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Association of a geminivirus with a leaf curl disease of sunn hemp (*Crotalaria juncea*) in India

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Abstract

Natural occurrence of a geminivirus causing severe leaf curl disease on sunn hemp (*Crotalaria juncea*) was recorded in India. The association of a geminivirus with the disease was demonstrated by whitefly transmission tests and polymerase chain reaction (PCR) amplification of DNA fragments of expected sizes with three pairs of degenerate geminivirus primers. The PCR-amplified viral DNA fragments were further characterized by Southern hybridization with a geminivirus probe consisting of the cloned coat protein (CP) gene of *Indian tomato leaf curl virus* (ITLCV). Restriction fragment length polymorphism analysis of a PCR-amplified CP fragment revealed that the geminivirus from sunn hemp was different than ITLCV.

Sunn hemp (*Crotalaria juncea*), a member of the family Fabaceae, has been cultivated in India since ancient time. Traditionally, sunn hemp has been considered as a good source of fiber for manufacturing twine and cord and has potential for use in pulp and paper. It is also an excellent green manure. Many pathogens including viruses, fungi and nematodes have been found to infect sunn hemp. There have been several reports of susceptibility of *C. juncea* to viral diseases. Strains of *Cowpea yellow mosaic virus* (Capoor and Varma, 1948); *Cowpea mosaic virus* (Lister and Thresh, 1955); strains of *Tobacco mosaic virus* (*Southern sunn hemp mosaic virus,* Capoor, 1962; cowpea strain, Sharma and Varma, 1975); *Crotalaria mosaic virus* (Kassanis and Varma, 1975); *Sunn hemp rosette virus* (Verma and Awasthi, 1976; 1978) and *Cowpea chlorotic spot virus* (Varma, 1986) have been reported to infect sunn hemp. *Crotalaria smalling virus* was regarded as identical to *Sunn hemp phyllody virus* rather than *Crotalaria mosaic virus* (Bos, 1964). *Cowpea mosaic virus* was also recorded in Nigeria (Ladipo, 1988) from naturally-infected *Crotalaria* exhibiting mosaic, distortion and puckering symptoms.

A severe leaf curl disease was observed on sunn hemp (*C. juncea*) plants grown for green manure in and around Lucknow during the rainy season, 2001. The severity of disease incidence was about 50–60% (based on data of three fields). Preliminary attempts to transmit a presumed viral pathogen from *C. juncea* to *Chenopodium amaranticolor*, *Nicotiana tabacum* cv. White Burley, *N. rustica* and *Lycopersicon esculentum* by sap inoculation were unsuccessful. In this communication, evidence is presented for association of a geminivirus with the disease based on virus transmission by whiteflies (*Bemisia tabaci*). Polymerase chain reaction (PCR) was used for its detection with total DNA from infected samples and three pairs of degenerate geminivirus primers. PCR products were characterized by Southern blot hybridization analysis with a probe prepared from cloned *Indian tomato leaf curl virus* (ITLCV)-coat protein (CP) and restriction fragment length polymorphism (RFLP) analysis.

Infected plants showed leaf curl and yellowing of young leaves (Figure 1). Sap transmission was attempted by inoculation of *C. amaranticolor*, *N. tabacum* cv. White Burley, *N. rustica* and

Diseased Healthy

Figure 1. Naturally-infected sunn hemp (left) and healthy sunn hemp (right).

L. esculentum plants with naturally-infected leaf tissue homogenized in a buffer (0.1 M sodium phosphate, pH 7.0 containing 1.0% sodium sulphate). None of the inoculated plants developed local or systemic symptoms 30 days post inoculation. Lack of sap transmission and a high population of whiteflies observed on sunn hemp plants indicated that the disease could be transmitted by the whitefly. Whitefly transmission tests were carried out using naturally-infected *C. juncea* plants as a source of virus inoculum, healthy *C. juncea* seedlings as test plants and *Bemisia tabaci* as the insect vector. Nonviruliferous whiteflies (*B. tabaci*) were maintained on *Clitoria ternatea* plants in a glasshouse. For whitefly transmission tests, approximately 100 *B. tabaci* were starved for 3 h and then allowed to feed on a twig and/or leaf of naturally-infected *C. juncea* for an acquisition access period of 24 h. These whiteflies were transferred to five healthy seedlings each of *C. juncea,* pigeonpea (*Cajanus cajan*), mungbean (*Vigna radiata*), blackgram (*V. mungo*) and tomato (*L. esculentum*) for an inoculation access period of 48 h. Whiteflies were killed by spraying test plants with 0.2% insecticide. The inoculated seedlings were maintained in insect-proof cages and observed for symptom development for a period of 30 days. *Crotalaria juncea*, *V. mungo* and *L. esculentum* seedlings developed yellow mosaic and leaf curl symptoms two weeks of post inoculation. *Cajanus cajan* and *V. radiata* did not develop symptoms. Symptomatic plants were confirmed to be infected by a geminivirus by PCR and Southern blot hybridization tests with a geminivirus probe. The virus was maintained on *C. juncea* seedling in a glasshouse via whitefly transmission.

Virus purification was attempted (Czosnek et al., 1988) using approximately 50 g of infected *C. juncea* leaf tissue powdered in liquid nitrogen and homogenized with 0.1 M sodium phosphate buffer, pH 6.5 containing 0.1% beta mercaptoethanol. Purified virus preparations as well as, leaf dip preparations were examined with an electron microscope at Central Institute of Medicinal and Aromatic Plants, Lucknow. A few geminate virus particles were observed in leaf dip preparations of infected *C. juncea* leaves. However, three attempts to purify the virus failed. Deng et al. (1994) mentioned that whitefly transmitted geminivirus (WTGs) are common in tropical countries, but that their virions were difficult to purify. Therefore, PCR detection is the preferred method for geminivirus identification. Rojas et al. (1993) and Deng et al. (1994) developed similar approaches for the PCR detection of WTGs (*Begomovirus*) in various plant species. They suggested that the development of such methods would be very useful for detection and identification of geminiviruses, and for study of the etiology and epidemology of geminivirus induced diseases. We attempted PCR detection of a WTG in naturally-infected *C. juncea* leaf samples using total plant DNA extracts and three pairs of degenerate primers previously designed for detection of WTGs.

Total plant DNA, extracted from infected *C. juncea* leaf samples (Srivastava et al., 1995), was used as a template in PCR reactions with three pairs of geminivirus specific primers. The three pairs of primers were A/B (Deng et al., 1994); C/D (ITLCV-CP specific, Srivastava et al., 1995) and E/F (PALIv1978 & PARIc496, Rojas et al., 1993). These primer pairs amplify about 550, 800 and 1100 bp, respectively, from the DNA-A component of most begomoviruses. These primers have proved to be useful for the detection of known, as well as, unknown geminiviruses. PCRs were set up in a 50 µl reaction mixture containing: template DNA (5 µl), dNTPs (10 mM each, 1 µl), two primers each (25 pMol μl^{-1}, 1 µl), Taq DNA polymerase (3U μl^{-1}), Taq Buffer (10×, 5 µl, Banglore Genei Pvt. Ltd.) and water (36 µl). The PCR conditions: PCR cycles 30, denaturation (94 °C for 5 min), extension (72 °C for 2–3 min) were the same except that the annealing temperatures varied for different pairs of primers. The annealing temperature for primers A/B, C/D and E/F were 52 °C, 47 °C and 50 °C, respectively, for 1 min each. The final extension cycle was 5 min at 72 °C. PCR products were analyzed by electrophoresis in 1.2% agarose gels and

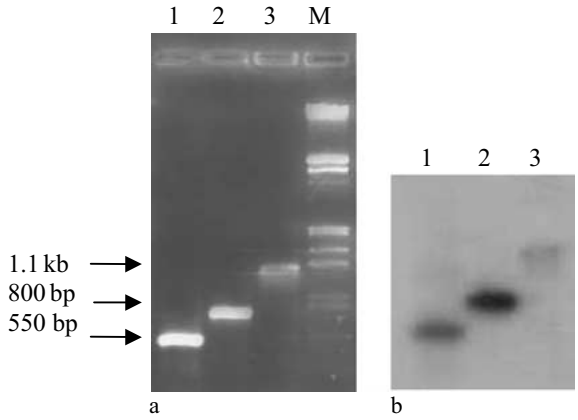

Figure 2. (a) Agarose gel electrophoresis of the PCR products amplified from DNA extracts prepared from sunn hemp plants with symptoms of geminivirus infection. Fragments amplified with Deng's primers (lane 1), PCR amplification from ITLCV-CP specific primers (lane 2) and PCR amplification from Rojas's primers (lane 3). (b) Southern blot hybridization analysis of the gel in (a) probed with the cloned ITLCV-coat protein gene in p*Bluescript* II SK+.

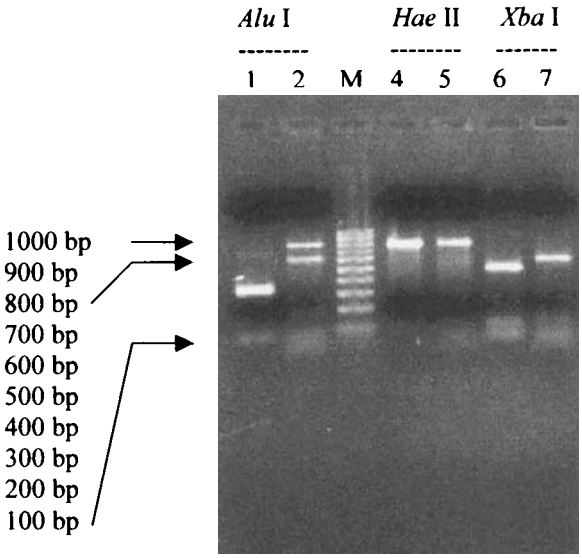

Figure 3. Restriction fragment length polymorphism analysis of coat protein DNA fragment, amplified by PCR with ITLCV-CP primers (C/D), from ITLCV and the geminivirus associated with sunn hemp. ITLCV fragments are in lanes 1, 4 and 6.

stained with ethidium bromide. All the three sets of primers directed the amplification of DNA fragments of the expected size from DNA of infected sunn hemp (Figure 2a). No such products were observed when nucleic acid from uninfected leaves was used as template. The results of PCR using three sets of geminivirus specific primers indicated the presence of geminivirus infection in naturally-infected sunn hemp.

To confirm identity of the putative DNA-A fragments, Southern blot hybridization analysis was carried out with a probe prepared from cloned coat protein (CP) gene of ITLCV (Srivastava et al., 1995). The ITLCV probe was used because all the three primers were expected to amplify all or part of the geminivirus CP gene. For Southern blot hybridizations, DNA fragment from agarose gels were transferred to Hy-bond membrane (Amersham-Pharmacia, UK) and hybridized with α P^{32} labeled probe. Pre hybridization and hybridizations were performed in a solution containing 50% formamide, 25% SSC (20×), 6% Denhardts (50×), 0.5% SDS (10%) and water. Hybridizations were done over night at 42 °C, washed at the moderate stringency conditions (as recommended by the company) and were then exposed to X-ray film. Developed X-ray film revealed strong hybridization signals between the PCR-amplified fragments from infected sunn hemp and the CP probe of ITLCV (Figure 2b). Because all the fragments amplified from the three different pairs of geminivirus primers hybridized with the CP probe of ITLCV, the association of a begomovirus with leaf curl disease of *C. juncea* was established.

Further evidence was provided by sequencing of the ~550 bp PCR product amplified from infected *C. juncea* DNA using primers A/B of Deng et al. (1994). DNA sequence analysis revealed 94% identity with a strain of monopartite *Tomato leaf curl virus* from South India (EMBL – Accession no. U38239).

RFLP analysis of the ~800 bp DNA fragments of the geminivirus from sunn hemp and ITLCV obtained from PCR using ITLCV-CP primers C/D of Srivastava et al. (1995), was carried out using *Alu* I, *Hae* III and *Xba* I restriction enzymes. The digested products were loaded into 1.5% agarose gels and electrophoresed in TBE buffer. The patterns obtained by digestion of the PCR products obtained from extracts of naturally-infected sunn hemp and ITLCV-infected tomato with *Alu* I, *Hae* III and *Xba* I restriction enzymes are shown in Figure 3. The DNA fragments, when digested with *Alu* I and *Xba* I, had different RFLP patterns. No RFLP were obtained with *Hae* III (Figure 3). Digestion with *Alu* I generated three fragments (two of same size 350 bp, which did not separate as two distinct bands) and one 100 bp in ITLCV where as three

bands, 800 (might be a partial digest), 650 and 150 bp were observed for the sunn hemp geminivirus fragment. *Xba* I generated three bands 550, 150 and 100 bp for ITLCV and three bands 600, 150 and 50 bp for the sunn hemp geminivirus fragment. *Alu* I and *Xba* I digestion of the CP fragment revealed a clear difference between the two viruses.

A survey of literature for viral diseases on sunn hemp (*C. juncea*) revealed the first record of *Sun hemp mosaic virus* from India by Capoor and Varma (1948). Indian sunn hemp (*C. juncea*) was also recorded as a susceptible host to *Apple mosaic virus*, *Cassia yellow spot virus*, *Cowpea mosaic virus*, *Elderberry virus, Okra mosaic virus, Sunn hemp mosaic virus* and *Tomato spotted wilt virus* (Brunt et al., 1995). However, there is no evidence available in literature for sunn hemp as a natural or susceptible host of any geminivirus. Therefore, our report is the first record of a geminivirus associated with leaf curl disease on *C. juncea.*

Geminiviruses are well suited to PCR detection and identification because they replicate via a double-stranded, circular DNA intermediate replicative forms (Stanley et al., 1991), which can serve as a template for amplification by PCR. Use of degenerate primers in PCR to detect WTGs was evaluated for amplification of geminiviral fragments from the DNA-A and/or DNA-B components of 15 geminiviruses from tomato, pepper, soybean, cassava and weed species (Rojas et al., 1993). In the present study, DNA fragments generated from infected sunn hemp tissues by PCR and three pairs of degenerate primers of geminiviruses were identified as those of a begomovirus. Hybridization with a ITLCV-CP probe confirmed the nature of the PCR-amplified fragments. Different restriction digestion patterns of PCR products from sunn hemp and ITLCV is consistent with these representing distinct viruses.

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