Molecular detection and partial characterization of a begomovirus causing leaf curl disease of potato in sub-Himalayan West Bengal, India

Aniruddha Saha*, Bikram Saha and Dipanwita Saha

1Department of Botany, University of North Bengal, Siliguri-734 013, India
2Department of Biotechnology, University of North Bengal, Siliguri-734 013, India

*Corresponding Author E-mail: asahanbu@yahoo.co.in

Abstract

The characteristic disease symptoms of apical leaf curl, crinkled leaves and conspicuous mosaic were observed in potato plants grown in areas of Coochbehar, one of the distinct agroclimatic zones of sub-Himalayan West Bengal. Whitefly (Bemisia tabaci) population was also observed in and around the infected plants. The characteristic disease symptoms and presence of whitefly (Bemisia tabaci) population indicated the possibility of begomovirus infection. Total DNA was extracted from infected samples and PCR was carried out using begomovirus specific primers. PCR product of 1539 nucleotide long containing pre-coat protein, coat protein, AC5, AC3, AC2 and partial AC1 gene of DNA-A segment and 1001 nucleotide long DNA-B containing BV1 and BC1 genes were submitted to the GenBank (accession numbers HQ597033 for DNA-A and JN390432 for DNA-B). The amplified DNA-A and DNA-B segment showed highest 98% and 99% nucleotide sequence similarity respectively with Tomato leaf curl New Delhi virus (ToLCNDV). In phylogenetic analysis also the virus sequence clustered with ToLCNDV isolates. The disease was successfully transmitted to healthy tomato plants using both whitefly vector, B. tabaci and mechanical sap inoculation using sap of infected potato leaves. It is the first record of begomovirus infection of cultivated potato in sub-Himalayan West Bengal of Eastern India.

Key words

Begomovirus, Potato, Whitefly

Introduction

Potato (Solanum tuberosum L.) is one of the important and widely cultivated vegetables crops of India. India’s total production of potato ranks fourth in the world although in cultivated area it ranks third (Bansal and Trehan, 2011). In India, West Bengal is the 2nd largest producer of potato. Cultivation of potato is often affected due to attack of various diseases caused by fungi, bacteria, viruses and nematodes (Paul-Khurana et al., 1998). Loss in potato yield due to one or more virus(es) infecting potatoes range from low to high. Severe mosaic caused by Potato virus Y (PVY) and Potato leaf roll virus (PLRV) alone can reduce the yields upto 70-80% while mild viruses, like PVX, PVS and PVM can also depress yield by 10-30% (Bhat et al., 2010). Cultivation of horticultural and agricultural crops is limited by begomovirus infection in India and many other parts of the world. Leaf curl disease of potato showed typical symptoms of begomoviruses. Leaf curl disease of potato has been reported from India and Pakistan (Usharani et al., 2004; Mubin et al., 2009). Begomovirus is the largest genus of Geminivirus family, with one or two genome (DNA-A and DNA-B) components which infect dicots following insect transmission route. Insect, Bemisia tabaci plays an important role in the transmission of begomovirus. The newly introduced Bemisia tabaci Biotype B was found to be a major vector for transmission of begomoviruses in India (Bank et al., 2001; Narayana et al., 2006, 2007; Maruthi et al., 2007; Mahesh et al., 2010). In the present study, apical leaf curl disease of potato from sub-Himalayan West Bengal were studied for detection of viruses, if any. Molecular characterization of the virus was also performed.
Materials and Methods

Ten samples of crinkled leaves from different regions of sub-Himalayan West Bengal were collected and immediately brought to laboratory. Subsequently, to avoid contamination, leaves of infected plants were cut with sterile blade. Cut leaves were poured in sterile plastic bags and washed with sterile distilled water. Similarly, healthy leaves of the same cultivars and from the same regions were also collected. Collected samples were washed and kept in plastic bags. Both the infected and healthy leaves, thus collected were kept in -20 ºC for further experiments.

Artificial transmission by whitefly : Artificial transmission studies were performed using white flies. For this purpose, virus free white flies were used. Technique of Ghanem et al. (2001) was followed for transmission of viruses. About twenty insects were allowed to feed on infected potato plants in an insect proof cage for 24 hr (acquisition access period). Thereafter, insects were transferred to healthy potato plants, duly covered by a mosquito net. The insects were allowed to feed for 72 hr on healthy tomato plants. Insects were removed carefully from the plant by shaking the plants and the insect-free plants were left for development of symptoms for 60 days.

Mechanical transmission : Leaves of infected potato plants were crushed in 0.1 M potassium phosphate buffer, pH 7.0 (1:2, w/v) containing 0.15% sodium sulphite following the method of Chakraborty et al. (2003). The resultant sap was used for inoculation of healthy tomato plants. Ten different plants were selected for transmission test. Initially, the young leaves of test plants were dusted with carborundum powder (400mesh), then a cotton-wrapped stick was soaked in the sap-inoculum. The stick was rubbed on the leaves where carborundum powder was previously applied. Inoculated plants were kept in a net house up to 60 days with proper care, for symptom development. The visible symptoms appeared after transmission experiment confirmed the success of mechanical transmission of the virus.

Extraction, amplification and cloning of total DNA : Total DNA were extracted from the infected and healthy plants. Cetyl trimethyl ammonium bromide (CTAB) method (Dellaporta et al., 1983) modified by Sharma et al. (2003) was followed for extraction of DNA. Plant tissue (1g 5ml -1 ) was homogenized with 2% CTAB-DNA extraction buffer of 60 ºC for 1hr and mixed with chloroform-isooamylic alcohol (24:1) and then centrifuged. Aqueous phase was subjected to precipitation by using 0.6 volume of isopropanol and precipitates were washed with 70% ethyl alcohol and dined overnight. DNA was treated with RNase and was dissolved in Tris buffer [pH 8] and stored at -20 ºC for further use. All extracted DNAs were diluted 10-fold in sterile distilled deionised water.

Primers used : The diseased samples, which showed leaf curl symptoms, were used for DNA extraction. From extracted DNA, amplification of begomovirus specific DNA sequence was done using PCR amplification technique. The primers as proposed by Reddy et al. (2005) were used in the present study. Four, different primer pairs were used in the study (Table 1).

Polymerase chain reactions (PCRs) were performed in 25 µl reaction mixture containing 2 µl of template DNA, 0.5 units of Taq polymerase (Genie, Bangalore), 1.5mM MgCl2, 0.2mM dNTPs, 1 pmol of each forward and reverse primers in 1X reaction buffer (Taq buffer B). The amplification was carried out using a GeneAmp 2400 thermal cycler PCR system (Perkin Elmer).

After PCR purification of amplified DNA fragment using Genei Quick PCR purification Kit (Genei, Bangalore), the PCR amplified DNA fragment was cloned into the pGEM-T vector using pGEM-T easy cloning kit (Promega, USA) following the method of Knoche and Kephart (1999). The vector also contained ampicillin resistant gene for antibiotic selection and lacZ gene that allowed blue/white selection of recombinant colonies by α-complementation. Ligation was carried out overnight at 4 ºC. The ligation mixture was used for transformation. The component cells of Escherichia coli strain JM109 were prepared by calcium

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence data</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deng universal primers</td>
<td>5'TAATATTACCKGWKGVCCSC3'</td>
<td>~530bp</td>
</tr>
<tr>
<td>Deng A</td>
<td>5'TGGACYTTYTRCAWGBBCTTCACA3'</td>
<td>~870bp</td>
</tr>
<tr>
<td>Deng B</td>
<td>5'ATGKCSAAGCGWCCRGCAAG3'</td>
<td>~1280bp</td>
</tr>
<tr>
<td>CP primers</td>
<td>5'TTWARAAATGTTAAWKAGAGGAG3'</td>
<td>~1120bp</td>
</tr>
<tr>
<td>CRv301</td>
<td>5'ACNGNARACNATGTTGGGC3'</td>
<td>~520bp</td>
</tr>
<tr>
<td>Crc1152</td>
<td>5'GNARATHTGGATGGA3'</td>
<td>~470bp</td>
</tr>
<tr>
<td>Rojas universal primers</td>
<td>5'GAGTTTCCGYYTTTGGAGAWCCCGGAAGT3'</td>
<td>~400bp</td>
</tr>
<tr>
<td>PALic1960</td>
<td>5'GYTKGAATTTYGCTTCTGK3'</td>
<td>~350bp</td>
</tr>
<tr>
<td>PARv722</td>
<td>5'YTGGCAATTTYGCAGTTTCA3'</td>
<td>~300bp</td>
</tr>
<tr>
<td>DNA-B primers</td>
<td>5'AGTGGAGTCGCCGAGTAAGTC3'</td>
<td>~250bp</td>
</tr>
<tr>
<td>BF518</td>
<td>5'CCTTCTCAGTTCCTGK3'</td>
<td>~200bp</td>
</tr>
</tbody>
</table>


Table 1 : Oligodeoxyribonucleotides used for DNA amplification and sequencing

Journal of Environmental Biology, May 2014
chloride method as described by Sambrook and Russel (2001).

**Screening and sequencing of clones:** White colonies were screened for positive insert and were grown in LB plates containing ampicillin, IPTG and X-Gal. PCR was carried out from the grown colony followed by boiling lysis using specific primers to confirm the colony carrying the insert. After successful confirmation of the presence of expected insert in the clone, the plates were directly sent for sequencing. The sequencing was done from automated DNA sequencing service from Genei, Bangalore. Sequencing was done in both directions using SP6 forward and T7 reverse primers.

**Sequence and phylogenetic analysis:** The sequence data were analyzed using BLAST at the NCBI website (http://www.ncbi.nlm.nih.gov/). The sequence data from three amplified PCR products were assembled and analyzed using CLUSTAL W from MEGA 4.0 version software. The sequence was submitted to GenBank with proper annotations. The accessions of GenBank were also received (Accession number HQ597029). The sequences were compared with equivalent sequences from a range of other geminiviruses present in GenBank. Multiple sequence alignment was carried out using the software clustaw in MEGA version 4.0 (Tamura et al., 2007). An evolutionary tree was constructed using Neighbor-Joining method (Saitou and Nei, 1997). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004).

**Results and Discussion**

**Symptoms and disease incidence:** An apical leaf curl disease similar to the disease caused by Begomovirus in potato in India and its surrounding countries was first noticed in potato fields of Coochbehar district of West Bengal state of India. The incidence of the disease was about 40%. The characteristic symptoms observed on naturally infected potato plants were apical leaf curl and crinkled leaves and a conspicuous mosaic (Fig. 1b). Infected plants produced tuber with reduced size.

**Transmission efficiency:** The disease was successfully transmitted to healthy potato plants using both whitefly vector, B. tabaci and mechanical sap inoculation using sap of infected potato leaves. In case of white fly transmission technique, all the 20 plants tested showed characteristic visible symptom of cupping of margins and upward curling of potato leaves after 10 days of inoculation. Severe symptoms of leaf curling were observed after 30 days of inoculation. The plants also showed distinct visible symptoms of leaf curl after 20 days of incubation when mechanical transmission technique was employed.

**PCR amplification:** DNA were extracted and specific genes were amplified in PCR. The amplified products were allowed to run on agarose gel. The PCR products of potato samples were amplified by specific primers. Molecular weights were determined by using standard DNA-molecular weight markers (500bp ladder). Three different types of primers for DNA-A and one primer for DNA-B were used for the purpose of amplification. The expected sizes of amplified DNAs by three primers as mentioned above were ~530bp, ~870bp and ~1280bp and ~1120bp. The results of the present experiments were compared with the standard results of Reddy et al. (2005). The samples were considered to contain virus when the molecular weight of the amplified products matched with standard reported molecular weights. The samples containing viral DNA were cloned and then sequenced.

**Sequencing and phylogenetic analysis:** After getting the amplicons of expected sizes, amplicons were cloned and sequenced. The sequences were analyzed using BLAST at the NCBI website (http://www.ncbi.nlm.nih.gov/). All the sequences thus obtained were again subjected to multiple sequence alignment using CLUSTAL W in MEGA version 4.0 software. The common regions from all the sequences were omitted and a sequence of 1539 nucleotide long containing pre-coat protein, coat protein, AC5, AC3, AC2 and partial AC1 gene of DNA-A segment and 1001 nucleotide long containing BV1 and BC1 genes (partial) were submitted to the GenBank after proper annotation. The accession numbers for these submissions were HQ597033 for DNA-A and JN390432 for DNA-B. Basic local alignment search tool (BLAST) analysis of the virus showed highest sequence identity with Tomato leaf curl New Delhi virus (ToLCNDV) isolates. In phylogenetic analysis also the virus sequence clustered with ToLCNDV isolates (Fig. 2).

The most conserved gene of Geminiviridae was CP gene. For provisional identification of virus and to infer geographic and vector relationship CP gene sequences can be used (Brown et al., 2001). But for virus identification complete sequence of at least DNA-A is necessary (Faquet et al., 2008). In the present study, phylogenetic analysis of CP sequences along with pre-coat protein (AV2), AC5, AC3, AC2 and partial AC1 gene of DNA-A segment and 1001 nucleotide long containing BV1 and BC1 genes (partial) were submitted to the GenBank after proper annotation. The above sequences were compared with 16 closely related sequences of begomoviruses of GenBank. Additionally, DNA-B of the virus containing partial BV1 and BC1 were also sequenced. All the above sequences were compared with 16 closely related sequences of begomovirus present in the GenBank. Amplified DNA-A showed highest 98% nucleotide sequences identity with that of Tomato leaf curl New Delhi virus (ToLCNDV). Amplified DNA-B showed highest 99% nucleotide sequence identity with ToLCNDV. In phylogenetic analysis also the virus sequence was found clustered with ToLCNDV isolates.

Lakra (2002) reported heavy yield losses in susceptible potato varieties due to a virus, which they reported as apical leaf curl virus. Similar observation was also recorded by Garg et al. (2001). Presence of 40-75% disease incidence of PALCV was reported by Venkatasalam et al. (2005) from some cultivars of Indo-Gangetic plains. Tomato leaf curl New Delhi virus from potato...
Fig. 1: Healthy potato plants in field (a) Mosaic and curling of potato leaves (b)

Fig. 2: Most parsimonious tree showing the relationship of DNA-A (a) and DNA-B (b) with other begomovirus sequences from GenBank. Numbers at the nodes represents the bootstrap percentage score out of 1000 replicates. Present isolates are represented by yellow rectangle
apical leaf curl diseased samples was detected by Gawande et al. (2007) following print capture PCR. The association of begomovirus with apical leaf curl disease of potato was reported by Venkatasalam et al. (2011), using ToLCNDV specific primer pair and ToLCNDV specific probe. The association of geminivirus (white fly transmitted begomavirus) with leaf curl disease of potato by using polyclonal antibody of Indian cassava mosaic virus in immunoelectro microscopy test has also been shown by Garg et al. (2001). They named the disease as potato apical leaf curl virus based on symptomology of the virus. Using DAS ELISA test, Singh and Venkatasalam (2005) reported the affinity of leaf curl disease of potato towards tomato yellow leaf curl virus. The affinity also led them to assume that the virus was Geminivirus.

Usharani et al. (2004) reported leaf curl disease in potato caused by a strain of Tomato leaf curl New Delhi virus having accession numbers AY286316 [DNA-A (partial)] and AY158080 [DNA-B (partial)]. Our virus isolate having accession number JN390432 (DNA-B) showed homogeneity with DNA-B sequence under same cluster. DNA-A (accession number HQ597033) of the virus showed unrelatedness while clustering. This was a first observation of begomovirus causing severe disease of potato in Eastern India. The nucleotide sequence data indicated that the causal organism was a virus closely related to ToLCNDV. The virus isolate showed sap transmissibility and could also infect tomato plants when artificial insect transmission test was conducted. Chandel et al. (2010) also showed that polyphagous pest, B. tabaci could infect numerous fibre, food, vegetable and ornamental plants in north western plains of India. With the above findings, it is likely that the present strain was, probably, a new strain of ToLCNDV which infects cultivated potato in sub-Himalayan Eastern India through whitefly vectors. While studying diversity of a bipartite begomoviruses, Jyothsna et al., (2013) showed that ToLCNDV could infect potato and cucurbits showing almost similar symptoms as found in tomato, growing in nine different places of northern India. It was also noticed that infection occurred by the present strain in a geographic region of Coochbehar which is infested with white fly vector. Among the ten geographic region of sub-Himalayan West Bengal, studied, Coochbehar being the farthest from the Himalayas and having slightly different climatic conditions than the other nine regions indicated that geographic condition was also a factor for virus infection and disease development in potato. In Coochbehar, tomato and potato are grown in nearby fields and place were infested with several strains of ToLCNDV (isolated from tomato leaves). Most likely the viruses from these tomato plants served as reservoirs for infection of potato plants in the area. Similar observation was reported by Sohrab et al. (2013) that ToLCNDV and Potato apical leaf curl disease (PALCD) in the northern India could easily be transmitted by whitefly to potato showing PALCD. The overlapping planting and harvesting of the crops was possibly the reason of transmission of the begomovirus in the present study.

Acknowledgment

Financial assistance received in the form of fellowship (Research Fellowship in Science for Meritorious Students) to B. S. from University Grant Commission (UGC), New Delhi is greatly acknowledged.

References


Mahesh, Y.S., K.S. Shankarappa, K.T. Rangaswamy, H.A. Prameela and D.S.A. Narayana: Detection and characterization of a...


