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RESEARCH ARTICLE

Molecular detection and characterisation of begomovirus causing bean yellowing disease in Sri Lanka

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Abstract: Bean (*Phaseolus vulgaris* L.) is one of the major vegetable crops cultivated in tropical, sub-tropical and temperate regions in the world. Bean yellowing disease (BYD), which was first reported in 1999 from Sri Lanka is still a serious viral threat to bean cultivation causing severe yield reduction. In this study, polymerase chain reaction (PCR) assays using degenerate (universal) primers were conducted with the aim of developing molecular techniques to detect the virus. Two degenerate primer pairs, namely, Deng 540/541 and AV494/AC1048 used in the PCR reactions confirmed the association of a begomovirus with the BYD by giving the desired core coat protein amplicons of 520 bp and 550 bp, respectively. The resulting amplicons were subjected to DNA sequencing and the sequence data were analysed to determine the phylogenetic and molecular evolutionary relationships with other related begomovirus sequences obtained from the GenBank. The analysis revealed that the virus associated with BYD (BYVD-GN-SL-Partial) is closely related to Horsegram [*Macrotyloma uniflorum* (Lam.) Verdc.] yellow mosaic virus isolate (HgYMV-LK: 09-Bean) reported in Sri Lanka. Further, the DNA sequence of BYVD-GN-SL-Partial was distinctively clustered with the Indian HgYMV sequences and positioned in between the Mungbean yellow mosaic virus (MYMV) sequences.

Keywords: Begomoviruses, Horsegram yellow mosaic virus (HgYMV), molecular characterisation, polymerase chain reaction (PCR).

INTRODUCTION

Bean (*Phaseolus vulgaris* L.) is one of the major vegetable crops cultivated in Sri Lanka up to an extent of 8000 ha and has an annual production of 40,000 mt. This accounts for 23 % of the total extent of up country vegetables (Anon, 2010). Among the biotic constraints of bean production, viral diseases have been identified as a major threat in Sri Lanka due to the spread of bean yellowing disease (BYD) in epidemic proportions. BYD is caused by the Horsegram yellow mosaic virus (HgYMV), which is a bipartite begomovirus first reported in 1999 (Monger *et al*., 2010) and continues to be the major threat for bean cultivation in Sri Lanka. Hence, accurate detection and characterisation of the virus is a crucial prerequisite when formulating management strategies. Early diagnosis of viral diseases along with accurate identification of their causal agents is immensely important in managing such outbreaks (Ozalan *et al*., 2006). Using symptomatology alone to detect viral diseases is not always accurate due to similar symptoms being caused by other pathogens. Polymerase chain reaction (PCR) technique using degenerate primers is a rapid, efficient, and a reliable method widely adopted in virus detection. Therefore in this study, two degenerate (universal) primer pairs, namely, Deng 540/541 and AV494/AC1048 were used to detect begomovirus associated with beans in Sri Lanka. Certain weeds act as alternate hosts of begomovirus, hence management of the viral diseases becomes more challenging. The present study also focused on using molecular methods to identify local weed species capable of harbouring BYD causing viruses. Begomoviruses have been categorised as major quarantine pests in the world (Hamilton, 2000). Comparing viral genome sequence information is vital in predicting possible evolutionary pathways and transboundary movements, which is ultimately important in executing appropriate quarantine measures. This is the first detailed study to reveal important molecular level information useful for effective management of the BYD in Sri Lanka.

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METHODOLOGY

Bean leaf samples for PCR

Tender bean leaf samples of various cultivars showing typical BYD symptoms were collected from farmer fields of different administrative districts of Sri Lanka, namely, Kandy (e.g. Kadugannawa and Yatinuwara), Matale (e.g. Naula), Nuwara Eliya (e.g. Seetaeliya) and Badulla (e.g. Bandarawela). Apparently healthy leaf samples of the same variety were also collected from the same locations as control samples. All the leaf samples were collected when the plants were at the early flowering stage and from the plants grown during the same growing season.

Weed plants for transmission studies

Seeds of *Hedyotis corymbosa* (Rubiaceae) and *Ageratum* spp. (Compositae) were sown in pots containing sterilised soil maintained in insect-proof cages. The seeds of *Ageratum* spp. were obtained from stock cultures maintained at the Division of Plant Pathology, Horticultural Crop Research and Development Institute (HORDI), Gannoruwa, Peradeniya and the seeds of *Hedyotis corymbosa* were collected from plants grown in experimental fields of HORDI, which did not show symptoms of viral infection.

Transmission of the virus into weed plants using whiteflies

Two-week old seedlings of the weed species were inoculated using viruliferous whiteflies that had been allowed to feed for a period of 24 hrs on bean plants showing characteristic symptoms and confirmed as infected with HgYMV. Using an aspirator randomly-picked whiteflies from the bean plants were transferred to individual weed plants grown in separate micro transmission cages at a rate of 10 whiteflies/weed plant. Three such weed plants per each weed species were inoculated with the whiteflies. After an inoculation access period of 24 hrs on the weed plants, the plants were sprayed with 200 g/L SL (1 mL/L) imidachloprid (Admire®, Bayer Crop Sci.) to eliminate the whiteflies. The weed plants were then maintained in the insect-proof cages for two weeks until symptom development. Tender leaf samples showing symptoms of viral infection were collected from the weed plants and subjected to extraction of DNA. Two other sets of the same weed species inoculated with non-viruliferous whiteflies were maintained as controls under the same conditions.

Selection of degenerate primers

Begomovirus specific degenerate primers (Promega, WI, USA), namely, Deng 540/541 (Deng *et al*., 1994) and AV 494/AC 1048 (Wyatt & Brown, 1996) were used in this study (Table 1). These primers amplify a part of coat protein gene in DNA-A component of the begomoviruses.

DNA extraction from leaf tissues

Total DNA was extracted from the leaves according to the CTAB (Cetyltrimethylammoniumbromide) extraction protocol by Lodhi *et al*. (1994) with a few modifications. Briefly, 150 mg of tender leaf tissues from each type of plant were homogenised with 1.5 mL of preheated (at 65 ˚C) DNA extraction buffer (0.1 M Tris HCl, 0.2 M EDTA, 1.4 M NaCl, 2 % CTAB, 1 % PVP, β-mercaptoethanol). The homogenised material was transferred into a 1.5 mL eppendorf tube and incubated at 65 ˚C for 30 min. The tube was next centrifuged at 2,000 rpm for 1 min and the supernatant was transferred to a new eppendorf tube. An equal volume of chloroform: isoamylalcohol (24:1) was added and mixed by gentle inversion and centrifuged for 10 min at 12,000 rpm. The resulting supernatant was transferred to another eppendorf tube, into which isopropanol was added. The tube was then inverted several times and incubated overnight at -20 ˚C. DNA was pelleted by centrifugation

Table 1: Sequences of selected degenerate primers

Primer	Sequence	Product	Reference
Deng 540	5'-TAATATTACC(K)G(W)(K)G(V)CC(S)C-3'		
Deng 541	5'-TGGAC(Y)TT(R)CA(W)GG(B)CCTTCACA-3'	~520 bp	Deng *et al*. (1994)
AV 494	5'-GCC(Y)AT(R)TA(Y)AG(R)AAGCC(M)AG-3'		
AC 1048	5'-GG(R)TT(D)GA(R)GCATG(H)GTACATG-3'	~550 bp	Wyatt & Brown (1996)

Ambiguity characters according to IUPAC nomenclature are represented as B = (C,G,T); D = (A,G,T); H = (A,C,T); K = (G,T); M = (A,C); R = (A,G); S = (C,G); V = (A,C,G); W = (A,T); Y = (C,T)

for 5 min at 12,000 rpm. The pellet was then washed with 70 % ethanol and dried at room temperature for 20 min. Finally the pellet was dissolved in 100 µL of 1× TE buffer and stored at -20 ˚C.

PCR amplification

Each PCR reaction was performed in the thermal cycler MultiGene (Labnet International Inc., NJ, USA) using 25 µL of reaction mixture. This mixture consisted of 2.0 µL of total DNA extracted from infected bean leaf tissues and diluted up to 1/25 (80 – 100 ng), 0.2 µL Taq DNA polymerase (5U/ µL), 2.5 µL of 10× PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), 0.5 µL of 25 mM $MgCl_2$, 2.0 µL each primer (10 mM), 2.0 µL dNTPs mix (2.5 mM each), and sterile water to make up the required volume.

The reaction mixture was subjected to one cycle of initial denaturation at 94 ˚C for 5 min followed by 30 cycles of denaturation at 94 ˚C for 30 s, annealing at 58 ˚C for 30 s, extension at 72 ˚C for 45 s and a final extension at 72 ˚C for 10 min for Deng 540 and Deng 541 primers.

Optimising PCR conditions for AV 494 and AC 1048 primers

The gradient PCR reactions were performed in the range of 55 – 65 ˚C under two dilutions of genomic DNA template [1/10 (200 – 250 ng) and 1/25 (80 – 100 ng)] to determine the optimum annealing temperature for AV 494 and AC 1048 and optimum dilution of DNA, respectively.

Analysis of PCR products

The PCR products were analysed on 1.0 % agarose gel at 60 V for 1 hr in 1× TBE buffer [100 mM Tris (pH 8), Boric acid, 0.5 M EDTA (pH 8)]. The gel was stained with ethidium bromide at 0.5 µg/mL. PCR product size was estimated using a 100 bp DNA size marker (Promega, WI, USA).

DNA sequencing of partial genome of BYD

A fragment of the core coat protein gene present on the genome A component of the causal virus of BYD was amplified using the above two primer pairs and sent for DNA sequencing to Genetech Pvt. Ltd., Sri Lanka. The sequence information obtained was subjected to homology search using the basic local alignment tool (BLAST) (Altschul *et al*., 1990) available at *www.ncbi.nlm.nih.gov/BLAST* (GenBank).

Phylogenetic and molecular evolutionary analysis

Thirty accessions showing higher homology with the DNA sequence obtained from the present study (designated as BYVD-GN-SL-Partial) were selected from the DNA databases available at *www.ncbi.nlm.nih.gov/BLAST* (GenBank). Using MEGA 4.0 software package, phylogenetic and molecular evolutionary relationships of the DNA sequences were determined by neighbour-joining method at 1000 bootstrap value (Tamura *et al*., 2007).

RESULTS

Appearance of symptoms on weed plants inoculated with viruliferous whiteflies

Virus-like symptoms appeared on the leaves of *H. corymbosa* after two weeks of inoculation using viruliferous whiteflies. However, the symptoms were different to the typical yellowing symptoms seen on beans and showed crinkling of both adaxial and abaxial surfaces of the leaves (Figure 1). In contrast, *Ageratum* spp. did not show any virus-like symptom (data not shown).

(A) (B) (C)

Figure 1: (A) Healthy *H. corymbosa* plant; (B) adaxial surface of inoculated *H. corymbosa* leaves and (C) abaxial surface of inoculated *H. corymbosa* leaves after 2 weeks of inoculation with viruliferous whiteflies

PCR by degenerate primers

Consistent amplification of ~520 bp fragments was obtained from DNA extracted from all bean leaf samples, which showed characteristic yellowing symptoms, and also in the two virus-inoculated weed species (i.e. *Ageratum* spp. and *H. corymbosa*) with Deng 540/541 primer pair (Figure 2). The amplification through gradient PCR using AV 494/AC 1048 primers showed faint bands of ~550 bp at the annealing temperature of 56 ˚C for 2 min (data not shown) and was found to be non-consistent over PCR assays performed within the range of 55 – 65 ˚C. No amplification resulted in the DNA extracted from apparently healthy leaves of bean plants and weeds with any of the above primer pairs.

Table 2: Sequence information of begomoviruses highly homologous to the DNA sequence (BYVD-GN-SL Partial) of the present study

SI no.	Accession no.	Description	Acronym	Origin	Maximum identity
1	GU323321.1	Horsegram yellow mosaic virus isolate LK:09 segment DNA-A, complete sequence	HgYMV-LK:09-Bean	Sri Lanka	96 %
2	AM932427.1	Horsegram yellow mosaic virus segment DNA-A complete sequence, isolate horse gram	HgYMV-CP-Ban-IN	India	91 %
3	AM932425.1	Horsegram yellow mosaic virus segment DNA-A complete sequence, isolate Frenchbean	HgYMV-A-Hg-Ban1-IN	India	91 %
4	AM932429.1	Horsegram yellow mosaic virus segment DNA-A complete sequence, isolate lima bean	HgYMV-A-Lb-Ban2-IN	India	91 %
5	AJ627904.1	Horsegram yellow mosaic virus AC4 gene, AV1 gene, AC1 gene, AC2 gene,AC3 gene and AV2 gene	HgYMV-A-Hg-Coi-IN	India	90 %
6	JX244175.1	Mungbean yellow mosaic virus isolate VN10 segment DNA-A, complete sequence	MYMV-Mb-VN10	Vietnam	84 %
7	JX244174.1	Mungbean yellow mosaic virus isolate VN7 segment DNA-A, complete sequence	MYMV-Mb-VN7	Vietnam	84 %
8	JX244173.1	Mungbean yellow mosaic virus isolate VN5 segment DNA-A, complete sequence	MYMV-Mb-VN5	Vietnam	84 %
9	AF314530.1	Mungbean yellow mosaic virus-Vigna [Maharashtra] A component, complete sequence	MYMV-Sb-Mhr-IN	India	84 %
10	AJ132575.1	Mungbean yellow mosaic virus-Vigna segment A, complete sequence	MYMV-Mb-TN-IN	India	84 %
11	JX244172.1	Mung bean yellow mosaic virus isolate VN1 segment DNA-A, complete sequence	MYMV-Mb-VN1	Vietnam	84 %
12	DQ865201.1	Mungbean yellow mosaic virus isolate Namakkal segment DNA-A, complete sequence	MYMV-mothb-Nam-TN-IN	India	84 %
13	DQ389150.1	Mungbean yellow mosaic India virus pre-coat protein (AV2) and coat protein (AV1) genes, complete cds	MYMV-Sb-Mhr1-IN	India	84 %
14	JX244176.1	Mung bean yellow mosaic virus isolate VN15 segment DNA-A, complete sequence	MYMV-Mb-VN15	Vietnam	83 %
15	AY271892.1	Mungbean yellow mosaic virus DNA-A, complete sequence	MYMV-Mb-CAMB	Cambodia	83 %
16	AJ421642.1	Mungbean yellow mosaic virus-Soybean [Madurai] segment DNA A, complete sequence, strain Madurai	MYMV-Sb-Madur-TN-IN	India	83 %
17	DQ389144.1	Mungbean yellow mosaic virus-[NAV] pre-coat protein (AV2) and coat protein (AV1) genes, complete cds	MYMV-Sb-Nav-Guj-IN	India	83 %
18	DQ400848.1	Mungbean yellow mosaic 19 virus segment DNA A, complete sequence	MYMV-Bg-Vam-TN-IN	India	83 %
19	AB017341.1	Mungbean yellow mosaic virus-Thailand DNA A, complete sequence	MYMV-Mb-TH	Thailand	82 %
20	D14703.1	Mungbean yellow mosaic virus DNA, segment DNA 1, complete sequence	MYMV-Mb-JAP	Japan	82 %
21	AY269991.1	Mungbean yellow mosaic virus - Soybean [Pakistan] segment A, complete genome	MYMV-Sb-PK	Pakistan	82 %
22	AY271896.1	Mungbean yellow mosaic virus isolate Haryana segment DNA-A, complete sequence	MYMV-Mb-Har-IN	India	82 %
23	FM242701.1	Mungbean yellow mosaic virus DNA A, complete sequence, clone MI65	MYMV-Rhynchosia capitata-PK	Pakistan	82 %

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Sl no.	Accession no.	Description	Acronym	Origin	Maximum identity
24	FN794200.1	Mungbean yellow mosaic India virus segment A, complete sequence, isolate Palampur	MYMV-Comn.Bn-Pala-IN	India	80 %
25	FM208845.1	Mungbean yellow mosaic India virus, segment DNA-A, complete sequence, clone MI13	MYMV-Mb-Is-PK	Pakistan	80 %
26	FM208834.1	Mungbean yellow mosaic India virus, segment DNA-A, complete sequence, clone A1E	MYMV-Sb-Fai-PK	Pakistan	79 %
27	FM208833.1	Mungbean yellow mosaic India virus, segment DNA-A, complete sequence, clone A1	MYMV-Sb-Fai 1-PK	Pakistan	79 %
28	AM992618.1	Mungbean yellow mosaic India virus, segment DNA-A, complete genome	MYMV-CP-ND-IN	India	79 %
29	JQ398669.1	Mungbean yellow mosaic virus [Urdbean:New Delhi:2011] clone MF2 segment A, complete sequence	MYMV-Mb(Ub)-ND-IN	India	81 %
30	FM955599.1	Mungbean yellow mosaic India virus, segment DNA-A, Vigna radiata av2 gene, AV1 gene, AC5 gene, AC3 gene, AC2 gene, AC1 gene and AC4 gene, clone MI72, complete genome	MYMV-Vig.rad-Fai-PK	Pakistan	79 %

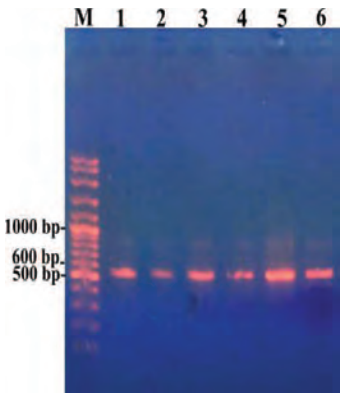

Figure 2: PCR amplification of virus infected beans and virus inoculated weeds using Deng 540/541 primers. (Lane M: 100 bp marker; Lanes 1, 2, 3, and 4: BYD infected beans collected from Kandy, Matale, Nuwara Eliya and Badulla Districts respectively; Lane 5: *Ageratum* spp.; Lane 6: *H. corymbosa*)

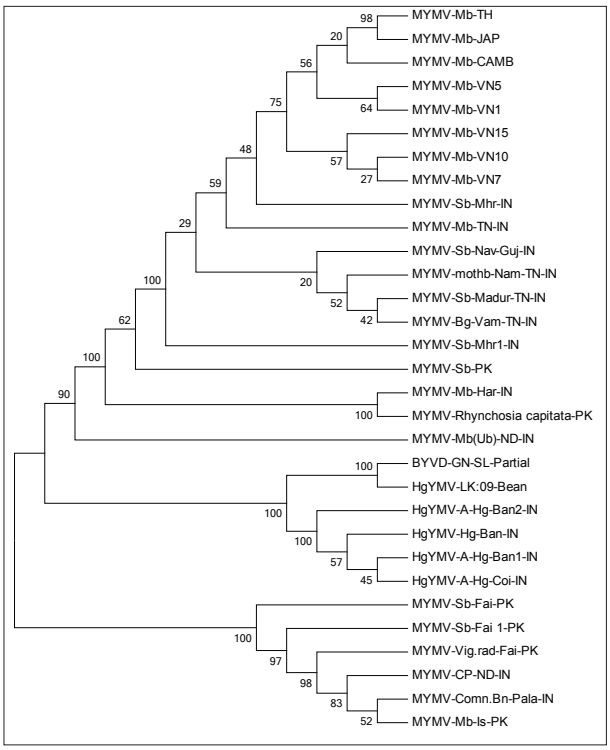

Figure 3: Molecular and evolutionary relationship of partial CP gene of begomoviruses associated with BYD in Sri Lanka (BYVD-GN-SL-Partial) to other homologous sequences

Phylogenetic and molecular evolutionary analysis

Closely related sequences to the DNA sequence of BYVD-GN-SL-Partial obtained through DNA homology search are given in Table 2. Phylogenetic and molecular evolutionary relationships obtained after analysing the 30 sequences using MEGA 4.0 software at 1000 bootstrap value are illustrated in Figure 3.

DISCUSSION

The present study confirmed the causal virus of BYD as a begomovirus, which can be successfully detected in infected plant tissues using Deng 540/541 primers and AV 494/AC 1048 primers. Findings of the present study agree with the existing research information that both Deng 540/541 (Rajeshwari *et al*., 2005; Govindappa *et al*., 2011; Bandaranayake *et al*., 2014) and AV 494/AC 1048 (Raj *et al*., 2008; Govindappa *et al*., 2011) primers are efficient tools to detect begomoviruses.

Although Wyatt and Brown (1996) have successfully detected different begomoviruses using AV 494/AC 1048 primers, no consistent amplification was obtained for BYD in the present study. The poor amplification could be due to less affinity of the primers towards template DNA and the presence of PCR inhibitors (Ghosh *et al*., 2009). The present study revealed the possibility of artificial inoculation of the begomovirus associated with BYD into *Ageratum* spp. and *H. corymbosa* through whiteflies indicating that they are possible alternative hosts that can harbour the virus naturally. According to Saunders *et al*. (2002) *A. conizoides* is a host of Sri Lankan cassava mosaic virus (SLCMV), which is also a begomovirus. Therefore, a detailed study on natural weed flora harbouring this virus is worthwhile.

The amplified DNA fragment of the BYD causal virus (BYVD-GN-SL-Partial) showed 96 % sequence homology to HgYMV-LK:09-Bean isolate, which has been isolated from beans in Sri Lanka by Monger *et al*. (2010). BYVD-GN-SL-Partial also showed 90 – 91 % sequence homology to several HgYMV isolates reported from India in Horsegram, Lima bean and French bean (Table 2). The DNA sequence of the present study formed a sub cluster with HgYMV-LK:09-Bean isolated by Monger *et al*. (2010) from beans in Sri Lanka, indicating the genetic similarity of BYVD-GN-SL-Partial with HgYMV-LK:09-Bean. Furthermore, both sequences (BYVD-GN-SL and HgYMV-LK:09-Bean) formed a distinct cluster with other HgYMV isolates, namely, HgYMV-CP-Ban-IN, HgYMV-A-Hg-Ban1-IN, HgYMV-A-Lb-Ban2-IN and HgYMV-A-Hg-Coi-IN reported in India suggesting the close genetic relationship of the begomovirus associated with bean yellowing disease in Sri Lanka with the HgYMV isolates in India. Thus phylogenetic analysis provides information that the begomovirus associated with BYD can be a pseudorecombinant of begomoviruses associated with the Horsegram yellow mosaic disease caused by HgYMV and mungbean yellow mosaic disease caused by mungbean yellow mosaic virus (MYMV). Similarly, Qazi *et al*. (2007) have suggested pseudorecombination as a possible reason for clustering of MYMV-(IN:Mad:Sb) along with HgYMV. Further, a higher degree of genetic similarity of the mungbean yellow mosaic virus with HgYMV-(IN:Coi) has been reported by Girish and Usha (2005).

The present study provides important information on rapid and precise detection of the BYD, its genetic characteristics and evolutionary relationships, and potential weed hosts. This information can be utilised to formulate future management strategies useful for effective management of the virus through crop management and quarantine practices.

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