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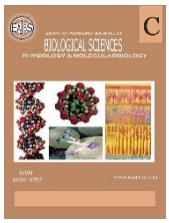

Molecular Detection and Phylogeny of The Bipartite Begomovirus *Pepper golden mosaic virus* Associated with Okra Leaf Curl Betasatellite in the *Iresine herbstii* Ornamental Plant in Egypt

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ABSTRACT

Pepper golden mosaic virus (PepGMV), a whitefly-transmitted bipartite begomovirus, is one of the most important viruses infecting pepper plants in the Western Hemisphere, including the United States and Latin America. PepGMV was detected in Giza, Egypt and identified as (PepGMV-EG-GZ), infecting the ornament *Iresine herbstii* and inducing mosaic and leaf curl symptoms. PCR analysis of the virus genome confirmed its bipartite begomovirus nature and its association with the defective okra leaf curl betasatellite (OLCB), namely PepGMB (FJ436005). The GenBank Blastn analysis, phylogeny, and nucleotide pairwise sequence identity (PSI) showed the clustering of PepGMV-EG-GZ DNA-A (FJ416867) and DNA-B (FJ416868) with PepGMV-Mo-US:TX (AY928512) and PepGMV-D-US:TX (AY928514), respectively. PepGMV-EG-GZ DNA-A had 98.2% PSI with PepGMV-Mo-US:TX, suggesting it was a variant of PepGMV isolates. PepGMB clustered with several Egyptian defective betasatellites. PepGMB had a PSI of 96.4% with OLCB-squash (FJ455515), indicating that it was an isolate of OLCBs. PepGMB had PSIs of 61.1% and 65.9% with the intact OLCB and cotton leaf curl betasatellite (CLCuB) from Pakistan (AJ316029) and India (AJ316037), respectively, an indication of its different nature from these two satellites. On the other hand, the intact OLCB(s) from Egypt clustered with the intact OLCB and CLCuB from Pakistan and India, respectively, an indication of their origin in the Indian subcontinent and not in the Middle East. Nucleotide sequence analysis on PepGMB showed the presence of a satellite-conservative region stem-loop structure similar to the other betasatellites. The possible role of defective PepGMB in affecting symptom development in *I. herbstii* is discussed.

INTRODUCTION

The family *Geminiviridae* circumvents plant-infecting viruses comprised of a circular ssDNA genome with ~2.7 kb in length encapsidated in twinned particles with average dimensions of 18×30 nm and exists in most parts of the world (Stanley 1985). According to their genomic identities, they have been classified by the International Committee of the Taxonomy of Viruses (ICTV) into fourteen genera, viz., *Becurtovirus*, *Begomovirus*, *Capulavirus*, *Citlodavirus*, *Curtovirus*, *Eragrovirus*, *Grablovirus*, *Maldovirus*, *Mastrevirus*, *Mulcrilevirus*, *Opunvirus*, *Topilevirus*, *Topocuvirus*, and *Turncurtovirus* (Mishra *et al.*, 2020; Walker *et al.*, 2021; Zerbini *et al.*, 2017).

Members of the genus *Begomovirus*, with >440 virus species, are among the most important viruses transmitted by different biotypes of the whitefly *Bemisia tabaci* Genn. (*Hemiptera*: *Aleyrodidae*) and inflicting heavy economic losses in various economic crops worldwide (Zerbini *et al.,* 2017; Rojas *et al.,* 2018; Fiallo-Olivé and Navas-Castillo, 2020). Begomoviruses can be divided according to their geographic distribution, genetic diversity, and genomic organization into the Old World (OW) and New World (NW) begomoviruses. The OW begomoviruses have monopartite and/or bipartite genomes. Monopartite begomoviruses, though, seem to outnumber those with bipartite genomes (Ha *et al.,* 2008). NW begomoviruses are mostly bipartite (Brown *et al.,* 2015). However, few monopartite begomoviruses were recently detected in the NW (Duffy *et al.,* 2007; Fiallo-Olivé and Navas-Castillo, 2020).

The genome of bipartite begomoviruses is divided into DNA A and DNA B of 2.6 kb in size, each with no sequence homology except for what is known as the common intergenic region (∼200 nt) and the stem loop motif (TAA TAT TAC). DNA A encodes the coat protein, replication initiation protein, replication enhancer, and transcriptional activator protein. DNA B is responsible for encoding the movement protein functions (Nawaz-ul-Rehman and Fauquet 2009). The monopartite begomoviruses have circular ssDNA (DNA A) of approximately 2.6 kb in size and contain the necessary genetic information on one DNA molecule (Rojas *et al.,* 2001).

Several types of globally distributed DNA satellites have been described to be associated with begomoviruses, *viz*., betasatellites (Briddon *et al.,* 2003), alphasatellites (DNA 1) (Briddon *et al.,* 2004; Lefeuvre *et al.,* 2010), and deltasatellites (Lozano *et al.,* 2016).

Betasatellites (family *Tolecusatellitidae*, Briddon *et al.,* 2016) are circular ssDNA (∼ 1.3 kb) that depend on their helper viruses for replication, encapsidation, movement, and vector transmission (Briddon *et al.,* 2003). Betasatellites have a highly conserved structure with highly divergent sequences. They contain a highly conserved structure with a single gene known as βC1 (encoding the βC1 protein in the complementary-sense strand), a high-rich adenine region, and a satellite-conserved region (SCR) with ∼150 nt in length. The βC1 protein is a pathogenicity determinant and suppressor of post-transcriptional gene silencing (Briddon *et al.,* 2003; Nawaz-ul-Rehman *et al.,* 2009). Briddon *et al.* (2003) and Akhtar *et al.* (2014) described deletion mutants of betasatellites with half the size of intact betasatellites that maintained the A-rich region and the SCR and could be trans-replicated by the helper begomovirus. These deleted mutants resembled non-coding DNA satellites associated with sweepoviruses (Lozano *et al.,* 2016). Association of betasatellites with OW begomoviruses leads to an increase in viral DNA accumulation, efficiency of transmission by *B. tabaci*, and severity of symptoms (Briddon *et al.,* 2003; Sharma *et al.,* 2010; Venkataravanappa *et al.,* 2011; Sivalingam and Varma, 2012; Jyothsna *et al.,* 2013; Leke *et al.,* 2015; Devendran *et al.,* 2022). A study by Nawaz-ul-Rehman *et al.* (2009) showed the adaptability of the OW cotton leaf curl Multan betasatellite to interact with the NW bipartite *Cabbage leaf curl virus*, leading to the enhancement of symptoms and satellite-DNA accumulation in *Nicotiana benthamiana.*

Alphasatellites (family: *Alphasatellitidae;* Briddon *et al.,* 2018) with circular ssDNA (∼ 1.3 kb) are autonomously replicating in the host but depend on their helper virus for their movement, encapsidation, and vector transmission (Briddon et al., 2004; Briddon and Stanley, 2006). Initially, it was thought that beta and alphasatellite complexes were associated with monopartite begomoviruses in the OW. Recently, however, alphasatellites have been detected with NW begomoviruses (Romay *et al.,* 2010) but without association with

betasatellites (Ha *et al.,* 2008; Rosario *et al.,* 2016).

Deltasatellites (family: *Tolecusatellitidae*, Briddon *et al.,* 2016), with 11 known species, are small non-coding DNA satellites associated with begomoviruses and distinct from betasatellites. Deltasatellites are about one-quarter the size of a begomovirus genome or genomic component and have all the features of *Tomato leaf curl virus*- satellite (ToLCV-sat) (Fiallo-Olivé and Navas-Castillo, 2020).

Several investigators have pointed out the possible movement of both beta and alphasatellites across different plant species as being promiscuous (Leke *et al.,* 2015; Abdel-Salam *et al.,* 2017). Furthermore, an alphasatellite and a betasatellite were found in association with the *wheat dwarf Indiavirus, a* monocot-infecting *Mastrevirus,* upon infecting wheat (Kumar *et al.,* 2020).

PepGMV, family *Geminiviridae*, genus *Begomovirus,* causes severe economic losses in both South America and the Western States of the USA (Brown *et al.,* 2005). PepGMV was first isolated in Texas in 1987 and in Mexico in 1989 from infected pepper plants and was early called Texas pepper geminivirus (TPGV) by Stenger *et al.* (1990). TPGV has geminate particles and a bipartite genome and was transmitted mechanically and persistently by *B. tabaci* to peppers and tomato plants (Stenger *et al.,* 1990). Based on nucleotide sequence comparisons between different isolates of TPGV and related viruses, the ICTV (Fauquet *et al.,* 2003) merged these virus isolates under the name PepGMV (Brown *et al.,* 2005). Diseases caused by PepGMV include members of the *Solanaceae*: *Capsicum annuum, C. frutescens, Datura discolor, Nicandra physaloides, Nicotiana glauca, Physalis ixocarpa,* and *Solanum lycopersicum* (Lotrakul *et al.,* 2000; Holguín-Peña *et al.,* 2004; Brown et al., 2005; Nakhla et al., 2005; Góngora-Castillo et al., 2012; Castro et al., 2013); members in the C*ucurbitaceae: Cucrbita moschata, C. pepo, Sechium edule;* and the *Fabaceae*: *Erythrina* spp. (Castro *et al.,* 2013).

The genus *Iresine* contains 20 to 25 species (Gledhill, 2008). *I. herbstii* (bloodleaf) is an ornamental perennial plant species in the family *Amaranthaceae* and native to South America. It has important medicinal and pharmacological properties. *Iresine* species plants are reproduced through seeds and stem cuttings (Dipankar *et al.,* 2011). *I. herbstii* was introduced to Egypt as an ornamental plant, probably in the last century. A survey for the presence of begomoviruses in vegetable crops, weeds, and ornaments was conducted in different locations in Egypt (Abdel-Salam *et al.,* 2017). Preliminary results based on symptoms, immunocapture-PCR, and rolling circle amplification (RCA) indicated that tested samples of *I. herbstii* were infected with a begomovirus of unknown nature. The purpose of the present study is to unveil the nature of this begomovirus infecting *I. herbstii* plants and its associated satellite using molecular tools used for virus identification and diagnosis.

MATERIALS AND METHODS

Sample Collection:

Leaves of *I. herbstii* showing virus–like symptoms of mosaic and associated leaf curling (Fig. 1**)** were collected, by the first author, from the Experimental Farm of the Faculty of Agriculture, Cairo University, in 2020. Permission for sample collection was obtained from the chairman of the Department of Plant Pathology, Faculty of Agriculture at Cairo University. A voucher specimen for collected infected leaves, preserved with silica gel, was deposited at the Department of Plant Pathology repository under "PepGMV: *I. herbstii* June, 2020, identified by Aly M. Abdel-Salam; email: ali.mamoun@agr.cu.edu.eg. Field studies on infected *I. herbstii* plants, including the collection of plant material, were compliant with relevant institutional, national, and international guidelines and legislation.

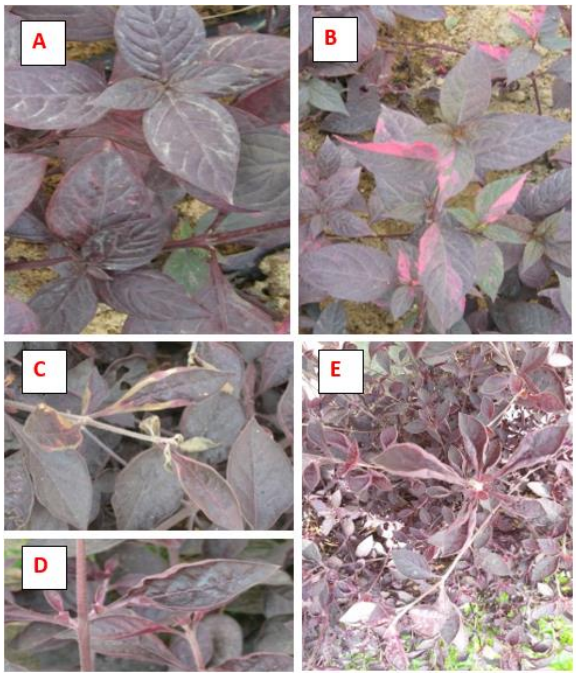

Fig. 1. Symptoms observed on *Iresine herbstii* plants upon infection with PepGMV. A, healthy plants; B, mosaic symptoms; C, development of leaf curl symptoms and mosaic; D, and E, leaf curling and mosaic symptom recovery.

DNA Extraction, RCA, cloning, and DNA Analysis:

Nucleic acid extraction for begomovirus and associated satellites was performed on fresh-leaf samples using the CTAB-based method (Haible *et al.*, 2006). Rolling circle amplification (RCA) was performed using Phi29 DNA polymerase (Inoue-Nagata *et al.*, 2004) according to the manufacturer's instructions (TempliphiTM, Amersham Biosciences). To enrich for circular genomic viral and betasatellite DNA molecules of amplified RCA, products were re-amplified to confirm the begomovirus presence and associated satellites using: 1- Av/Ac degenerate primers (Brown's laboratory, Tucson, AZ, USA), designed to amplify ~579 nt of the DNA A of the core coat protein gene (AV1) of begomoviruses; 2- primers BV1855 (Idris and Brown, 1998) and BV2571 (Idris and Brown, 2004) designed to yield ~665 bp of DNA B; and 3- β01/β02 primers (Briddon *et al.*, 2002) designed to amplify the full length of DNA betasatellies. For details of the DNA sequences of the three primer pairs mentioned above, their annealing temperatures, and PCR protocols, please refer to Supplementary Table 1, and Abdel-Salam *et al.* (2017).

The different DNA amplicons were examined by electrophoresis using a 1% agarose gel prepared in 1X TAE buffer at 100 V for 40 min and then stained with 0.5 µg ml^{-1} ethidium bromide. DNA fragments of interest were cut, purified, ligated with the pGEM T-easy vector (Promega), and used to transform cells of *Escherichia coli*, strain DH5α, according to Sambrook *et al.* (1989). Plasmids were recovered from the bacterial cells. The presence of DNA inserts of viral DNA-A, DNA-B, and DNA betasatellites in plasmids was confirmed using the corresponding primer pairs and PCR testing before sending plasmids for DNA sequencing using Sanger's method.

The obtained DNA sequences from the GenBank were analyzed using Blastn at the NCBI website to find species with high similarity according to the recommendation of the ICTV-Geminiviridae Study Group (Brown *et al.*, 2015). Sequences of PepGMV and other corresponding sequences (Table 1) were aligned by MUSCLE using Mega11 (Tamura *et al.*, 2021). The phylogenetic relationships of the begomovirus sequences were analyzed by the Maximum Likelihood

method and Tamura-Nei model (Tamura and Neil, 1993) in MEGA11 (Tamura *et al.*, 2021). Initial trees for the heuristic search were obtained automatically by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model and then selecting the topology with a superior log likelihood value. The trees were drawn to scale, with branch lengths measured in the number of substitutions per site (next to the branches).

Table 1. New and Old World begomoviruses and their GenBank accession numbers used in phylogenetic and/or SDT analyses.

Virus/isolates	Acronyms	Hosts	Origin	Begomovirus Access. #	
				DNA-A	DNA-B
New World viruses					
Abutilon mosaic virus-US:Hwa	AbMV-US:Hwa	*Abutilon theophrasti*	Hawaii, USA	U51137	U51138
Bean golden mosaic virus-Br	BGMV-Br	*Phaseolus vulgaris*	Brazil	M88686	M88687
Cabbage leaf curl virus-US:FL	CaLCV-US:FL	*Brassica oleracea*	Florida, USA	U65529	U65530
Pepper golden mosaic virus isolates					
Pepper golden mosaic virus-Distortion-US:TX	PepGMV-D-US:TX	*Capsicum frutescens*	TX, USA	AY928514	AY928515
Pepper golden mosaic virus-Serrano-MX:Si	PepGMV-Ser- MX:Si	*Capsicum annuum*	Sinaloa, Mexico	AY928516	AY928517
Pepper golden mosaic virus-Mo-US:TX	PepGMV-Mo-US:TX	*Capsicum frutescens*	TX, USA	AY928512	AY928513
Pepper golden mosaic virus-Costa Rica	PepGMV-CR	*Capsicum frutescens*	Costa Rica	AF149227	NA**
Pepper golden mosaic virus Guanajuato-MX	PepGMV-GU:MX	*Solanum lycopersicum*	Guanajuato Mexico	GU128148	GU128147
Pepper golden mosaic virus-Egypt:Giza*	PepGMV-EG:GZ	*Iresine herbstii*	Giza, Egypt	FJ416867	FJ416868
Potato yellow mosaic virus-isolate Venezuela-US	PYMV-Ven-US	*Nicotiana benthaminiana*	USA	D00940	D00941
Squash leaf curl virus MX:BCS:La Paz	SLCuV-MXBCS:La_Paz	*Cucurbita pepo*	Baja California Sur,	MF187211	MG544926
Squash leaf curl virus-WAZ-US:Arizona	SLCuV-WAZ-US:AZ	*Cucurbita pepo*	Arizona, USA	DQ285016	DQ285018
Squash leaf Curl virus-Egypt:Cairo*	SLCuV-EG:Cai	*Cucurbita pepo*	Cairo, Egypt	DQ285019	DQ285020
Tomato mottle virus-US:FL	ToMoV-US:FL	*Solanum lycopersicum*	Florida, USA	L14460	L14461
Old World viruses					
African cassava mosaic virus-Kenya	ACMV-KE	*Manihot esculenta*	West Kenyan	J02057	J02058
Ageratum leaf curl virus-Ind	ALCuV-Ind	*Ageratum conizoides*	India	KU376491	
Cotton leaf curl Gezira virus-Egypt:Ash	CLCuVGeV-EG:Ash	*Capsicum annuum*	Ashmoun, Egypt	MK947932	
Cotton leaf curl Gezira virus isolate OLCV-Egypt	CLCuVGeV-(OLCV)-EG	*Abelmoschus esculentus*	Giza, Egypt	AY036010	
Cotton leaf curl Multan Virus-G-Ind:Rajasthan	CLCuMuV-G- Ind:Raj	*Gossypium hirsutum*	Punjab, India	NC_003199	
Hollyhock leaf crumple virus-Egypt:Cairo	HLCrV-EG:Cai	*Alcea rosea*	Cairo, Egypt	NC004071	
Sweet Potato Leaf curl virus-Egypt:Giza	SPLCV-EG:GZ	*Ipomoea batatas*	Giza, Egypt	FJ455517	
Tomato leaf curl virus-Australia	ToLCV-Aus	*Solanum lycopersicum*	Australia	S53251	
Tomato yellow leaf curl virus-Egypt:Giza	TYLCV-EG:GZ	*Solanum lycopersicum*	Giza, Egypt	FJ030876	
Tomato leaf curl New Delhi virus-Ind	ToLCNDV-Ind	*Solanum lycopersicum*	India	EF068246	EF408038

*bipartite begomoviruses isolated in Egypt, **NA=not available.

The Sequence Demarcation Tool (SDT v1.2) (Muhire *et al.,* 2014), based on muscle alignment, was used to measure the percentage of DNA-sequence identities for begomoviruses and associated satellites (Tables 1 and 2). Analysis of the betasatellite stem loop containing the TAATATTAC sequence was carried out using Clustal Omega (http://ebi.ac.uk/Tools/msa/clustalo/).

Table 2: DNA beta- and deltasatellites involved in the present study.

Betasatellites					**References**
GenBank Access. #	**DNA-satellite Names**	**Host**	**Length (nt)**	**Country**	
AF397215	OLCB	Okra	1305	Egypt (EG)	Idris et al. (2002)
AF397217	OLCB	Okra	1350	Egypt (EG)	Idris et al. (2002)
AJ316039	OYVB	Okra	1307	Egypt (EG)	Briddon et al. (2003)
AJ316029	OLCB	Okra	1361	Pakistan (PK)	Briddon et al. (2003)
AJ316037	CLCuB	Cotton (*Gossybium hirsutum*)	1353	India (IN)	Briddon et al. (2003)
Defective betasatellites					
FJ436005	PepGMB	*Iresine herbstii*	653	Egypt (EG)	Present study
FJ187796	OLCB	Okra	668	Egypt (EG)	Abdel-Salam et l.(2017)
Af397216	OLCB	Okra	774	Egypt (EG)	Idris et al. (2002)
FJ455515	OLCB	Squash	704	Egypt (EG)	Abdel-Salam et l.(2017)
AF397214	HLCrB	Cotton (*Gossybium hirsutum*)	741	Egypt (EG)	Idris et al. (2002)
AJ316044	HLCrB	Hollyhock	660	Egypt (EG)	Briddon et al. (2003)
AJ316043	OYVB	Okra	759	Egypt (EG)	Briddon et al. (2003)
Deltasatellites					
FJ914391	SPLCD1	Sweet potato	622	Spain (SP)	Lozano et al. (2016)
KF716173	SPLCD2	Sweet potato	733	Venezuela (VEN)	Lozano et al. (2016)
U74627	ToLCD	Tomato	682	Australia (AUS)	Dry et al. (1997)

CLCuB= Cotton leaf curl betasatellite, HLCrB =Hollyhock leaf crumple betasatellite, OLCB= Okra leaf curl betasatellite, OYVB=Okra yellow vein betasatellite, PepGMB=Pepper golden mosaic betasatellite, SPLCD=Sweet potato leaf curl deltasatellite, ToLCD=Tomato leaf curl deltasatellite

RESULTS AND DISCUSSION

Symptomatology, Analysis of Virus DNAs, and Associated Satellite:

Symptom Development And Role Of Betasatllite In Symptom Aggravation:

Naturally infected *Iresine herbstii* plants with PepGMV-EG-GZ showed a mosaic pattern on young leaves (Fig. 1-B), which mostly disappeared, and the plants showed a recovery of symptoms. Later on, most of the mosaic symptoms were replaced by leaf curling (Fig. 1-C). Finally, all infected leaves showed only leaf curling symptoms (Fig. 1-D and 1-E). Similarly, symptom remission or host-recovery phenomenon noticed in infected iresine plants with PepGMV was previously described in PepGMV infecting pepper plants, where PepGMV was reported to induce symptom-remission phenomenon in peppers (Carrillo-Tripp *et al.*, 2007; Rodríguez-Gandarilla *et al.*, 2020).

PCR experiments using β01/β02 primers indicated the association of a defective betasatellite with PepGMV-infected *I. herbstii* plants (Table 2). The association of defective PepGMB with PepGMV infection in *I. herbstii* and its role in symptom aggravation represent an important issue worth discussion. Most of the associated intact betasatellites containing the βC1 gene intensify leaf curl-symptom expression upon

association with mono and bipartite begomoviruses (Briddon *et al.,* 2003; Sharma *et al.,* 2010; Venkataravanappa *et al.,* 2011; Sivalingam and Varma, 2012; Jyothsna *et al.,* 2013; Leke *et al.,* 2015; Agnihotri *et al.,* 2018; Devendran *et al.,* 2022). On the other hand, defective DNA betasatellites, with partial deletion of viral genomes, were referred to as defective interfering molecules alleviating symptom expression, as suggested by Patil and Dasgupta (2006). Previously, Briddon *et al.* (2003) considered defective betasatellites as entities not to influence the symptoms caused by their helper begomovirus. In the present study, however, severe leaf curling symptoms in iresine leaves were associated with defective betasatellite presence, an indication of their possible effect on intensifying symptom development. Usahrani et al. (2004) indicated that the bipartite ToLCNDV caused a severe disease form upon its association with defective betasatellite DNA molecules. Abdel-Salam *et al.* (2017) showed that several monopartite begomoviruses, such as OLCV, HLCrV, and OYVV, and the bipartite begomovirus, *Squash leaf curl virus* (SLCuV), were associated with defective betasatellites; where infected plants developed typical symptoms of leaf curling, vein swelling, vein greening, and/or leaf enations. Furthermore, the *Cotton leaf curl Gezira virus* (CLCuGV) associated with defective cotton leaf curl Gezira betasatellites was recently detected in Texas, USA, for the first time, infecting okra plants, resulting in the total loss of the okra crop (Villegas *et al.,* 2019). Additionally, Fiallo-Olivé and Navas-Castillo (2020) demonstrated that agro-inoculation of the helper virus *Chorchorus yellow vein Cuba virus*, a monopartite begomovirus containing a recombinant DNA-A and associated with the non-coding desmodium leaf distortion deltasatellite, enabled this virus to infect non-host common bean plants, thus extending the host range of this virus. From the above results, one would suggest that though the defective DNA satellites are lacking the βC1 gene, coding for the pathogenesis determinant protein, other factors such as the DNA sequences of the A-rich region and the SCR, required for maintaining trans-replication (Saunders *et al.,* 2008) by the helper virus, may also be involved in determining pathogenesis (Gnanasekaran *et al.,* 2019). With the presence of the promoter of the βC1 gene in the A-rich region (Gnanasekaran *et al.,* 2019), the loss of the βC1 gene in defective betasatellites could be compromised through mixed infection with other helper begomoviruses, associated with full-length betasatellites, through recombination, pseudo-recombination, and DNA sharing (Kumar *et al.,* 2015; Gnanasekaran *et al.,* 2019; Leeks *et al.,* 2019; Mubin *et al.,* 2020). In fact, all the defective betasattellites from Egypt mentioned in the present study were detected with their corresponding full-length betasatellites (Idris *et al.,* 2002; Briddon *et al.,* 2003), although the frequency of the intact betasatellites was far less than that of defective betasatellites, an indication of DNA sharing that enabled these defective satellites to acquire the necessary sequence necessary for igniting pathogenesis.

Phylogenetic Analysis And Pairwise Sequence Identity Comparisons:

DNA-A Analysis:

The NW and OW begomoviruses used for phylogenetic analysis, their acronyms, origins, and accession numbers in the present study, are described in Table 1. Evolutionary analysis by the Maximum Likelihood method for DNA-As of the coat protein (AV1) gene for PepGMV-EG-GZ with other begomoviruses and percentages of PSI, measured by SDT, of these sequences are illustrated in Figures 2 and 3. In the phylogenetic tree depicted in Figure 2, two major branches separate the DNA sequences under study. The first major branch circumvented the Egyptian monopatite begomoviruses HLCrV and CLCuGeV, TYLCV, and the sweepovirus SPLCV. SPLCV was separated into a monophyletic branch apart from the rest of the Egyptian viruses. Interestingly, TYLCV-EG-GZ was segregated with ACMV-KE from the rest of the Egyptian begomoviruses, and this may

indicate the unnoticed introduction of ACMV into Egypt, probably through the illegal introduction of cassava root stocks carrying ACMV-KE for propagation purposes. It is important to mention that a locally induced polyclonal antiserum for a begomovirus, isolated from a cassava experimental field in Egypt years ago, had cross-reacted serologically with several begomoviruses in Egypt (Abdel-Salam, unpublished results). The second major branch of the phylogenetic tree circumvented two sub-branches of begomoviruses belonging to OW and NW. The first sub-branch was for viruses in the Indian sub-continent (CLCuMV, ToLCNDV, and ALCuV) and Australia (ToLCV). It is worth mentioning that the Middle East begomoviruses and the Indian sub-continent ones are distinctive, probably due to geographic barriers (Tahir *et al.*, 2011). In the second sub-branch containing the NW begomoviruses, PepGMV-EG:GZ was segregated within a clade of five isolates of PePGMV from the USA, Mexico, and Costa Rica. PepGMV-Mo-US:TX, apart from other NW begomoviruses, was the closest isolate to PepGMV-EG:GZ. The previous introduction of SLCuV from the NW into Egypt (Idris *et al.*, 2006), namely SLCuV-EG:Cai, was segregated into a clade containing SLCuV-US:AZ and SLCuV-MX. BCS:La Paz. Interestingly, the latter virus was closer to the Egyptian virus than SLCuV-US:AZ, as previously stated by Medina-Hernández *et al.* (2019).

The above phylogenetic analysis for the coat protein (AV1) gene of the DNA-A of PepGMV-EG-GZ and its relationship with other bipartite begomoviruses in the present study (Fig. 2) was confirmed with the PSI measured by the SDT v1.2 program (Fig. 3 and Supplementary Table 2). PepGMV-EG-GZ had PSI with PepGMV-Mo-US:TX (98.6%), PepGMV-D-US:TX (94.7%), PepGMV-GU:MX (94%), PepGMVSer-MX:Si (93.6%), and PepGMV-CR (92.2%). These PSIs are above the threshold cut-off value for species demarcation (≥91%) based on the ICTV for begomovirus taxonomy criteria (Brown *et al.* 2015) and indicate that all the studied PepGMVs in this study are isolates of PepGMV. PepGMV-EG:GZ had a PSI of 84.2% with SLCuV-EG:Cai, and therefore these two viruses are considered different species. Based on the strain demarcation cut-off value of ≥94% for nucleotide PSI (Fauquet et al., 2008; Brown et al., 2015), PepGMV-EG-GZ could be considered a variant of PepGMV-Mo-US:TX.

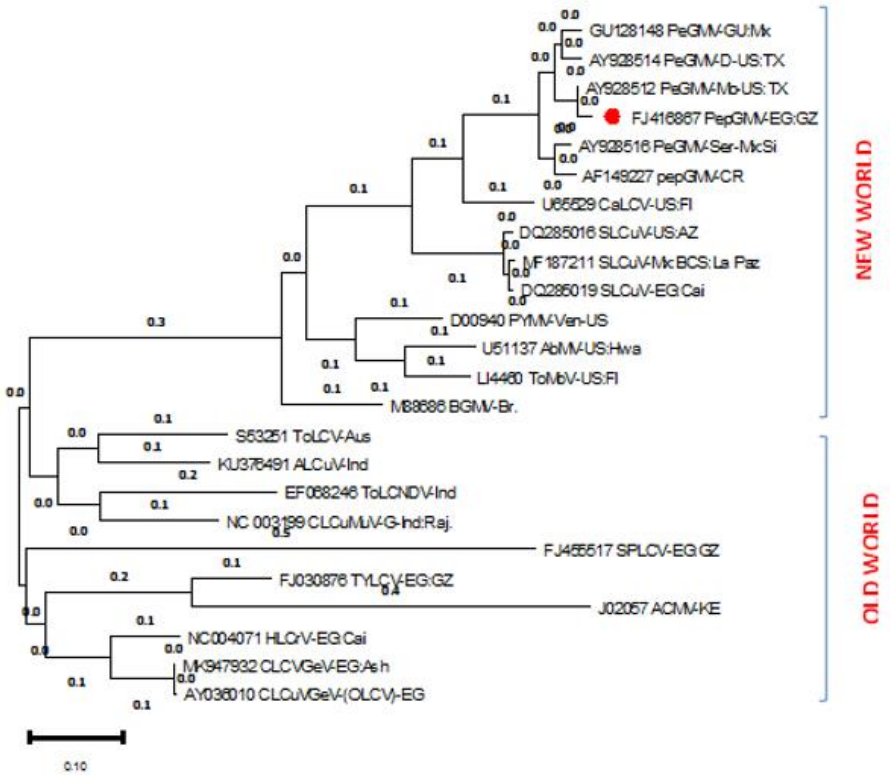

Fig. 2. Evolutionary analysis by Maximum Likelihood method for PepGMV-EG-GZ DNA-A nucleotide substitutions, for the core coat protein AV1 gene with other corresponding begomoviruses, mentioned in Table 1. The tree with the highest log likelihood (-37407.69) is shown. This analysis involved 24 nucleotide sequences. There were a total of 4966 positions in the final dataset. PepGMV-EG-GZ was marked with red-filled circle.

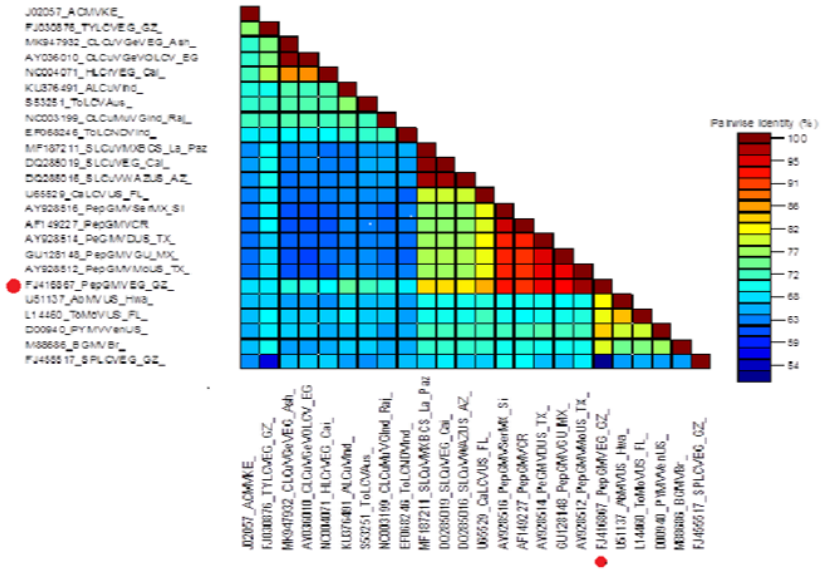

Fig. 3. A graphical representation of percentage pairwise genome scores and nucleotide identity plot of 24 DNA-A genomes (see Table1) using SDTv1.2 (Species Demarcation Tool) (Muhire et al., 2014). PepGMV-EG:GZ was marked with filled red circle.

DNA-B Analysis:

As shown in Figure 4, phylogenetic analysis carried out with the DNA-B components confirmed the close phylogenetic relationship between PepGMV-EG-GZ and the other NW PepGMV isolates. PepGMV-EG-GZ DNA-B was closer to PepGMV-D-US:TX than PepGMV-Mo-US:TX. The segregation of PepGMV-EG:GZ DNA-A and B with their corresponding DNAs in Figures 2 and 4 confirmed its identity as a bipartite begomovirus introduced from the NW into the OW.

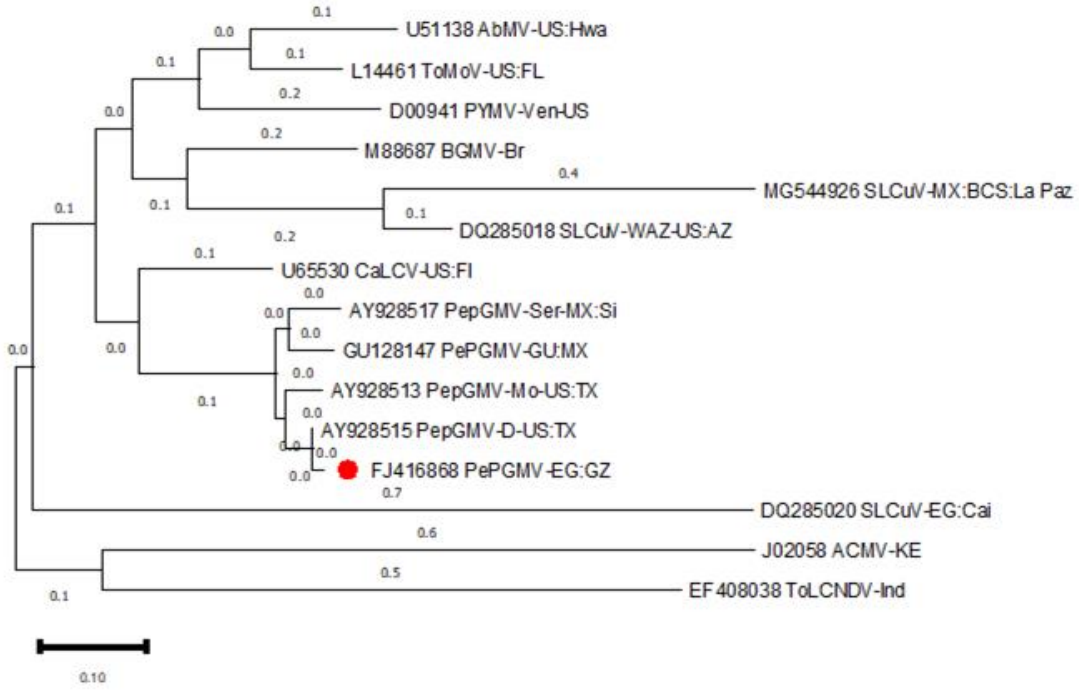

Fig. 4. Evolutionary analysis by Maximum Likelihood method for PepGMV-EG-GZ DNA-B with other begomoviruses mentioned in Table 1. The tree with the highest log likelihood (-20735.98) is shown. There were a total of 3435 positions in the final dataset. PepGMV-EG-GZ was marked with red-filled circle. This analysis involved 15 nucleotide sequences.

DNA-Associated Satellites:

Phylogenetic analysis carried out on DNA satellites, mentioned in Table 2, indicated the segregation of defective betasatellies, delta satellites, and intact betasatellites into separate monophyletic clades where deltasatellites stood between defective and intact betasatellites (Fig. 5). Similar results by Briddon *et al.* (2003), analyzing the A-rich and SCR regions of betasatellites, indicated the segregation of defective betasatellites from Egypt apart from intact betasatellites from Pakistan and India. Intact betasatellites from Egypt (Fig. 5), namely AJ316039 OYVB-EG:Okra, AF397215 OLCB-sat10-EG:Okra, and AF397217 OLCB sat3-EG:Okra, were segregated with AJ316037 CLCuB-IN:Cotton and AJ316029 OLCB-PK:Okra in a major sub-branch, an indication of their origin to the Indian sub-continent and not to the Middle-East ones. Probably these intact betasatellites were introduced into Egypt through plants contaminated with eggs of viruliferous whiteflies, infected stem-cutting plants, or through seed-transmitted begomovirus infection, as reported in similar cases worldwide (Kandito et al., 2023; Gomathi Devi et al., 2023). Migrating viruliferous whiteflies is of little concern herein because of the geographic barriers that hinder whitefly transmission (Tahir *et al.*, 2011).

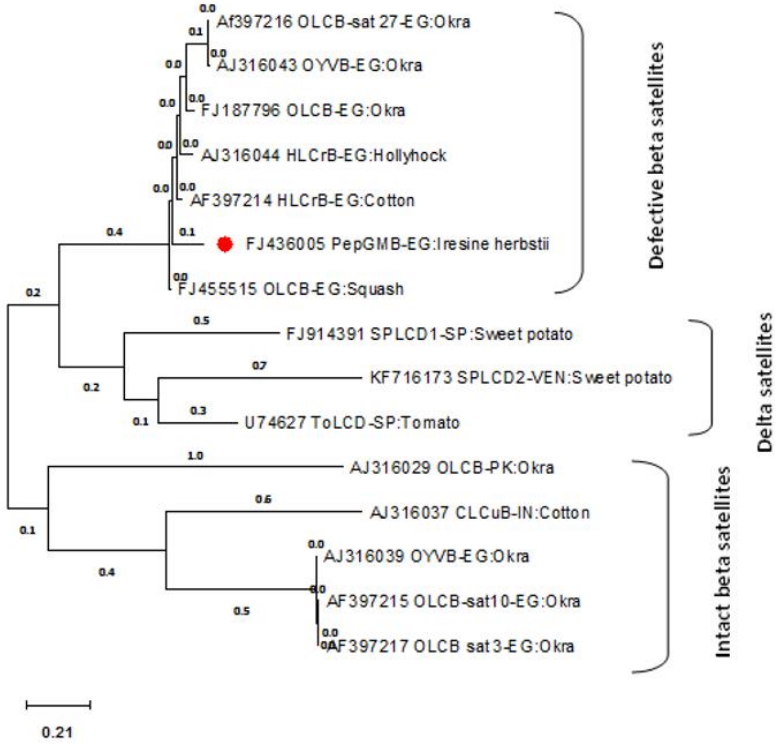

Fig. 5. Evolutionary analysis by Maximum Likelihood method for betasatellites and deltasatellites involved in the present study. The tree with the highest log likelihood (-6227.52) is shown. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 1031 positions in the final dataset. This analysis involved 15 nucleotide sequences. PepGMB-EG:Iresine herdstii was marked with red-filled circle.

The results of the phylogenetic analysis in Figure 5 were solidified by SDT analysis. Comparisons between PepGMB and other betasatellites in the present study (Fig. 6 and Supplementary Table 3) revealed a PSI range from 96.4% down to 86.1% with the Egyptian defective: okra leaf curl betasatellite (OLCB-Squash: FJ455515), hollyhock leaf crumple betasatellite (HLCrB-Hollyhock:AJ316044), and okra yellow vein betasatellite (OYVB-Okra:AJ316043). This indicates that all these defective satellites are isolates of OLCB since 78% represents the species demarcation threshold for betasatellites (Briddon et al., 2008). Furthermore, defective OLCB isolates from Egypt are different from those from Pakistan and India, with PSIs of 61.1% and 65.9%, respectively (Supplementary Table 3). The PSI between PGMB and deltasatellites indicated distant identity, with a PSI range of 52.9% to 54.3 (Supplementary Table 3). This latter result is confirmed by the finding of Fiallo-Olivé and Navas-Castillo (2020) that noncoding betasatellites differed from deltasatellites.

PCR analysis of PepGMV-associated betasatellite produced a 653 bp defective betasatellite molecule that lost the βC1 gene but retained most of the SCR and the A-rich region, which renders them capable of trans replication by cognate and non-cognate begomviruses (Briddon et al., 2003; Lozano et al., 2016). Multiple sequence alignments of the SCR of betasatellites in Figure 7, show the presence of conserved nonanucleotide sequences (5′-TAATATTAC-3′) of the SCR in both defective and intact (full length) OLCB satellite species. Similar results by other investigators indicated the presence of both defective and intact betasatellites for the same begomovirus species associated with virus infection in malvaceous hosts in Egypt (Idris *et al.*, 2002; Briddon *et al.*, 2003).

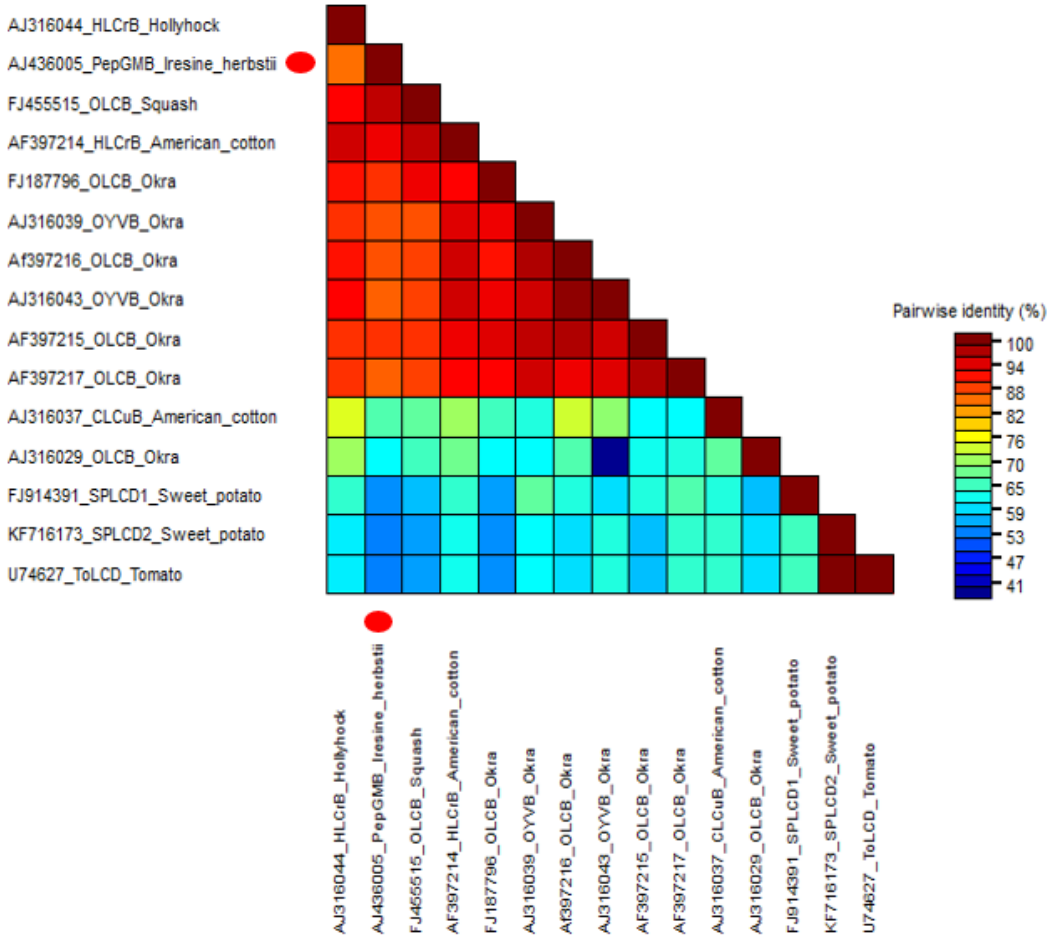

Fig. 6. A graphical representation of percentage pairwise genome scores and nucleotide identity plot of 12 beta-and 3 deltasatellites, including PepGMB, (see Table 2) using SDTv1.2 (Species Demarcation Tool) (Muhire et al., 2014). PepGMV-EG:GZ:Iresine herdstii was marked with filled red circle.

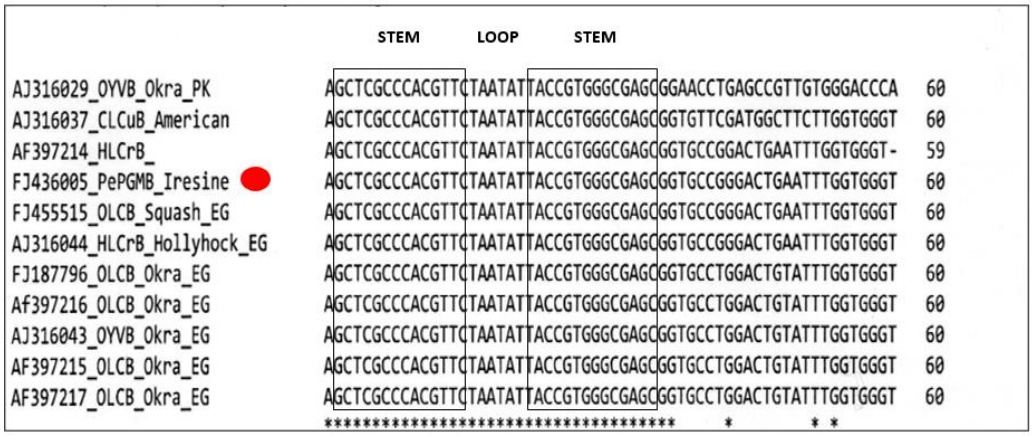

Fig. 7. Nucleotide sequences shows the presence of satellite conservative regions, which include TAATATTAC stem-loop structure in PepGMB compared with the betasatellites (see Table 2) from GenBank. Analysis of sequences was carried out using Edit Sequence – DNASTAR and sequence alignment using Clustal Omega (http://ebi.ac.uk/Tools/msa/clustalo/). PepGMB-Iresine was marked with red-filled circle.

Expected Role of Whitefly, Betasatellites, and Ornamentals in The Pathogenesis of PepGMV and Other Bipartite Begomoviruses in Egypt:

Possible PepGMV infections in pepper in Egypt need to be investigated in depth. Most of the virus symptoms observed in pepper plants include leaf curling and stunting, but without associated mosaic symptoms. The previous survey by Gambley *et al.* (2020) in Egypt showed the presence of the *Cotton leaf curl Gezira virus* in pepper (*Capsicum annuum*) and melon (*Cucumis melo*). No bipartite begomovirus and associated betasatellites, nevertheless, were reported by those investigators on peppers in the surveyed fields. So it seems that PepGMV is not causing considerable disease damage to the pepper plant in Egypt so far. Experimental whitefly transmission under greenhouse conditions would give us valuable information about the efficiency of whitefly insects to transmit PepGMV-EG:GZ from *I. herbstii* to pepper plants. In terms of epidemiology, previous results suggested that the association of bipartite ToLCNDV with betasatellites (Sivalingam and Varma, 2012; Jyothsna *et al.*, 2013) increases pathogenesis and virus accumulation. Further, Abdel-Salam et al. (2017) pointed out that the introduction of the bipartite SLCuV into Egypt and its association with betasatellites might increase the biodiversity in such a limited-biodiversity area and modify viral virulence and fitness.

The present study confirms the role played by ornamental plants as secondary hosts and reservoirs for begomoviruses, as exemplified by the PepGMV infecting *I. herbstii* in Egypt. Most likely, PepGMV was introduced into Egypt from Latin America through contaminated stem cuttings of infected *I. herbstii*. Previously, ToLCNDV was isolated from *Eclipta prostrata* in Pakistan (Haider *et al.*, 2006), and TYLCV was isolated from *Lamium amplexicaule* in Korea (Eui-Joon *et al.*, 2014). Such previous examples indicate the unnoticed worldwide role of ornamentals in spreading out begomoviruses and, hence, increasing begomovirus biodiversity through recombination and re-assortment events. Strict quarantine measures should be applied to limit the introduction of new ornamentals into foreign countries.

The introduction of the bipartite PepGMV from the NW into Egypt is not the first introduction of bipartite begomoviruses into Egypt. A previous introduction was recorded for the bipartite begomovirus SLCuV (Abdel-Salam *et al.*, 2006; Idris *et al.*, 2006). The association of these bipartite begomoviruses with betasatellites in Egypt may lead to tri-genomic relationships that may modify viral virulence and fitness through genetic recombination and re-assortment.

Supplementary Tables:

Table 1 A list of primers and its amplification conditions used for testing PepGMV EG GZ genome and associated betasatellites.

Primer Name	Primer sequence (5' to 3')	Amplified region of genome	Annealing temp. (°C)	Amplicon size bp	References
Av core	GCCHATRTAYAGRAAGCCMAGRAT	DNA A	58	579	J.K. Brown's laboratory. Tucson Az., USA (unpublished)
Ac Core	GGRTTDGARGCATGHGTACANGCC				
BV1855	AC(A/G)CAA(A/G)TG(A/G)TC(A/T/G)A T(C/T) TTCAT	DNA-B	50	665	Idris, A.M. and J.K. Brown, 1998
BC2571	GGTAATATTATA(A/C/T)CGGATGG				Idris, A.M. and J.K. Brown, 2004
βeta 01	GGTACCACTACGCTACGCAGCAGCC	DNA betasatellite	58	1350	Briddon, R.W., S.E. Bull, S. Mansoor, I. Amin and P.G. Markham, 2002
βeta 02	GGTACCTACCCTCCCAGGGGTACAC				

Table 2 Pairwise sequence identity of PepGMV EG GZ with several mono and bipartite begomoviruses* involved in the present study.

ACMV KE	100																							
TYLCVEG GZ	77.3	100																						
CLCuVGeV EG Ash	73.1	76.7	100																					
CLCuVGeV OLCV_EG	72.9	77	99.4	100																				
HLCrV EG Cai	74	79	87.5	87.4	100																			
ALCuV Ind	72.7	71.9	74.6	74.2	74.8	100																		
ToLCV Aus	73.2	73.9	74.4	74.3	74.8	77.6	100																	
CLCuMuV G Ind_Raj	73.5	73.5	74.6	74.3	74	75.6	73.9	100																
ToLCNDV Ind	70.3	70.3	70.6	70.5	70.8	73.3	72.8	73.7	100															
SLCuV MX BCSLaPaz	66.1	71.0	64.9	65.6	64.3	65.3	66.7	66,5	67.1	100														
SLCuV EG_Cai	66.3	71.4	65.3	65.9	64.8	65.4	67.1	67.3	66.9	98.9	100													
SLCuV WAZ US AZ	65.9	71.4	64.7	65.1	64.8	65.9	66.3	66.4	66.5	98.2	98.2	100												
CaLCVUS_FL	64.9	70.5	64.3	64.6	65.1	66.1	66.2	66.1	63.9	80.2	80.2	80.3	100											
PepGMV Ser MX_Si	64.5	70.0	63.0	63.0	63.5	65.3	65.2	63.9	65.3	77.5	77.4	77.4	81.8	100										
PepGMV CR	64.5	71.0	62.9	63.2	62.7	65.8	65.4	64.3	65.3	77.6	77.7	77.7	81.3	96.0	100									
PeGMV D US TX	64.6	68.7	63.5	63.4	63.5	65.5	65.4	65.0	64.3	78.4	78.3	78.3	82.2	93.0	92.5	100								
PepGMV GU MX	64.5	69.8	62.7	62.6	64.2	65.1	66.1	64.5	64.0	79.1	78.9	78.7	81.6	92.7	92.20	95.6	100							
PepGMV Mo US TX	64.5	69.6	63.1	63.1	63.2	66.6	65.6	65.2	65.1	78.2	78.0	77.9	81.8	93.0	92.2	95.1	95.0	100						
PepGMV EG GZ	69.8	69.9	72.2	72.5	71.1	75.1	73.8	72.5	70.4	84.0	84.2	83.5	86.0	93.6	92.2	94.7	94.0	98.6	100					
AbMV US Hwa	67.8	68.9	67.0	66.7	66.1	68.5	68.3	67.9	66.8	71.4	71.5	72.0	72.4	71.5	71.6	71.3	71.0	71.3	82.6	100				
ToMoV US FL	68.4	71.2	68.4	68.5	68.5	70.0	68.8	68.8	67.0	71.8	71.8	71.9	73.6	71.4	71.1	71.7	71.7	72.6	82.1	85.7	100			
PYMV Ven US	67.5	70.0	68.6	68.6	67.5	68.3	68.9	67.0	67.4	73.7	73.8	73.7	73.8	73.6	73.9	74.8	73.7	73.9	84.9	80.1	80.4	100		
BGMVBr	67.7	71.6	66.7	66.8	67.6	68.6	68.7	67.4	67.6	73.3	73.4	73.5	73.5	71.8	72.1	72.0	72.0	71.8	81.2	76.0	76.7	78.5	100	
SPLCV EG GZ	66.0	66.0	68.3	69.1	68.5	66.9	65.8	67.3	67.9	69.1	70.2	70.2	68.6	71.9	71.3	67.7	68.2	70.1	54.3	66.5	67.5	66.4	66.8	100
	ACMV KE	TYLCV EG GZ	CLCuVGeV EG Ash	CLCuVGeV OLCV_EG	HLCrV EG Cai	ALCuV Ind	ToLCV Aus	CLCuMuV G Ind_Raj	ToLCNDV Ind	SLCuV MX BCSLaPaz	SLCuV EG_Cai	SLCuV WAZ US AZ	CaLCVUS_FL	PepGMV Ser MX_Si	PepGMV CR	PepGMV D US TX	PepGMV GU MX	PepGMV Mo US TX	PepGMV EG GZ	AbMV US HWA	ToMoV US FL	PYMV Ven US	BGMV Br	SPLCV EG GZ

Table 3 DNA pairwise sequence of 15 DNA sequences of beta and delta satellites·.

HLCrB_Hollyhock: Egypt AJ316044	100.0														
PepGMB_Iresine_herbstii: Egypt AJ436005	85.5	100.0													
OLCB_Squash: Egypt FJ455515	91.9	96.4	100.0												
HLCrB_American_cotton: Egypt AF397214	95.1	93.4	96.3	100.0											
OLCB_Okra: Egypt FJ187796	91.1	89.0	92.8	92.1	100.0										
OYVB_Okra: Egypt AJ316039	89.1	87.1	87.6	94.0	92.9	100.0									
OLCB_Okra: Egypt Af397216	91.2	87.3	88.6	95.5	91.6	96.7	100.0								
OYVB_Okra: Egypt: AJ316043	91.7	86.1	88.1	94.6	92.7	95.1	98.8	100.0							
OLCB_Okra: Egypt AF397215	89.2	89.3	88.9	93.6	94.3	96.2	97.0	95.6	100.0						
OLCB_Okra:Egypt AF397217	89.7	86.1	88.1	88.1	91.9	94.7	93.6	94.2	96.6	100.0					
CLCuB_American_cotton: India AJ316037	74.4	65.9	66.5	70.7	70.7	63.2	73.3	69.8	61.0	61.3	100.0				
OLCB_Okra: Pakistan AJ316029	70.5	61.1	65.1	68.1	61.1	61.0	66.1	38.4	61.6	63.0	66.5	100.0			
SPLCD1_Sweet_potato: Spain FJ914391	63.8	54.3	56.5	63.7	54.7	66.7	63.0	58.5	63.0	65.8	63.3	**56.8**	100.0		
SPLCD2_Sweet_potato: Venezuela KF716173	59.6	52.9	55.2	62.1	53.6	61.0	59.0	62.8	57.0	63.4	64.2	**58.6**	64.7	100.0	
ToLCD_Tomato:Australia U74627	59.6	52.9	55.2	62.1	53.6	61.0	59.0	62.8	57.0	63.4	64.2	58.6	64.7	100.0	100.0
	HLCrB_Hollyhock AJ316044	PepGMB_Iresine_herbstii AJ436005	OLCB_Squash FJ455515	HLCrB_American_cotton AF397214	OLCB_Okra FJ187796	OYVB_Okra AJ316039	OLCB_Okra Af397216	OYVB_Okra AJ316043	OLCB_Okra AF397215	OLCB_Okra AF397217	CLCuB_American_cotton AJ316037	OLCB_Okra AJ316029	SPLCD1_Sweet_potato FJ914391	SPLCD2_Sweet_potato KF716173	ToLCD_Tomato U74627

Declarations:

Ethical Approval: Not applicable.

Author Contributions: Dr. Aly M. Abdel-Salam (first author): Contributed to virus-filed isolation, serologic identification (through IC-PCR), RCA-genome amplification, DNA submission to GenBank, DNA molecular and phylogenetic analysis, and writing and reviewing the manuscript. Dr. Malik Mujaddad-ur-Rehman (second

author): Contributed to RCA-genome amplification, PCR studies, cloning, and DNA-sequence purification.

Dr. Doaa Z. Soliman (third author): Contributed to PCR studies

Conflict of interests: The authors declare no conflicts of interest.

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Availability of Supporting Data: The datasets used and analyzed during the current study are available from the corresponding author at reasonable request.

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