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Original Research Article

CONSTRUCTION OF THE INFECTIOUS MOLECULE OF BETA SATELLITE ASSOCIATED WITH AGERATUM YELLOW VEIN DISEASE OF *AGERATUM CONYZOIDES*

AMJID N[1], REHMAN MSNU[1],*KHALID MN[2], AMJAD I[2]

[1]*Centre of Agricultural Biochemistry and Biotechnology, University of Agriculture Faisalabad, Pakistan*
[2]*Department of Plant Breeding and Genetics, University of Agriculture Faisalabad, Pakistan*
**Correspondence author email address:noumankhalidpbg@gmail.com*

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Abstract: *Several dicot plant species are mostly infected by the vast variety of begomoviruses in different areas around the globe. Begomoviruses exhibit association with two satellites molecules, alphasatellites and betasatellites which are involved to cause severe viral infection. Whitefly plays a vital role as a vector in the transmission of virus from one plant to another. These viruses use weeds to cause infections when the major crop plants are absent in the field. Ageratum conyzoides is very important weed plant, infected with a unique virus complex. The study is intended to evaluate the satellite molecule diversity associated with yellow vein disease of A.conyzoides. Symptomatic leave samples of field grown ageratum plants were collected from different areas of Faisalabad. Betasatellites were extracted from infected ageratum plants.To amplify the beta satellite component clones, the rolling circular amplification was applied on extracted DNA-β isolated from infectious weed plants.The confirmation of DNA-β was done with the help of restriction by different suitable enzymes. The betasatellites were mainly focused in this study. The DNA-β was completely sequenced and infectious molecule was made.*

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Keywords:*Ageratum conyzoides,*beta satellite, Ageratum

Introduction

Ageratum conyzoides belongs to the family Asteraceae, it is a type of weed plant that occurs in many parts of world annually. It is estimated thatCentral America and the Caribbean are the main center of its origin from where the distribution extended to thevariouscontinents of the world. *A. conyzoides* is regularly distributed in large scale and toxic weeds in South Asia .it is most challenging to control the diverse species present in the different cultivated zones and natural forests, range/pasture areas, uneasy shrubs/scrubs, and wet lands(Saunders et al., 2008). *A. conyzoides* have ability and resistance in growth at sea level to higher levellike upto 2600 m altitude(Saunders et al., 2000). It can grow more efficiently in very rich, muddy soils and having the capability to bear shade. It hasconsistent growth in grasslands, forests, crop fields, farminglands and generally present in uncultivated lands, different railway lines along the roads(Saunders et al., 2004). *A. conyzoides* is capable of having a very much rate ofseed productionwithin a period ofplant maturity about 5 to 9 months in agreat capacity of plants appearance(Ueda et al., 2012). Besides that, it exhibits its astonishingfunctional stability in agricultural lands. The impact of *A conyzoides* is very significant over the crop fields and the soil from where they can easily grow(Xiong et al., 2007).

Many reports illustrated that weed plants are actively act as hosts forseveral diseases(Leke et al., 2012). There are the hosts of differentdesignated begomoviruses i.e.,*Ageratum*yellow vein virus from China, Pakistan, America, Brazil.Those type of herbs interfering in the growth and crop production, and thus expressivelyeffects on the environment(Andou et al., 2010), Ageratum enation virus from Pakistan(R W Briddon et al., 2002), the pepper yellow curl Indonesia leaf virus from Indonesia(Kumar et al., 2013). *Ageratum* yellow vein Taiwan virus belonging to China as well as India(Huang et al., 2013), tomatoes and yellow paper curl Tanzania virus belonging to Singapore(Saunders et al., 2001), Ageratum vein yellow China virus belongs to China and the Philippines(Jose & Usha, 2003).

Material and methods

Collection of Sample

This experiment was conducted on *Ageratum conyzoides* in the locality of Agriculture University Faisalabad. First of all, selection of newly emerging leaves on the basis of symptoms i.e., downward cup leaf curling. Cleaning of Leaves with the help of

tissue paper; three leaf discs were put in a tube and placed on ice and labelled properly. DNA was extracted after the collection of samples.

DNA extraction

Extraction of DNA was done by using CTAB method. Sample was completely crushed with blue pestles and added 1ml buffer of CTAB and 5µl β-mercapthoethanol. It was to be incubated at 60ºC for 40 minutes and mixed after every 15 min. rpm at room temperature. Supernatant was then put into new tube and washed with absolute (95%) ethanol. 700µl of absolute ethanol was added, incubated at –20ºC for 20 min and centrifuged for 15 min. Ethanol was discarded and washed with 70% ethanol. For this 200µl of 70% ethanol was added and spin for 5 min. Supernatant was discarded and pallet was air dried by placing in 37ºC incubator for 10 min. dissolve the Pallet in 50µl of ddH2O and stored at –20ºC.

Rolling circle amplification (RCA) and PCR

Circular viral molecules were amplified through rolling circle amplification (RCA) Phi 29 Kit Fermentas (GE Healthcare, formerly Amersham biosciences) following the manufacturer's protocol. For this purpose, 13ul of Phi mixture (50µl of 500µM hexanucleotides, 200µl of 10mM dNTPs, 100µl of 10X reaction buffer, 600µl of ddH_2O) was mixed in 0.5µl of concentrated DNA, boiled at 85ºC and immediately put on ice. Then sample was incubated for 16-20 hours at 30ºC after adding Phi 29 DNA polymerase enzyme. Next morning 10µl of ddH2O was added in mixture. Master mixture ishaving all the things except template DNA. Then aliquots of same volume were pipette into different tubes, and DNA template was added separately in each PCR tube. Total of 25µl PCR reactions in which 0.2µl Taq polymerase, 2.5µl Taq Buffer, 1.5µl MgCl2, 0.5µl dNTPs, o.5µl forward and reverse primer each, remaining volume makeup is balanced by water, and different volume of DNA was used. Tubes were placed in thermal cycler. The final reaction mixture was prepared in 25 µl mixture containing reagents (Table 1).

Table 1: PCR ingredients used for PCR reaction (25 µl)

INGREDIENTS	QUANTITY	INGREDIENTS	QUANTITY
Taq polymerase	0.2 µl	Reverse Primer	0.5 µl
Taq buffer	2µl	DNA	2.0 µl
MgCl2	1.5 µl	Water	16.8 µl
dNTPs	0.5 µl	Total	25 µl
Forward Primer	0.5 µl		

Purification of samples

The PCR clones was to be purified by using phenol chloroform extraction. Phenol-Chloroform (1:1) procedure was normally used to purify the samples from enzymes and proteins. The PCR product was diluted with d_3H2O to make 200µl volume. 23µl of remaining PCR products was taken and volume was made up to 200µl by adding 177µl of ddH2O. 200µl of phenol-Chloroform was added, shake gently and centrifuged for 2-4 min at room temperature. There were two clear layers in the Eppendorf. Supernatant was taken in a new Eppendorf tube, 1.5 volume (500µl) of absolute ethanol and 1/10th volume (10µl) of 3M Na acetate was added into the tube and placed into –20ºC for 15 min. Then samples were spin in refrigerated centrifuge machine at 4ºC for 15 min at 14000 rpm. DNA was pelleted at the bottom of the Eppendorf and ethanol was discarded. Samples were then washed with 200µl of 70% ethanol to remove excess salts if any. The centrifugation of mixtureand pellet was air dried at 37ºC for 10 min. 20µl of ddH2O was added in pallet, dissolved well and 2µl of phenol product was load on the 1% (w/v) agarose gel to check the concentration of DNA or were quantified with a spectrometer. it was stored at–20ºC until ligation.

Ligation

Purified PCR products (insert) were ligated into pTZ57/RT vector for cloning and sequencing as described by manufacturer's instructions (InsT/AcloneTM PCR Product Cloning Kit, Fermentas). 20µl of ligation mixture is shown in table 3.5. Ligase buffer. The ligation mixture was incubated at 30ºC for 3 hours (Table 2).

Table 2: Ligation mixture ingredients

Ingredients	Volume (µl)	Ingredients	Volume (µl)
Insert	6 µl	PEG	2 µl
Vector	1 µl	Water	8.5 µl
Enzyme (T4 DNA ligase)	0.5 µl	Total	20 µl
Ligation Buffer	2 µl		

Preparing of heat shock *E. coli* competent cells

The preparation of competent cells was proceeded according to the published protocol (Cohen *et al*., 1972) with little modifications. A one colony from plate was picked and transferred into 50 ml LB medium in a 50 ml flask and incubated at 37°C overnight with vigorous shaking. 1 ml of the overnight culture was taken and 250 ml liquid LB in a 1-liter flask was inoculated with inoculum. This 1-liter flask was placed at 37ºC with vigorous shaking until OD of 0.5-1.0 at 260 nm. Before spinning down the cells, culture was cooled by placing on ice for 10 minutes. The culture was then reassigned to sterile nonrefundable 50 ml propylene tubes. The cells were done by centrifugation 3000 rpm at 4ºC for 10 minutes and re-suspend in 10 ml of 0.1 M MgCl2 very gently.

Transformation in *E. coli*

Transformation in *E. coli* Top10 cell was done by applying heat shock method. Frozen, cells (100 µl) were thawed on ice for 10 min. 5µl ligating mixture added to the competent cells, mixed quietly and placed on ice for 20 min. Heat shock was known to these cells placing the tubes in a 42°C water bath for 30 secs and then again placed on ice for further 2 min. LB broth (200 µl) was added, mixed and incubated at 37°C for 45 min-1hour (Table 3).

Table 3: LB media composition for bacterial growth

Tryptone	10 g/Litre
Yeast extract	5 g/Litre
NaCl	10 g/Litre
pH	7.0
Agar (only for solid LB)	15 g/Litre

Collection of cell by centrifuged at 12,000 rpm for 1 min and in 100 µl LB. the spreading of transformed cells by using an sterilized plastic or steal spreader onto the surface of LB medium agar X-gal for blue white selection and selective antibiotics. These were incubated overnight at 37°C.

Composition of Solution I150µl solution II add to eppendorf tube and mixed by inverting tube 4-5 times. 150µl of solution III was added to tube, shaked well

Table 4: Composition of Solution II and Solution III

Solution II		Solution III	
Ingredients	Volume (ml)	5M KoAc	60 ml
10% SDS	1 ml	96%Acetic Acid	11.5 ml
5M NaOH	0.4 ml	dH_2O	28.5 ml
dH2O	8.6 ml		

The supernatant was taken into a fresh tube and adding 1.5 volumes of 100 % ethanol precipitated DNA. The eppendorf tube was then kept at –20°C for 15 minutes and centrifuged at 14000 rpm for 15 minutes. After centrifugation the 70 % ethanol was discarded and pellet was air-dried. Finally, 40-50µl of d3H2O was added to dissolve DNA and stored at –20°C.

Screening for clones

The isolated DNA digested with suitable enzymes by using suitable buffers to confirm the clones. For restriction, the 20µl total reaction mixture was used. Reaction mixture for enzymatic reaction is shown in Table 5.

Table 5: Reaction mixture for enzymatic reaction

Template	2 µl	RNase	0.1 µl
Enzyme (BamH1+EcoR1)	0.2 µl + 0.2 µl	Water	15.5 µl
Tango Buffer	2 µl	Total	20 µl

The incubation of reaction mixture was done at 37ºC for 3 hours. Fast digest enzymes were also used, in those cases incubation time was changed to 15 to 16 min. The incubation time and temperature could be different for different enzymes so it was adjusted according to the guidelines by supplier (Fermentas). Similarly, different buffers were to be used for different enzymes for example to confirm full length DNA molecule (1.4kb), plasmid was cut with *BamH1* (cloning site) with its own buffer, but for double digestion with *BamH1* and *EcoR1*, 1X yellow Tango buffer was used. For clone's confirmation of Beta and Alpha satellite molecules were double restricted with *EcoR1* and *Hind111* enzymes, 2X yellow Tango buffer was used. The digested DNA samples were resolved on 1% (w/v) agarose gel in 0.5X TBE along with the standard 1Kb DNA ladder to compare the size. The DNA samples having the correct inserted fragment were selected and used for further analysis.

Partial dimer construction

To generate tandem repeat, full length betasatellites genome of begomovirus was cloned in pGreen vector (pGreen0029-FL) at *Kpn*1 site. Two bands were obtained by double digestion (*Kpn*1-*Bam*H1) of full-length monomer, cloned in pTZ vector (pTZ-Partial clone, 0.9kb) and (pTZ-Partial clone, 0.5kb). Both Partial clones were digested with *Kpn*1 (by adding 3µl (~3µg) of DNA-B, 2µl Kpn1 buffer, 0.2µl *Kpn*1 restriction enzyme, 0.1µlRNase, 14.7µl ddH2O and placing at 37ºC for 3 hours). CIAP treatment was given to partial fragment by adding 17ul of restricted fragment, 2µl of CIAP buffer and 1µl of CIAP enzyme. This treatment was given to remove phosphate end to prevent self-ligation of vector. Both samples were purified using Phenol: chloroform method, checked on 1% (w/v) agarose gel. Monomeric sequence was introduced into each partial fragment by following ligation, transformation, plasmid DNA isolation and confirmed following restriction (methods described above).

Results

Symptom detection

Leaves from weed plant *'Ageratum conyzoides'* were taken showing typical symptoms of begomovirus impurity from thearea of University of Agriculture Faisalabad. The infections was noticed onweeds, which exhibiting symptoms of vein yellowing and swelling and leaf enation. A weed plants was also found infected by the begomoviruses*'*. Newly growing leaves were selected with vein yellowing, vein thickening and leaf curling. It was looking very interesting with lot of leaf swelling on the infected plant

DNA extraction

Total DNA was then extracted effectivelyfrom leaves of infected plants and DNA concentration was analyzed after running on 1% agarose gel

Detection of begomoviruses

Firstly to confirm begomoviruses presence diseased plants, DNA was diluted and subjected to PCR amplification by applying universal primers (sequence given**)** (Briddon et al., 2002). Primers were planned at satellite-conserved regions and supposed to give bands of 1.4kb if begomoviruses present in the sample. Theses primers amplified the fragments of 1.4kb size showing presence of begomoviruses.

5′ GGTACCACTACGCTACGCAGCAGCC 3′
3′ CACATGGGGACCCTCCCATCCATGG 5′

Sequence of universal beta primers

PCR Analysis

for the sake of amplification,we generally use the PCR machine to make the copies of our respective clones. it is very simple to identify our clones

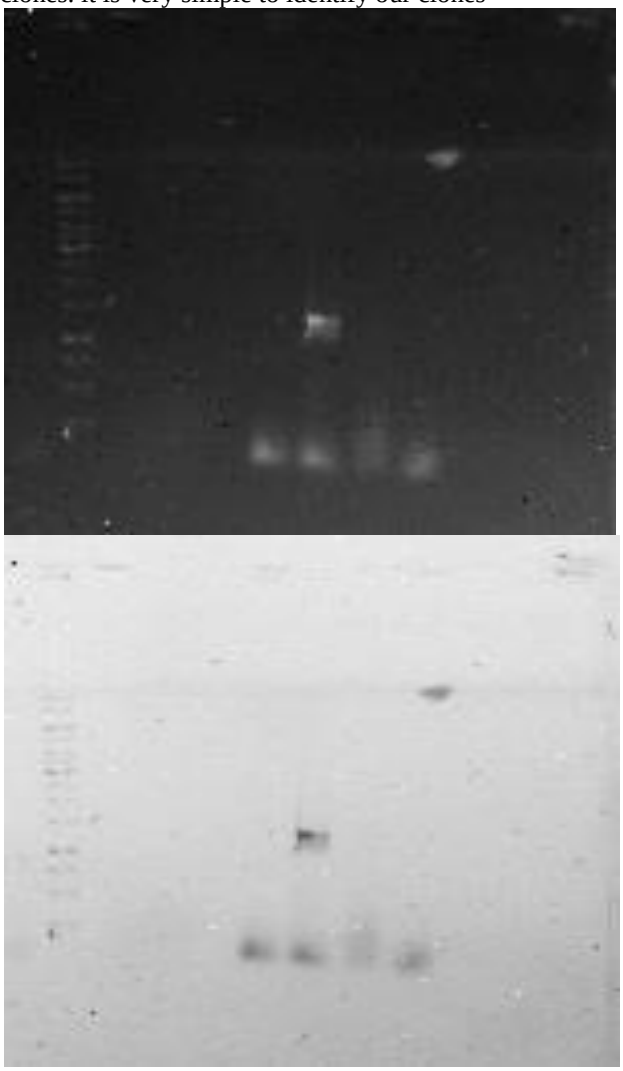

Figure 1: Gel picture

Clones of restricted fragments

The extraction of plasmid vector was proceeded effectively by applying alkaline lysis method (Bimboim & Doly, 1979; Ish-Horowicz & Burke, 1981). Following restriction with *Kpn*1 and *Pst*1, the vector was purified with the help of phenol:chloroform (1:1) method and concentration. Amplified fragments of 2.8kb and 1.4kb were successfully ligated into pTZ vector at *Kpn*1 and *Pst*1 site respectively.

Screening of cloned fragments

Succeeding alteration in E-coli Top 10 cells, the picking of six white colonies for plasmid isolation. These samples were taken to restraint digestion analysis for screening and three of them were seemed to be required inserts. The clones of 1.4kb size were double digested with Kpn1 and *Bam*H1 and give 2 bands if restriction site for *Bam*H1 is present in the fragment. Two bands of ~0.9kb and ~0.5kb cut out from the pTZ. Another single digestion was performed with *Kpn*1 (cloning site), which gave single band of ~1.4kb confirmation of the cloned fragments. All the restricted fragments were compared with gene ruler 1kb marker.

Partial dimer construction

1.4kb size clone was double digested with enzymes like*Kpn*1 and *Bam*H1 to cut into 2 separate fragments. 2 fragments of ~0.9kb and ~0.5kb were obtained and successfully cloned into pTZ vector. Full-length molecule was introduced in pGreen0029 vector at Kpn1 site. Following ligation and transformation, head to tail joining of full-length molecule into partial fragments was successfully done and dimeric constructs were confirmed by restriction analysis. Single digestion with either by the enzyme like*Kpn*1 or *Bam*H1 cut the fragment of 1.4kb, while double digestion with *Kpn*1 and *Bam*H1 cut out 2 fragments of ~500bp and ~700bp as were in restriction of full-length molecule. Both partial clones were appeared to be harboring full-length molecule

Discussions

Begomoviruses can be considered dangerous plant viruses' group and it became risk to agricultural crops in tropical regions around the globe(Saunders et al., 2002). Satellites have enlarged the complication of disease infecting plants(Tahir et al., 2015). There is high mutation rate found in begomoviruses that contribute towards the more diversification in population within a short time(Shahid et al., 2014). My work focuses on a virus ageratum yellow vein virus' infecting weed plant *Ageratum conyzoides it* is distributed in all over the world. I have cloned full-length molecule of Ageratum yellow vein virus (AYVV) by using PCR technique. To fulfill Koch's postulates and confirmation of causative agent of disease, partial dimers were made, genetic variability within speciesto made dimeric constructs of Ageratum yellow vein virus (AYVV). Full-length molecule was then restricted by suitable enzymes into two fragments making partial clones. as reported before(Saunders et al., 2002). To fulfill Koch's postulates and final characterization of isolated virus, I'll transfer this construct into an expression vector pGreen.At the same time full dimer was also tried to synthesize using partial restriction of RCA product and infectivity analysis will be completed. Detailed analysis will be possible aftergetting infection on the host plants that will fulfill the Koch's postulates. I have taken *Ageratum conyzoides* as host plant of AYVV Previously *e*. Such anextensive host range will construct were introduced in pTZ. in wider increase in impending pool hosts that may provide viruses to invade properly in crops in different seasons.

Recombination in geminiviruses rises that can involve to symptom on it has been reported in Singapore(Tao 2008). Its closely related viruses not only infect plants of family *Asteracea.* In addition the recombination may factor that changed the host range with respect to evolution of viruses. Vast range of virulence and distribution of begomoviruses can be involved to uninhibited populationthe host, occurrence of recombination within these viruses but this is the answers after sequencing. After sequencing and it has longer effect in begomoviruses. In this study; as a result of this a novel viral genome has progressed that has capability to persist in extreme circumstances.

Moreover, AYVV begomovirus is transmitted by vector whitefly not with any other insects, this can introduce a new direction of research that how. As recombination is not process *B. tabaci* populations develop spreading of begomoviruses. I amplified fragment of ~1450nt from *Ageratum conyzoides* through PCR that provide basic evidence to evaluate potential impact of different populationsIt is projected that it is a frequent process that plays vital role in the evolution of viruses because of no modern methods, progress has been sluggish to track the population. but from last few years this has become focus of extensive research. That will also give valuable information to study or limit the host range. Monopartite begomoviruses also associated with betasatellites primers that was successfully cloned. by sequencing we are able to illustrateMoreover, existence of beta satellite is rather conceivable in this host, this study has caused in discovery of a host and.; it can deliver useful information to study begomovirus evolution and scheming long term and broad-spectrum control strategies.

Conclusion

Begomoviruses hold single stranded circular DNA and belong to family *Geminivirida*). Many reports from Asia, Africa and Australia revealed that highly pathogenic begomoviruses can cause diseases in many plant species, Recently, the weed plant was detected that displaying the symptoms of yellowing vein in the locality of Faisalabad. Thus, in this study samples were collected from symptomatic plants to discover the presence of begomoviruses. The extraction of DNA was done by using the CTAB method and for quality checkingpurpose, run on the gel. The viral molecules amplification was proceeded either by applying PCR technique or by using universal primers in the PCR reaction. The molecules were cloned in pTZ and restriction digestion by suitable enzymes was applied for the sake of confirmation. Restriction by using different enzymes pTZ vector at *Kpn*1 and *Pst*1 site of MCS (multiple cloning site) respectively. PCR amplified product was directly ligated into pTZ. Transformation in *E. coli*, Top10 cell was performed by using heat shock method. Colonies was cultured and picking of white colonies after Blue white screening was done that supposed to be sheltering inserts of required size. Required band 1.4kb was obtained through restriction process with *Kpn*1 and *Pst*1. Circularized pTZ vector was restricted in a same way with the help of *kpn*1 and *Pst*1 separately. After the restriction, purification of restricted products was done by phenol-based purification. With the help of Purified restricted fragment of 1.4kb were then ligated into cloning vector confirming the clones of ~1.4kb size. Another single digestion was performed with *Kpn*1 (cloning site), which gave single band of ~1.4kb reconfirmed the cloned fragments. The cloned molecules were then under confirmation by performing plasmid DNA isolation and restriction digestion. The clones of expected 1.4kb size were double digested with *Kpn*1 and *Bam*H1 and supposed to give 2 bands (combine ~1.4kb size). Two bands of ~0.5kb and ~0.9kb were cut from the pTZ. The bands were appeared on the 1% agarose gel, ~1.4kb showing pTZ vector. The cloned molecules were sequenced and analyzed for the presence of any mutation or recombination. After partial sequencing, finally. To fulfill Koch's postulates partial dimer were constructed by digestion of full-length molecule in two smaller fragments (0.4kb and 0.9kb), each was then cloned in pTZ and. Full molecule was re-ligated in both partial clones. Dimeric constructs were confirmed by restriction analysis. Single digestion with either *Kpn*1 or *Bam*H1 cut out the fragment of 1.4kb, while double digestion with *Kpn*1 and *Bam*H1 cut out 2 fragments of ~0.5kb and ~0.9kb as were in restriction of full-length molecule.

Conflict of interest

The authors declared absence of conflict of interest.

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