Molecular Characterization and Transmission Study of the Begomovirus on Hollyhock Plant Causing Leaf Curl Disease for the First Time in Province Punjab, Pakistan

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Research Article

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Abstract

I. Background: Hollyhock (*Alcea rosea*) is an ornamental plant belonging to the *Malvaceae* family and has a remarkable aesthetic and medicinal value. Previously in Pakistan, the hollyhock plant was not found to be infected by begomovirus and the plant first time showed the symptoms of typical leaf curling, puckering as well as thickened veins.

II. Methods and Results: During the year 2018, symptomatic samples of the hollyhock plants were collected that exhibited characteristic typical leaf curling, puckering as well as thickened veins. DNA was extracted from the samples and the PCR technique was optimized for the detection of begomovirus followed by sequencing. The samples were detected to be infected with begomovirus by using Av/Ac core, Begomo 01/02, and CLCV 01/02 primer showed positive results with 579bp, 2.8kb, and 1.1kb nucleotide respectively. The betasatellite was amplified by using beta01/02 and CLCuMuBF11/R33 showed positive results with 1400bp and 481bp respectively. Sequencing results showed that diseased hollyhock plants were associated with *Cotton leaf curl Multan virus*-Rajasthan strain along with Cotton leaf curl Multan betasatellite.

III. Conclusion: Hollyhock plants infected by begomovirus has been reported for the first time as a possible source of virus inoculum from Pakistan.

1. Introduction

Hollyhock (*Alcea rosea*) is a famous decorative annual herb cultivated in South Asia and other temperate regions as a valuable resource for food, medicine, fabric, dyes, and the cosmetic industry. This study included isolation and characterization of *Cotton leaf curl Multan virus*-Rajasthan (CLCuMV-Raj) from hollyhock. The Cotton leaf curl Multan betasatellite (CLCuMB) was also associated with *A. rosea* samples identified for the first time in this study from Multan, Pakistan. Significance was discussed of these findings. Begomoviruses (family *Geminiviridae*) are whitefly transmitted, icosahedral particles that are found in pairs and possess single-stranded circular DNA genome, and is known to cause cotton leaf curl disease. The genome of begomovirus ranges from 2.4 to 2.8 kb in size [1]. Family, *Geminiviridae* has been divided into nine genera on the basis of their characters like transmission by insect vectors and the genome organization [2]. Begomoviruses have appeared as a devastating pathogen of several vegetables, fiber, and food crops that are mostly cultivated in tropical and sub-tropical regions. Enormous losses were caused by begomovirus infection on cash crops [3, 4]. The role of weeds and alternative hosts play a significant role in the dispersal and survival of viruses during the unavailability of the primary crop [5–8]. The development of Begomoviruses is mainly due to the whitefly population and changes in the genetic makeup of planting material. Recombinations in the genome of viruses cause the emergence of new virulent strains that may have the ability to infect existing and new host plant species with more severity [9], increasing the host range and disease causing ability of pathogen.

Begomoviruses are either monopartite (DNA-A) or are bipartite (DNA-A and DNA-B). Monopartite begomoviruses have only a single genomic component, enough to cause disease. The genome of monopartite begomoviruses is generally comprised of the coat protein (CP) as well as movement protein (V2) in the virion sense region, while replication-linked protein (Rep), transcriptional interlinked activator protein (TrAP), replication-associated enhancer protein (REn) as well as pathogenicity protein (C4) in the complementary sense (Sharma and Ikegami 2010). Old World monopartite begomoviruses are associated with two sorts of satellite molecules named as alphasatellite [10] and betasatellite [11, 12]. Betasatellite possesses a molecular size almost half of the size of the genome of begomovirus itself (approximately 1.4 Kb), encoding βC1 gene and depends on a helper virus for replication, vector

transmission, and movement. In the host plant, betasatellite molecules are responsible for the severity of disease symptoms [13] whereas, alphasatellite encodes replication-related protein analogous to nano viruses, and so are proficient in self-governing replication in host plant cells; however, the involvement of helper begomovirus is obligatory for its movement within the host plant [10, 14].

2. Material And Methods

2.1. Sample Collection

During the summer season of the year 2018, a total of 49 symptomatic hollyhock samples showing typical symptoms such as enation, vein thickening, stunted growth, leaf curling, and puckering were collected from Multan. All the samples were stored and processed at Molecular Plant Virology Laboratory, Department of Plant Pathology, Bahauddin Zakariya University (BZU), Faculty of Agriculture Sciences and Technology, Multan, Pakistan.

2.2. Detection test by Polymerase Chain Reaction (PCR)

Molecular detection of the begomoviruses and the associated betasatellite was done by polymerase chain reaction using the primers (Table 1). For the execution of PCR, the DNA of infected samples was isolated by the CTAB process [15]. To identify the isolates of begomovirus and betasatellite, degenerate and universal primers, were used on hollyhock samples.

Table 1
Primers employed during the study.

Primer ID	Forward sequence	Reverse sequence	References
AV/AC Core	GCCHATRTAYAGRAAGCCMAGRAT	GGRTTDGARGCATGHGTA CANGCC	[16]
CLCuMuBF11/R33	GGTCCCACTGCTTGTCTTGA	GGTTCATAGTCGACGTTCGC	[17]
Begomo 01/02	ACGCGTGCCGTGCTGCTGCCCCCATTG TCC	ACGCGTATGGGCTGY CGA AGTTSAGAC	[18]
Beta 01/02	GGTACCACTACGCTACGCAGCAGCC	GGTACCTACCCTCCCAGG GGTACAC	[19]
CLCuV 01/02	CCGTGCTGCTGCCCCCATTGTCCGCGTCAC	CTGCCACAACCATGGATT CACGCACAGGG	[20]

2.3. PCR reaction and Thermocyclic conditions

PCR reaction of 25 µl was performed containing 2.5 µl of 10X PCR Buffer, 0.3µl of Taq DNA polymerase, 1µl of dNTPs (10 mM), 1.5µl of $MgCl_2$ (25 mM), 15.70µl of Nuclease free water, 1µl of Primer Forward (10 µM), 1µl of Primer Reverse (10 µM) and 2µl of DNA template. Incubation was performed for all these reactions at 95°C for 5 min prior to 35 cycles. The amplification parameters of the Beta 01/02 (95°C; 30sec, 63°C; 45sec, and 72°C; 2min), Av/Ac core (95°C; 30sec, 50°C; 30sec, and 72°C; 45sec), CLCuMuBF11/BR33 (95°C; 30sec, 56°C; 30sec, and 72°C; 45sec), CLCuV 01/02 (94°C; 30sec, 63°C; 45sec, 72°C; 1min) and Begomo 01/02 (94°C; 1 min, 63°C; 2 min, 72°C; 3 min). The final extension was performed at 72°C; 10 min. 1% agarose gel was used for visualization of the PCR product.

2.4. Viral transmission studies

2.4.1. Virus source

Diseased cotton plants were collected from cotton fields cultivated in the Alipur region of the Multan district. They were maintained in the insect-proof cages at the glasshouse established at the Department of Plant Pathology, Bahauddin Zakariya University, Multan. Leaves of the symptomatic cotton plant were positively tested using AV/AC core and Beta 01/02 primers. These infected plants were maintained in a controlled insect free environment so that they can be used as a source of inoculum for the transmission experiments. A total of fifteen hollyhock plants, including five hollyhock seedlings as control plants, were raised in an insect free environment using dome cages.

2.4.2. Whitefly Colony development

Whiteflies were collected from cucumber plants grown in the open fields in the Multan district. The collected whiteflies were reared on cucumber as they are non-host plant species for begomoviruses. After a month, nymphs were collected from cucumber plants and were shifted to a new set of cucumber plants to obtain virus-free whitefly colony. After three months, the 3rd generation of whitefly was received that is non-viruliferous and was used as a vector for the transmission studies.

2.4.3. Transmission experiment

For the acquisition of the virus, a set of 100 non-viruliferous whiteflies were allowed to feed on the leaves of the positively tested cotton plant for a period of 72 hours [21]. On completion of the acquisition access period, these whiteflies were shifted to healthy hollyhock seedlings at the five-leaf stage with the purpose of inoculation. For successful inoculation, the viruliferous whiteflies were allowed to feed on the healthy hollyhock seedlings for a period of 72 hours. The set of five hollyhock seedlings were inoculated by non-viruliferous whiteflies following the transmission parameters followed for the inoculation of test plants. Whiteflies were killed by diafenthiuron (POLO) after 72 hours of inoculation access period (IAP) on hollyhock kept in cages. Symptoms were observed after 13 days in our trial studies [22]. However, incubation of 1 month was allowed for symptom development. Association of begomovirus to the hollyhock leaf curl disease was confirmed after transmission studies, and PCR using primers [15].

2.5. Purification, Sequencing and Phylogenetic analysis

FavorPrep PCR Clean-up Mini Kit (Favorgen) was used to purify the amplified PCR products. Purified PCR products was submitted for two way Sanger sequencing to the Macrogen address. The obtained sequence of the amplified PCR product were submitted to Genbank for the purpose of obtaining the accession numbers. The cleaned sequence were submitted to BLAST analysis for the identification purpose. Good quality sequences were subjected to Clustal W alignment and neighbour-joining phylogenetic analysis keeping the bootstrap value of 1000 with MEGA X software [23].

3. Results

3.1. Field observation and molecular study

Symptoms of begomovirus were observed on the hollyhock plant that showed upward / downward leaf curling, puckering, and vein thickening (Fig. 1).

Different detection rate was recorded by using different primers. Seventeen samples collected from auditorium area of BZU, 13 showed positive result by using three primers (Av/Ac core, Begomo 01/02 and CLCV 01/02), but 14 samples have showed positive result using CLCuMuBF11/R33 with higher detection rate. Out of total fifteen samples collected from Vehari, 8 were detected to be infected with betasatellite and begomovirus by using Beta

01/02, CLCuMuBF11/R33, Av/Ac core, Begomo 01/02 and CLCV 01/02 primers, with 53% detection rate. Seventeen samples was collected from Shujabad out of which eleven showed positive result having 65% incidence by using five different primers (Tab. 2).

Table 2
Detection of infected hollyhock samples through PCR technique

Sr. No.	Location	Number of Sample	Positive for Av/Ac core (%)	Positive for CLCuMuBF11/R33 (%)	Positive for Beta 01/02 (%)	Positive for Begomo 01/02 (%)	Positive for CLCV01/02 (%)
1	Auditorium BZU	17	13/17 (76)	14/17 (82)	14/17 (82)	13/17 (76)	13/17 (76)
2	Vehari chowk	15	8/15 (53)	8/15 (53)	8/15 (53)	8/15 (53)	8/15 (53)
3	Shujabad	17	11/17 (65)	11/17 (65)	11/17 (65)	11/17 (65)	11/17 (65)
	Total	49	32/49 (65)	33/49 (67)	33/49(67)	32/49 (65)	32/49 (65)

The amplicon of 1.1 kb obtained through CLCuV 01/02 (Fig. 2A). The 1.4 kb amplicon through Beta 01/02 primer confirmed the association of betasatellite (Fig. 2B). The amplicon of 2.8 kb with a universal primer of begomovirus (Begomo 01/02) confirmed the association of begomovirus on hollyhock samples (Fig. 2C). However, PCR product of 579 bp and 478 bp was obtained from Av /Ac core and CLCuMuBF11/ R33 primers, respectively (Fig. 2D, E).

3.2. Virus transmission studies

Symptoms were observed after thirteen days of incubation after treatment with diafenthiuron to kill the whitefly. All the plants are other than the control treatment developed typical symptoms like leaf curling, vein thickening, and puckering as a result of successful infection of the virus by the viruliferous whiteflies (Fig. 3). Moreover, all these symptomatic plants were tested positive using specific primers Av/Ac core (579 bp), Beta 01/02 (1.4 kb), and CLCuMuBF11/R33 (481 bp) (Fig. 4).

3.3. Sequencing and phylogenetic analysis

The amplified PCR product was sequenced of CLCuMuBF11/R33 primer and Av/Ac core primer. After sequencing, a product of 416bp was obtained through CLCuMuBF11/R33 primer. Moreover, a nucleotide sequence of 564bp was achieved after the sequencing of the Av/Ac core primer. Sequences were submitted on NCBI for getting their accession number. BLAST analysis was done to download similar sequences. The dendrogram was created for their comparison with already submitted sequences is given below.

Dendrogram constructed with a nucleotide sequence alignment of CLCuMB obtained from hollyhock plant along with available sequences present on NCBI by Neighbour-joining method. Every sequence accession number is mentioned along with geographical origin and host. Node number presents the bootstrap value (1000 replicates). Hollyhock's sequence of this study was highlighted with a purple circle. The ChiLCuV is used as an outgroup, a sequence that is a far related sequence having a green circle in a tree.

This phylogenetic tree was created by sequence alignments submitted in the blast along with our two sequences of CLCuMV. Every accession number is stated with geographical origin and host. The bootstrap value used to construct this tree was 1000 replicates. Our sequences of this study were highlighted with red circles. The outgroup Citrus greening bacteria (CGB) is highlighted with a blue circle.

4. Discussion

Hollyhock is one of the attractive ornamental plants, and its association with begomoviruses works as a limiting factor for its growth and aesthetic beauty. Begomovirus emerges as a damaging pathogen by decreasing the crop yield and market value. The spread of begomoviruses was credited to several biotypes of the whitefly vector and significantly affected the global trade of agricultural host plant species. Begomovirus has a great ability to adapt to the changing environmental conditions due to its capability to recombine and evolve very quickly [24, 25]. Begomoviruses can survive on alternate hosts like weeds and ornamentals during the unavailability of the primary host crops. Numerous factors influence its establishment as a key participant of disease complex on new hosts, especially the capacity of different betasatellites to interact with a different begomovirus. The role of betasatellite assumes to be crucial for symptom expression on a variety of hosts and associated with Old World monopartite Begomoviruses [26]. The product size of 416 bp was obtained by using CLCuMuBF11/R33 primer, and the purified product of PCR was sequenced. The sequence of betasatellite was submitted to the NCBI database with accession number OL407060. Amplified products of 519 bp and 526 bp were amplified from infected hollyhock samples with Av/Ac core primer were also sequenced and partial sequences of CLCuMV-Raj submitted in the database with accession number MT668932 and MT668933 respectively.

In our study, obtained 519 bp and 526 bp are partial sequences of begomovirus isolated from hollyhock having coat protein region processed in the database have shown 99.79% and 99.58% identity with already reported CLCuMV-Raj strain having isolate PK129 (MG904816) of Pakistan. Moreover, a sequence of betasatellite showed 98.80% identity with previously reported CLCuMB from soybean crop of Pakistan (isolate Mad-6, accession no. MN910267). So, it is cleared that CLCuMV and CLCuMB associate with each other and infected the hollyhock plant in Multan, Pakistan. Hence, this result confirmed the association of monopartite begomovirus.

Previously CLCuMV–Raj was found to be associated with cotton plants in Pakistan, India, and China. Begomovirus (CLCuMV) found to infect the wide range of host plant including cotton [27], chilli [28, 20], rose [29], tomato [30], okra [31], *Vinca minor* [32] and *Digera arvensis* [33] concerned with major diseases of the different crop. The host range was increasing day by day, and begomovirus was becoming a serious problem because whitefly plays a role in its spread. In Pakistan, Malvaceous plants were grown commonly like, cotton, okra, *Hibiscus* sp, and hollyhock, which are characterized as a leading cost-effective resource for the Pakistan economy. CLCuMV proved to be critical for cotton production in the world as transmitted by the whitefly population [34–35] (Rashida *et al*. 2010; Rashida *et al*. 2005). CLCuMV is also problematic in hollyhock, as an alternate host, acts as a survival point of begomovirus during the offseason of the main crop.

Our study revealed hollyhock as a new host of begomovirus in Pakistan. As the conversation began related to host plant, previously begomovirus on hollyhock was reported on the United States of America, India, and Egypt. Hollyhock plant was found to be associated with Hollyhock yellow vein mosaic virus (HoYVMV) along with Ludwigia leaf distortion betasatellite in India [36]. Hollyhock leaf crumple virus (HLCrV) was identified to be associated with hollyhock in the USA [37]. In Egypt, the hollyhock plant was found to be associated with begomovirus *Althea rosea enation virus* (AREV) [38].

Conclusion

Hollyhock act as a begomovirus reservoir for the first time in Pakistan. This virus was appeared as a threat to many crops, being linked with, as revealed here, leaf curl in hollyhock, by means of an alternative host. The study discussed here displayed that viruses survive on ornamentals as they need, during the absence of main seasonal crops, so more effort is required for virus management. We concluded that more focus is needed for controlling its vector to prevent its spread to ornamentals and main seasonal crops in the future.

Declarations

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Author contributions

Conceptualization: RP, HA; methodology: HA, MNT, UDU, FÖ, AA; investigation: RP, MNT, UDU, FÖ; funding acquisition: RP, UDU; Supervision: RP, MNT; writing—original draft: HA, AA; Writing—review and editing: RP, MNT, UDU, FÖ.

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Declarations

Conflict Of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Consent to participate and publish

All authors reviewed and approved the final version for publication.

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Figures

Figure 1

A. Leaf curling **B**. Puckering **C**. Vein thickening.

Figure 2

The extracted DNA of hollyhock were tested with 5 different primers (**A**. CLCV 01/02 primer **B.** Beta 01/02 primer **C.** Begomo 01/02 primer **D**. CLCuMuBF11/BR33 **E.** Av/Ac core primer) required amplicon was obtained.

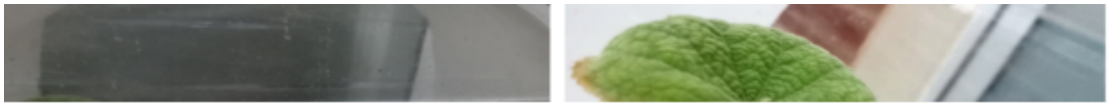

Figure 3

Virus transmission study of hollyhock plant (A) hollyhock plants in the cage (B) Symptoms observed after 13 days are puckering and vein thickening (C) Clear vein thickening on leaves after 14 days (D) Control plant of hollyhock.

Figure 4

Samples of inoculated plant were tested through Beta 01/02 (1400 bp), Av/Ac core (579 bp) and CLCuMuBF11/R33 (481 bp).

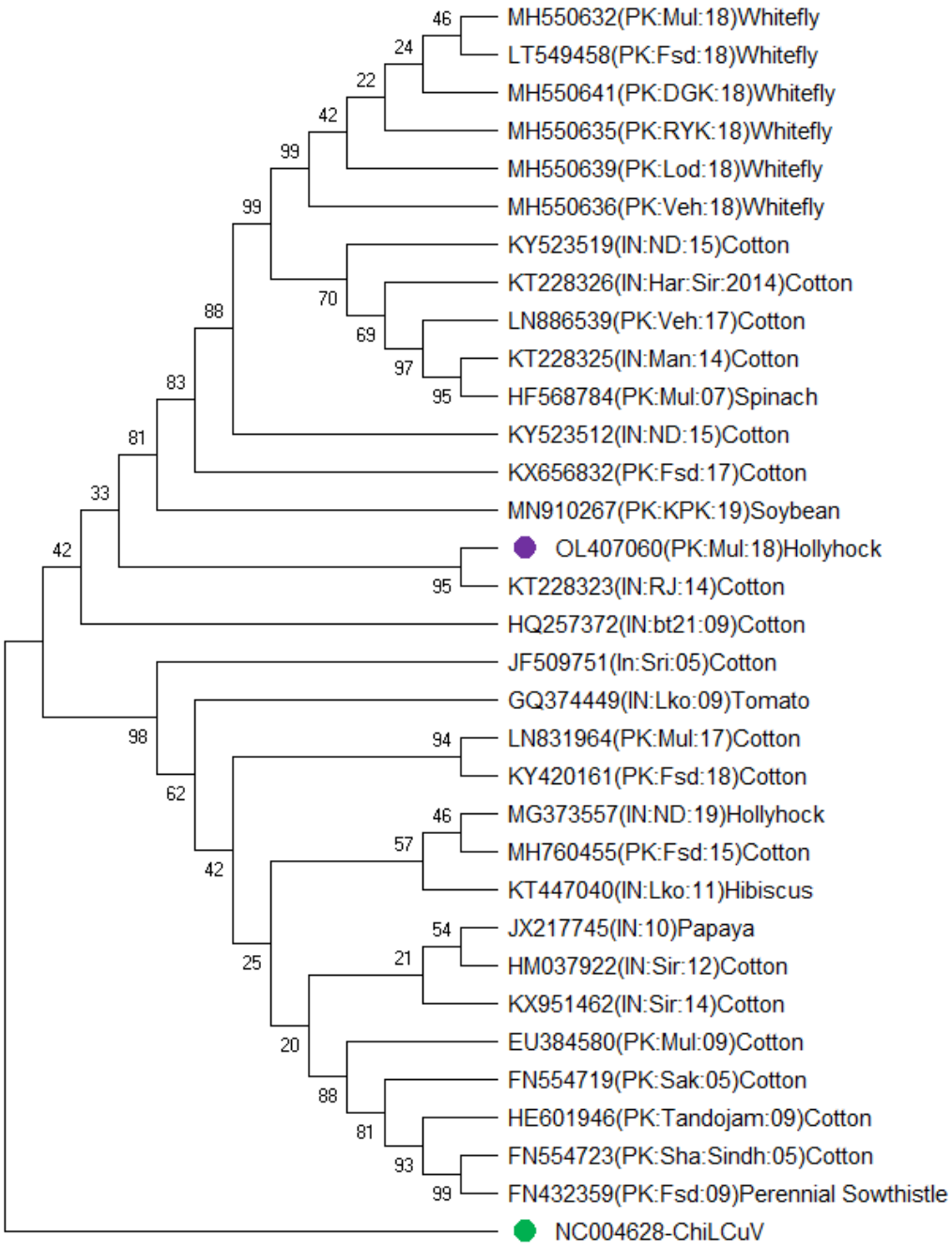

Figure 5

The phylogenetic tree of CLCuMB was constructed with outgroup Chilli leaf curl virus (ChiLCuV).

Figure 6

The phylogenetic tree by the neighbor-joining method was constructed with a nucleotide sequence of CLCuMV-Raj isolated from the hollyhock plant with other existing sequences in the database.