floret petals and reduction of number of corms as compared to the leaves, stem; inflorescence; florets and corms of the healthy gladiolus plants (Figure 1).

The yield loss, in terms of height of plant, number of tillers per

plant, length of spike, number of florets per plant, number of cormels per plant and fresh weights of mother corms attributes was estimated to assess the impact of virus disease on various gladiolus cultivars and the results has been summarized in Table S1. The highest and the lowest disease incidence was 97.5 and 40% in Tiger Flame and Snow Princess, respectively (Table 1). There was a reduction of 7.7-42.1%, 17.6-50.0% and 7.2-57.6% in number of florets per spike, cormels and FW of mother corms, respectively, due to virus infection as compared to healthy ones in 2011, which subsequently increased to 14.3-50.0%, 20-62.5%, 9.7-63.7%, respectively in 2012 (Table S1). Cumulatively, these results suggested that virus caused significant deterioration in quality.

Virus transmission by sap inoculation and hos

T- range studies: Mechanical inoculations using crude sap obtained from leaf tissue of diseased gladiolus (showing severe mosaic symptoms) induced necrotic local lesions on leaves of *C. amaranticolor*,



Figure 1: Symptoms on various parts: leaves, inflorescence, florets and corms of naturally infected gladiolus plants (a, c, e and g) as compared with their healthy plants (b, d, f and h).



Figure 2: Transmission electron micrographs of partially purified preparation of naturally infected gladiolus showing flexuous rod shaped virus particles of size 720 × 11 nm (a). Ultrathin sections of infected gladiolus leaves observed under TEM showing laminated inclusions (b) and scrolls (c). Virus detection by western immuno blot assay (d) using BYMV antiserum showing positive bands of ~35 kDa in naturally infected gladiolus, experimentally inoculated gladiolus and *V. faba* (lanes 2, 3 & 4) similar to BYMV infected *P. peruviana* taken as a positive control (lane P), PageRuler™ Prestained Protein Ladder as protein marker (Lane M). Virus detection by RT-PCR (e) 1% Agarose gel electrophoresis of RT-PCR products of potyvirus infected gladiolus samples (1-5) using potyvirus degenerate primers Pot I/II. M = λ-DNA EcoRI/HindIII as DNA marker.

V. faba and *V. radiata* at 7-10 dpi (Figure S1). Sap also induced systemic mosaic symptoms on *V. faba*, *P. hybrida*, *C. annuum* at 25-30 dpi (Figure S1). Healthy gladiolus plantlets (obtained from Floriculture Laboratory, CSIR-NBRI) when mechanically inoculated with the leaf sap of naturally infected gladiolus, developed mosaic symptoms at 25-30 dpi which were similar to those of naturally infected gladiolus collected from fields. Some of the inoculated test plants did not show any local or systemic symptoms at 25-30 dpi, these plants were confirmed by back inoculation to *V. faba* which did not produce any symptoms up to 25-30 dpi, indicating non hosts of the virus infecting gladiolus.

Transmission of virus by aphids

During virus transmission by *A. craccivora* Koch as vector when *V. faba* as recipient host and gladiolus plants as donor plants were taken, the inoculated *V. faba* plants developed systemic mosaic symptoms (7/10) at 25-30 dpi (Figure 2).

Virus particle morphology and study of by TEM

Flexuous rod-shaped virus particles of 720 nm x 11 nm were observed in the partially purified virus preparation (Figure 3a). The size

and shape of the virus particles were similar to those reported earlier for potyviruses [15]. Sections of infected gladiolus leaves, when observed under TEM, showed the presence of laminated and scrolls cytoplasmic inclusion bodies (Figure 3b and c), characteristic of potyvirus infection [16,17].

Serological detection of potyvirus by western immuno blot assay

Western immuno blot assay using antiserum of BYMV showed the expected ~35 kDa band in naturally infected gladiolus, experimentally inoculated gladiolus and *V. faba* similar as in BYMV infected plants taken as a positive control (Figure 3e). However, no such band was obtained in any of the tested plant sample with antisera of narcissus potyvirus and ScMV, indicating absence of narcissus potyvirus and ScMV potyvirus in all tested samples of gladiolus and *V. faba*.

RT-PCR detection of BYMV infecting gladiolus

RT-PCR using potyvirus degenerate primer pairs from partial NIB to 3'-UTR region (Pot I/II, 10) gave amplicon of ~1.5 kb (Figure 3d) in all five infected gladiolus samples. The sequence data obtained had 99-100% nucleotide identity with each other and 86-89% with other BYMV isolates reported worldwide. Hence the sequence was identified as an isolate of BYMV and the sequence data were submitted in NCBI GenBank under the accession: JQ686721 (1549 bp) as BYMV isolates from gladiolus.

Molecular identification of BYMV isolates by sequence analysis of the complete genome

For complete genome amplification of BYMV from gladiolus, four primer pairs of four overlapping BYMV regions were used in RT-PCR (Figure 4). RT-PCR resulted in the expected size amplicons of ~1.5 kb, 4.0 kb, 3.0 kb and 1.6 kb from four infected samples of *G. dalenii* cv. Sylvia (Figure S2). The strategy for complete genome sequencing of BYMV was to amplify, clone and sequence the overlapping fragments of BYMV and then to analyze, assemble and submit the complete genome sequence data to GenBank under the accessions: KM114059 (*G. dalenii* cv. Sylvia: CK-GL2, 9532 nt). The genome details are given in Table S3. BLASTn analysis of isolate CK-GL2 showed 93-96% sequence identity with several known BYMV isolates (Table 3).

Pairwise sequence similarities of CK-GL2 by CLUSTAL-W

During Genomatix DiAlign analysis, the RNA genome of CK-GL2 isolate shared 88-94% nucleotide sequence similarities with BYMV

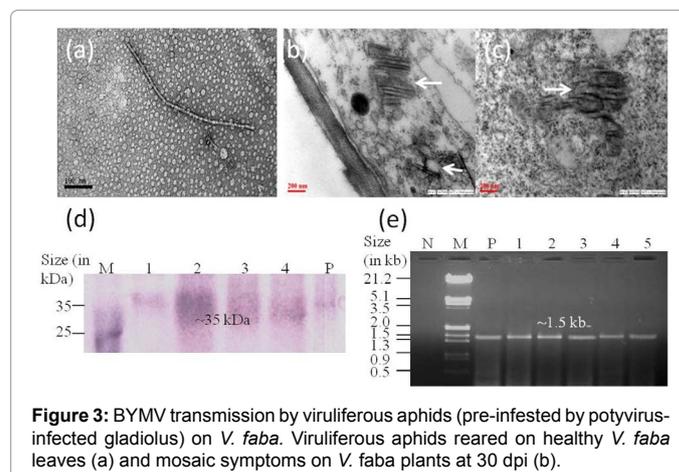


Figure 3: BYMV transmission by viruliferous aphids (pre-infested by potyvirus-infected gladiolus) on *V. faba*. Viruliferous aphids reared on healthy *V. faba* leaves (a) and mosaic symptoms on *V. faba* plants at 30 dpi (b).

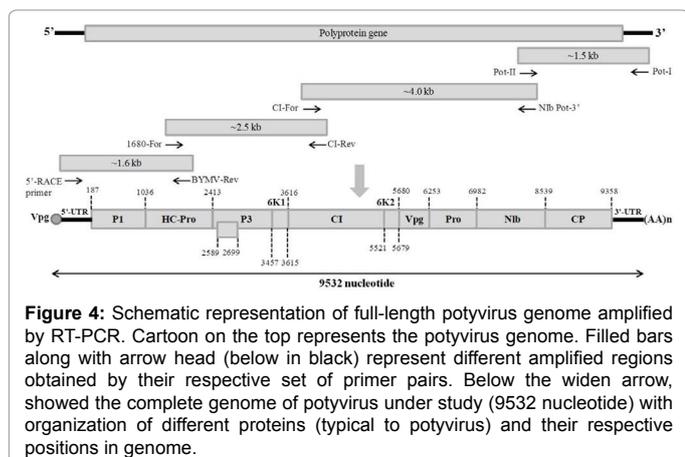


Figure 4: Schematic representation of full-length potyvirus genome amplified by RT-PCR. Cartoon on the top represents the potyvirus genome. Filled bars along with arrow head (below in black) represent different amplified regions obtained by their respective set of primer pairs. Below the wider arrow, showed the complete genome of potyvirus under study (9532 nucleotide) with organization of different proteins (typical to potyvirus) and their respective positions in genome.

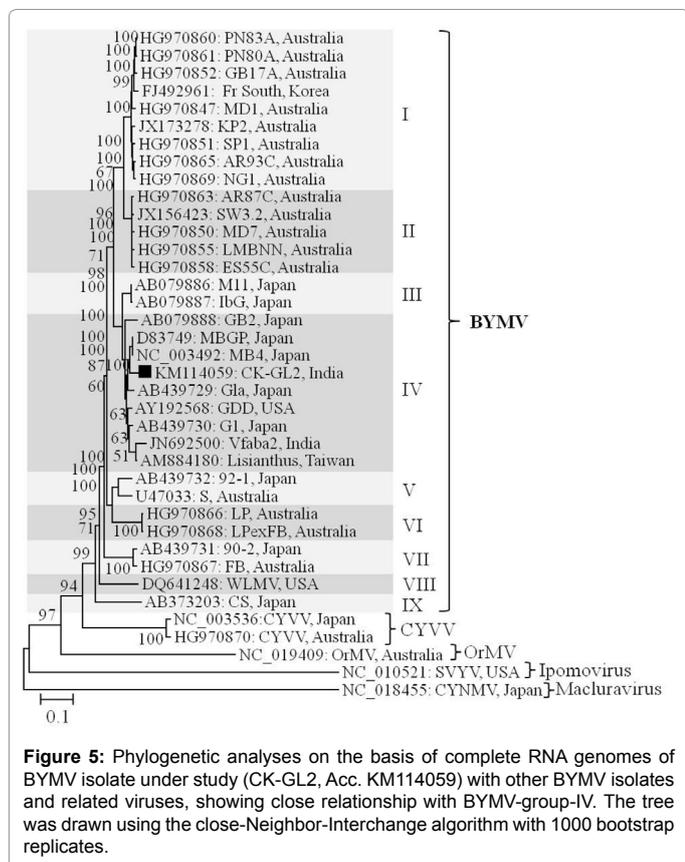


Figure 5: Phylogenetic analyses on the basis of complete RNA genomes of BYMV isolate under study (CK-GL2, Acc. KM114059) with other BYMV isolates and related viruses, showing close relationship with BYMV-group-IV. The tree was drawn using the close-Neighbor-Interchange algorithm with 1000 bootstrap replicates.

isolates of phylogenetic group IV (MBGP, MB4, G1, GDD, *Lisianthus*, VFaba2, GB2 and Gla) reported from Japan, USA, India and Taiwan (Table 3). CK-GL2 shared 63-85% sequence similarity with BYMV phylogenetic groups I, II, III, V, VI, VII, VIII and IX (Table 3). Based on deduced amino acid sequence of translated long polypeptide, the CK-GL2 isolate shared the highest (90-94%) identity with BYMV strains of phylogenetic group-IV and a lower (89-92%) with group-III, 88% with group-I and -II, 86-87% with group-V, 86% with group-VI; 85% with group-VII, 82 and 80% with group-VIII and -IX, respectively. The alignment analysis of deduced amino acid residues of various proteins translated within the long polypeptide of CK-GL2 isolate showed variable sequence identity with individual proteins to other phylogenetic groups, however, overall similar results were obtained with the complete nucleotide and deduced amino acid sequence of the

polyprotein (Table 3).

The analyses of various genes of CK-GL2 isolate showed variability in the amino acid sequence similarity and the results indicated that the conserved regions of CK-GL2 isolate lie in proteins (in decreasing order): CI > 6K1 > 6K2 > HC-Pro > NIa-Pro > CP > VPg > P3 > NIb > P1 with respect to other closely related BYMV isolates (Table 3).

Phylogenetic analyses of CK-GL2 isolate

The phylogenetic analysis using nucleotide sequences of complete RNA genomes and amino acid sequences of various genes of CK-GL2 isolate under study was performed with the selected potyvirus, macluravirus and ipomovirus sequences (Figure 5). The complete genome of CK-GL2 clustered with BYMV isolates of phylogenetic group IV, reported from Japan (MBGP, MB4, GB2, Gla and G1); India (VFaba2); Taiwan (*Lisianthus*) and USA (GDD). Other BYMV isolates reported from Australia, Japan, South Korea and USA clustered in I-IX different phylogenetic groups, two closely related CYVV isolates grouped in a separate cluster, while OrMV (NC_019409), macluravirus (NC_018455) and ipomovirus (NC_010521) were taken for rooting (Figure 5). Based on BLASTn analysis, pairwise sequence similarity and phylogenetic relationship analysis with closely related potyvirus isolates, CK-GL2 was found to be the member of phylogenetic group IV of BYMV. The pairwise amino acid sequence and phylogenetic analysis of CK-GL2 showed differences in their various genes.

Recombination analysis of CK-GL2 isolate

When CK-GL2 isolate sequence was analyzed with thirty seven complete genomes of potyviruses (BYMV, CYVV and OrMV) retrieved from NCBI database (Table 3), eight recombination events were detected by two or more than two methods (Figure 6 and Table S4). The major recombination events of CK-GL2 were detected with six phylogenetic groups (I, IV, V, VI, VII and IX) of BYMV from region HC-Pro to 3'UTR. In HC-Pro region of CK-GL2, recombination was identified with phylogenetic groups I, IV and V; in PIPO, parental phylogenetic groups were I and V; in P3, CI, 6K2, VPg and NIa-Pro genes of CK-GL2, recombination was detected with parental phylogenetic groups: I, IV and V. The groups V and IV were major and minor parents in 6K1, and highest number of recombination events was detected in NIb region with phylogenetic groups: I, IV, V, VI, VII and IX (6). In CP gene of CK-GL2, the recombination events were identified with groups I, IV, VI and VII. Isolates of groups IV and VI were major and minor parents respectively, which lie in 3'-UTR region, and no recombination was detected in the 5'-UTR, and P1 gene of CK-GL2 isolate (Table S4).

Discussion

Viruses are a major problem in gladiolus because the plants are propagated by corms that may harbor the virus which may cause significant plant mortality [6]. *Gladiolus* is reported to be a natural host of several RNA viruses such as AMV, BYMV, BBWV, CMV, INSPV, OrMV, SLRSV, TAV, TMV, TRV, TRSV and TSWV [5,9,18-24].

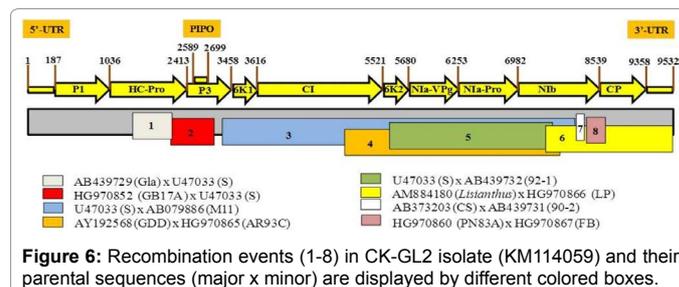


Figure 6: Recombination events (1-8) in CK-GL2 isolate (KM114059) and their parental sequences (major x minor) are displayed by different colored boxes.

Initially CK-GL2 isolate was detected in several plants of all ten cultivars, however sequence analysis of clones of ~1.5 kb gene showed 99.9-100% nucleotide identity and, therefore, an isolate from Sylvia cultivar which also has high cut-flower value was further used for full length genome amplification. The genome of CK-GL2 was of 9532 nucleotide containing 5'-UTR, a large ORF, a putatively small overlapping ORF, and 3'-UTR region, and genomic organization was very similar to several BYMV strains [25,26]. Both large and small ORFs encode all the 10 mature reported gene products: P1 proteinase; helper component proteinase (HC-Pro); P3 protein; 6K1 protein; cylindrical inclusion (CI) protein; 6K2 protein; nuclear inclusion a (NIa) protein, a polyprotein that is further processed into, the viral protein genome-linked (VPg) and the NIa proteinase (NIa-Pro); nuclear inclusion b (NIb) protein, the viral RNA dependent RNA polymerase; and coat protein (CP) [27]. An additional gene product "P3N-PIPO" was also found [28].

The existence of seven phylogenetic groupings of BYMV has been proposed earlier [26] on the basis of CP gene sequences of isolates of original hosts. A general group with a broad host range including monocots and dicots, and six other special groups designated as isolates of original hosts (broad bean, canna, lupin, monocot, pea, W). Further, Kehoe et al. [12] analyzed 40 complete BYMV genomes, covering all the original hosts of BYMV (except canna because its complete genome was not available), and suggested nine phylogenetic groups (I-IX). The general group of BYMV [26] was from a diverse origin, which was split into three groups: I, II and IV [12].

Fairly, the CK-GL2 isolate showed high sequence homology and clustered with the BYMV isolates infecting mainly gladiolus and *E. russellianum* [26] and recently proposed as BYMV phylogenetic group-IV [12]. The CK-GL2 isolate was closely related to the BYMV isolates of broad bean, *E. russellianum* and gladiolus reported from Japan and USA, indicating the introduction of BYMV with infected plant material from different locations in different time beings. The infected plant material could be the cause of introduction of a virus in new habitat or in new hosts, this might be one of the reasons that the isolates from different locations clustered together.

The complete genome of CK-GL2 isolate showed 90-96% identity with all 36 closely related BYMV isolates [29] and besides this, the HC-Pro, P3 and NIb proteins within the genome also showed high sequence diversity (lesser nucleotide identity) with them, possibly due to recombination in the CK-GL2 genome. Recombination in RNA viruses permits rapid evolution and adaptation [30] and has been well studied in potyviruses [31,32]. Recombination is found to play a major role in BYMV host specialization as suggested earlier [33]. Kehoe and colleagues also found extensive recombination occurring amongst diverse BYMV genome sequences which is likely to have significant evolutionary implications for the virus [29]. Recombination analysis revealed a total of eight firm recombination events scattered in P3 to 3'-UTR region, however, no recombination was detected in 5'-UTR and P1 gene. We report here the characterization of a new isolate of BYMV group-IV associated with mosaic disease of gladiolus for the first time in India.

Acknowledgements

Thanks are due to the director, Council of Scientific and Industrial Research-National Botanical Research Institute, Lucknow for facilities under the Council of Scientific and Industrial Research funded PMSI project "BSC0117" and Dr. R. K. Roy, Head Floriculture, CSIR-NBRI, Lucknow for providing gladiolus cultivars. The author C. Kaur is

thankful to University Grants Commission, India for fellowship.

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