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Dot blot hybridization and PCR based detection of begomoviruses from the cotton growing regions of Punjab, Pakistan.

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Abstract

Total DNA extraction was carried out from the five virus infected host plant species namely, *Zinnia elegans, Eclipta prostrata, Solanum nigrum, Capsicum annuum and Ageratum conyzoides* with suspected Geminivirus symptoms. By using universal primers for the DNA A genome of whitefly-transmitted geminiviruses, approximate full length DNA A genome was PCR amplified for four of the viruses Zinnia leaf curl virus (ZiLCV), Solanum yellow leaf curl virus (SYLCV), Pepper leaf curl virus (PeLCV) & Ageratum yellow vein virus-Pakistan (AgYVV-P) except *Eclipta prostrata* yellow vein virus (EPYVV). Nucleic acid hybridization was used to compare homologies with four different probes of the begomoviruses namely african cassava mosaic virus (ACMV), watermelon chlorotic stunt virus (WCSV), cotton leaf curl virus (CLCuV) and SYLCV (DNA A). Relative levels of cross hybridization were analyzed under similar optimized conditions for each test. EPYVV showed least homology with any other Begomovirus from Pakistan, PeLCV found to be more closely related with ACMV and WCSV as compared to the probes from Pakistan viruses ie., SYLCV & CLCuV.

Key words: Dot blot, PCR, Begomoviruses.

Introduction

The taxonomy of the Geminiviridae divides the virusers into three genera. The division is on the basis of insect vector (either whitefly [Begomovirus] or leafhopper [Mastervirus or Curtovirus]) and genome arrangement. In addition to these three genera, a fourth genus has been proposed, to accommodate the treehopper-transmitted, tomato pseudu-curly top virus (Briddon and Markham, 1995).

DNA probes have been used for the detection of many plant viruses and viroids (Baulcombe *et al*., 1984; Maule *et al*., 1983), including geminiviruses (Czosnek *et al*., 1980; Haber *et al*., 1987; Polston *et al*., 1989). DNA probes made from partial or full length clones of either of the two genomic components (DNA A & DNA B) of several whitefly-transmitted geminiviruses have been used in hybridization assays for virus detection, and in some cases, for virus identification. DNA hybridization assays incorporating individual probes or cocktail of probes of DNA A components are useful for virus detection (Brown and Poulos, 1990). In contrast, the nucleotide sequences of the DNA B components of whitefly-transmitted geminiviruses are nearly unique for each virus and can serve as virus or strain specific probes. (Brown and Poulos, 1990a; Harrison, 1985).

Comparisons among nucleic acid and protein sequences of viral origin, along with comparisons among structural and biological criteria have long been used to identify and classify plant viruses (Shukla & Ward, 1988, 1989). In addition, nucleic acid hybridization and Polymerase chain reaction (PCR) techniques have been used to study the molecular variability of some of these viruses (Gilbertson *et al*., 1991a; Hughes *et al*., 1992; Polston *et al*., 1989).

PCR is a highly sensitive and reliable technique for the detection of plant viruses. Geminiviruses are small, single stranded DNA viruses with circular genome and thus are well suited for the detection of these viruses by PCR.

Comparative hybridization studies using a panel of DNA A component probes of whitefly-transmitted geminiviruses indicated that there are greater differences among the DNA A component nucleotide sequences of geminiviruses in regions defined by geographic barriers within or between continents than among those of geminiviruses on the same continent or in the same geographic region. Furthermore DNA B component probes tested in the same manner have proved to be highly virus specific (Harrison, 1985). There are

limitations in the utility of DNA hybridization assays for virus diagnosis, imposed primarily by the need to maintain a broad array of virus clones. However, reproducible and differential patterns resulting from cross hybridization with a defined panel of probes are potentially indicative of the degrees of similarities or differences among the isolates tested.

The Begomoviruses are transmitted by the whitefly *Bemisia tabaci* infecting dicotyledonous host plants only, cause a variety of symptoms; leaf curling is very common among the members of this genus, other symptoms include, stunting, distortion and vein yellowing; mosaic, vein thickening and enations can also be observed in few cases. Most of the economically important geminiviruses fall into the genus Begomovirus. Cotton leaf curl viral disease, a serious cause of crop loss in Pakistan during the last one decade also produces vein thickening and enations along with leaf curling. It is estimated that during the year 1992-97 the disease has caused loss of 7.4 million bales of cotton with an estimated value of 4.98 billion US dollars.

Present studies were aimed to detect Begomoviruses from other than cotton plants that were found infected in and around the cotton fields and to determine their relationship with CLCuV.

Materials and Methods

Origin of virus infected plant materials

Five host plants (*Zinnia elegans, Eclipta prostrata, Solanum nigrum, Capsicum annuum and Ageratum conyzoides*) showing Begomovirus like symptoms were collected from the fields of Punjab (Multan and Bahawalpur zone), Pakistan. These infected samples were brought to the United Kingdom in the year 1993, and studies were conducted at John Innes Centre, Norwich during the years 1993-96. However some of the results were reconfirmed at the School of Biological Sciences, University of the Punjab, Lahore, Pakistan in the year 2003-04 (where ever it was required). The viruses were maintained on the original/wild host plants or suitable alternate host plants (Table 1).

DNA extraction from plant material

Total DNA extraction from healthy or infected plant tissue was carried out essentially as described by Parish & Kirby (1966), commonly known as the Kirby method. Tissues (0.5 mg) was ground in a pestle and mortar in liquid nitrogen, followed by adding 1 ml of Kirby buffer consisting of 1% TNE [Triisopropylnaphthalenesul-fonic acid sodium salt], 6% PMS [p-Minosalicylic acid (SIGMA)], 50 mM Tris-HCl (pH8.3), and 6% (v/v) buffer saturated phenol, and 1 ml of phenol/chloroform was then added. The homogenate was transferred into an eppendorf tube and spun at 10 K for 10 minutes. The supernatant was transferred into a new tube, followed by two phenol/chloroform extractions. The DNA was ethanol precipitated, dried and resuspended in sterile distilled water.

Dot blot hybridization

Relative levels of cross-hybridization were determined by dot blot hybridization as described previously Maule *et al*., (1983), however total DNA from infected plants was used instead of plant sap. Probes were produced from full length clones of african cassava mosaic virus (ACMV) DNA A (Kenyan isolate; Stanley and Gay, 1983), watermelon chlorotic stunt virus (WCSV) DNA A (Bedford *et al*., 1994), and PCR amplified cotton leaf curl virus (CLCuV) DNA A (Mansoor *et al*., 1993) and SYLCV DNA A (Haider, 1996) obtained by PCR amplification using universal primers (Briddon and Markham, 1994). Preparation of probe and hybridization was performed as described by Feinberg and Vogelstein (1984) and Southern (1975) respectively.

Polymerase Chain Reaction

Amplifications were performed in volumes of 100 µl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2) 0.01% (w/v) gelatin, 200 µM each of dATP, dTTP, dGTP, dCTP (Pharmacia), 5 µM of each primer, 100 ng of total nucleic acid, 2.5 U of *Taq* polymerase I (Amplitaq, Perkn Elmer Cetus) and were overlayed with 50 µl of mineral oil (Sigma) to reduce evaporation. Reactions were carried out in a Techne PHC-3 Thermal Cycler programmed for 40 cycles of 1 min at 94^{o}C, 1 min at 50^{o}C and 4 min at 72^{o}C, using the fastest available transition between each temperature. Universal primers (F5' GTCGGGGTCGACGTCATCAATGACGTTGAT AC 3') (R3' CGGTCAGAGAGAAGCCCG GGGTGTAC-TTAAGAGAA 5') for the PCR-mediated amplification of dicot-infecting geminiviruses (Briddon and Markham, 1994) were used to amplify a nearly full-length genome of approx 2.7 kb length (DNA A genomic component). PCR product was detected by agarose gel electrophoresis using 1Kb ladder as marker.

DNA Cloning and Manipulation

PCR amplified fragment of SYLCV DNA A full length genome was ligated into pGEM-T cloning vector according to the suppliers instructions, the clones in DH5α cells were screened for the correct type of insert through restriction digestion of the isolated plasmids. Restriction endonucleases were obtained mainly

from Gibco BRL or New England Biolabs. Digestion was performed under the conditions recommended by the manufacturer. Digestions were performed in a water bath for 1 hour or overnight. The digested DNA was normally checked by agarose gel electrophoresis.

Ligation of DNA inserts and vectors

Ligations of DNA inserts and vectors were carried out in 20 ul 1x T4 DNA ligase reaction buffer containing 66 mM Tris-HCl (pH7.5), 10 mM MgCl, 1mM dithiothreitol (DTT), 1 mM (EDTA), 0.5 mM ATP at 14^{O}C overnight using 1µl (5 units) of T4 DNA ligase (Gibco BRL). Reaction mixture typically contained 50-100 ng cloning vector and 100-200 ng of the fragment to be cloned.

Transformation of *E. coli* cells

Competent cells 100 µl were added to 10-20 µl of the ligation reaction mix, uncut plasmid control, or ligation control and allowed to stand on ice for 45 minutes. The cell/plasmid mixtures were heat shocked for 2 minutes at 42^{O}C and placed on ice for a further 15 minutes. The transformed cells were directly spread onto the surface of LB medium agar plates (1.5% Bacto tryptone, 1.0% Bacto yeast extract, 0.5% NaCl, 1.2% Agar) containing 130 µg/ml IPTG, 270 µg/ml X-gal and 200 µg/ml carbenicillin. Plates were incubated upside down at 37^{0}C overnight. The white clones (plasmids) were selected and reinoculated for small scale preparation as described by Ish-Horowitz and Burke (1981).

Results

Identification of the viral nucleic acid

To obtain evidence of the identity of the begomovirus for each isolated virus, total plant DNA was extracted from whitefly-inoculated: *Nicotiana benthamiana* plants infected with each individual virus: EPYVV, SYLCV and PeLCV; tomato for ZiLCV and *Datura stramonium* for AgYVV. Total DNA from healthy plants (*N. benthamiana*, tomato and *Ageratum* was also included as a negative control. Total DNA extracted from infected plants for each of the four viruses, from which a probe was made, served as a positive control for each hybridization test.

Nucleic acid hybridization

The results are shown in Fig. 1 & 2 and summarized in Table 2. All five viruses cross-hybridized to the CLCuV probe with ZiLCV showing the strongest response. EPYVV showed low levels of hybridization to all four probes with a moderate response to CLCuV. All the Pakistan viruses showed relatively good hybridization to SYLCV and WCSV except EPYVV, PeLCV was exceptional in that it gave a marginally better response to ACMV then to the probes from Pakistan viruses ie., SYLCV and CLCuV.

PCR amplification of the full length DNA A genome

Further confirmation of the identity of the viral nucleic acid was obtained by PCR amplification. The viral DNA was amplified from the DNA preparations as mentioned above. DNA from healthy plants was used as a negative control. Amplified virus fragments of the expected size, approx. 2.7-2.8 kb, were obtained in all the preparations except EPYVV, and there was no amplification from the healthy plants DNA (Fig. 3).

Identification and restriction mapping of the cloned DNA A genome

Cloned SYLCV genome was further treated (restricted) with a set of randomly selected restriction enzymes namely *Bgl II, Sma I, Cla I, Kpn I, Hind III, Nru I* and *Xba I*, that do not cut pGEM-T vector. Only two restriction enzymes (*Nru I & Xba I*) could cut SYLCV DNA A, but the single cutter was found to be *Xba I*. The expected band size ~ 5.5 kb of the lenearized DNA along with the vector was detected on ethidium bromide stained agarose gel (Fig. 4).

Table 1: Virus codes, original host plants, symptoms and maintenance methods.

Code	**Host Plant**	**Symptoms**	**Maintenance Method**
ZiLCV	*Zinnia elegans*	Leaf curl	(Gt)(It)
EPYVV	*Eclipta prostrata*	Yellow vein	(Gt)(Vp)(It)
SYLCV	*Solanum nigrum*	Yellow leaf curl	(Gt)(It)(Mi)
PeLCV	*Capsicum annuum*	Leaf curl	(Gt)(It)
AgYVV	*Ageratum conyzoides*	Yellow vein	(Vp)(It)

Abbreviations: ZiLCV = Zinnia leaf curl virus; EPYVV = Eclipta prostrata yellow vein virus; SYLCV = Solanum yellow leaf curl virus; PeLCV = Pepper leaf curl virus; AgYVV = Ageratum yellow vein virus; Gt = Graft transmission; Vp = Vegetative propagation; It = Insect transmission; Mi = Mechanical inoculation.

Table 2: Relative levels of cross hybridization using four probes against six viruses from Pakistan and compared to ACMV (Africa) and WCSV (Yemen).

	--Probe--			
Viruses	**CLCuV**	**SYLCV**	**ACMV**	**WCSV**
ACMV	3.0	2.5	6.0	4.0
WCSV	2.5	3.0	4.0	6.0
ZiLCV	5.0	4.5	4.0	4.5
EPYVV	2.0	1.0	0.5	0.0
SYLCV	4.0	6.0	2.5	3.0
PeLCV	3.0	3.0	4.5	3.5
AgYVV	4.5	4.0	3.5	4.0
CLCuV	6.0	3.5	3.0	2.5

Scores range from 1 (very weak) to 6 (homologous) reaction. **Bold** figures showing reciprocal reactions. *Italic* letters showing viruses from Pakistan.

Discussion

The results confirmed that all the five viruses belong to the family Geminiviridae and genus Begomovirus (Briddon and Markham, 1995). The transmission characteristics and morphology of the viruses has already been described (Haider *et al*., 2003; Haider *et al*., 2002) respectively, that support our present findings.

DNA cross hybridization tests have shown that EPYVV seemed to have less sequence homology to all the four probes tested, (Fig.1 and 2) and the only significant hybridization was with CLCuV. Since EPYVV showed a very narrow host range in comparison with all the other four viruses from Pakistan and also gave a different symptom pattern on *Zinnia elegans* and *N. benthamiana*, common host plants of at least three of the other Pakistan viruses, suggested that EPYVV is probably a different virus from the other Pakistan viruses.

Dot blot hybridization tests also proved that PeLCV has different sequence homology from the viruses to which it was compared. Begomoviruses infecting pepper in the new world have shown different biological properties; two of those reported were mechanically transmitted and the third one was shown to have a different symptom pattern (Stenger *et al*., 1990; Brown and Poulos, 1990; Garzon-Tiznado *et al*., 1993).

Some other host plant species have also been found infected under natural conditions from Pakistan. Harrison *et al*., (1997) reported five whitefly-transmitted geminiviruses based on their epitope profile. These viruses were associated with, tobacco leaf curl, squash yellow blotch, tomato yellow leaf curl, watermelon leaf crinkle and soybean yellow mosaic diseases. Mansoor *et al*., (1998) found four plant species (China rose, Tomato, Chillies and Okra) based on PCR strategy, infected with begomoviruses from the cotton growing areas of Sind Province that has also been found infected from cotton growing areas of Punjab Province as well, previously.

Among begomoviruses in general, it is becoming increasingly difficult to decide whether two virus isolates are best considered strains of the same virus or distinct viruses. The association of different begomoviruses with similar diseases of the same plant species in different countries, and the possibility that begomoviruses can adapt more readily than other plant viruses to different host species, raises the question of how begomoviruses should be distinguished and what criteria should be used to justify naming individual virus species.

Accepting that the function of some viral gene products, and possibly of some viral non-coding nucleotide sequences, necessitates interactions with host components, the occurrence of related viruses in the same plant species provides an opportunity to assess the evidence of adaptation of virus to host species.

The comparisons made in the present studies and elsewhere (Padidam *et al*., 1995b) suggests that begomoviruses are evolving rapidly and may be able to adapt more readily to new host species than plant viruses with RNA genomes.

Exciting progress has been made in the study of several begomoviruses in recent years, and knowledge of the biological, biochemical, serological and molecular nature of the pathogens has increased with the emergence of new technologies.

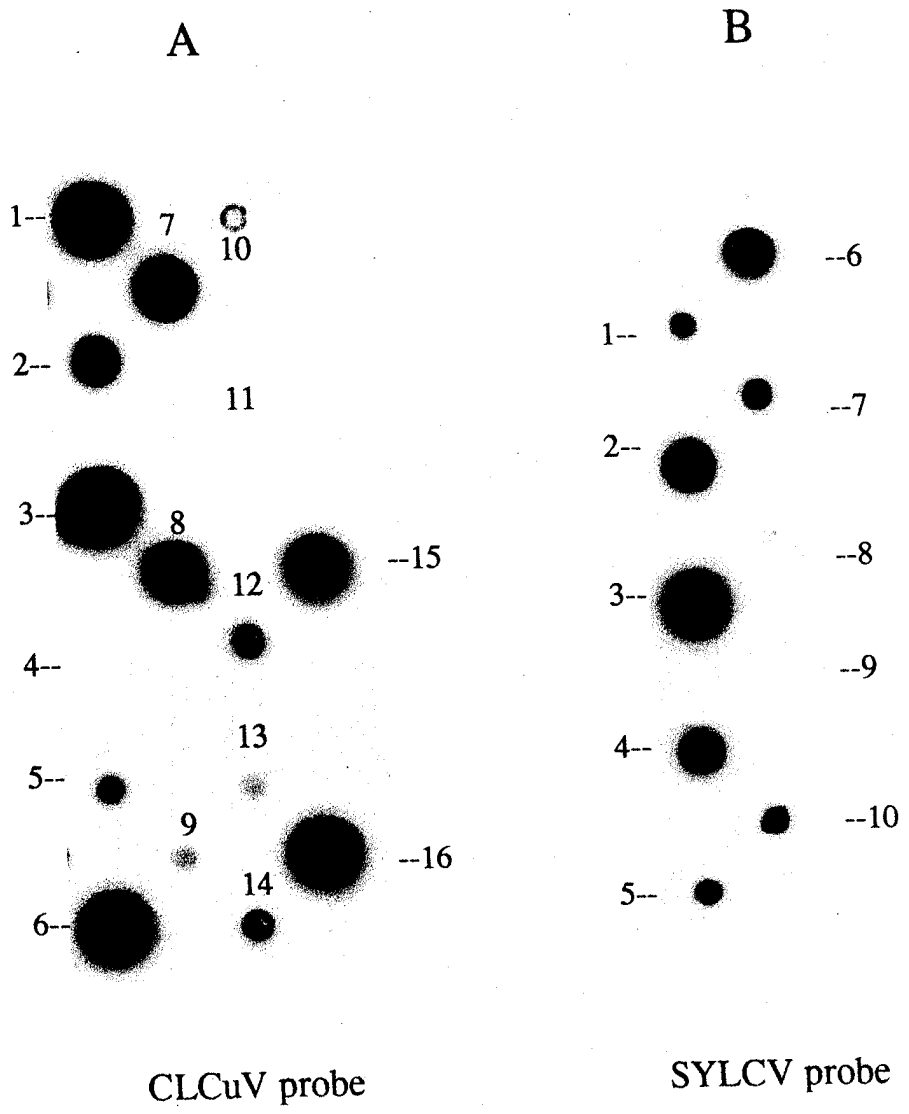

Fig.1: Dot blot hybridization tests, radioactively labeled DNA A of CLCuV (A) and of SYLCV (B), used as a probe. Input samples were the total nucleic acid (10 µg/sample) extracted from (A), ZLCV & TLCV-Aus infected tomato (1 & 2) respectively, CLCuV infected and healthy *N. tabaccum* (3 & 4) respectively, BcaMV infected *Phaseolus vulgaris* (5), AgYVV infected *D. stramonium* (6), ACMV infected *N. benthamiana* (7), WCSV infected watermelon (8), healthy tomato, *Ageratum* and *N. benthamiana* (9, 10 & 11), EPYVV infected *N. benthamiana* (12), healthy and AGMV infected *Asystasia* (13 & 14) respectively, PLCV & SYLCV infected *N. benthamiana* (15 & 16) respectively. (B), EPYVV infected *N. benthamiana* (3), CLCuV infected *N. tabaccum* (4), WCSV infected watermelon (5), AgYVV infected *D. stramonium* (6), PLCV infected and healthy *N. benthamiana* (7 & 8) respectively, healthy tomato (9), ACMV infected *N. benthamiana* (10). Washing was done under medium stringent conditions.

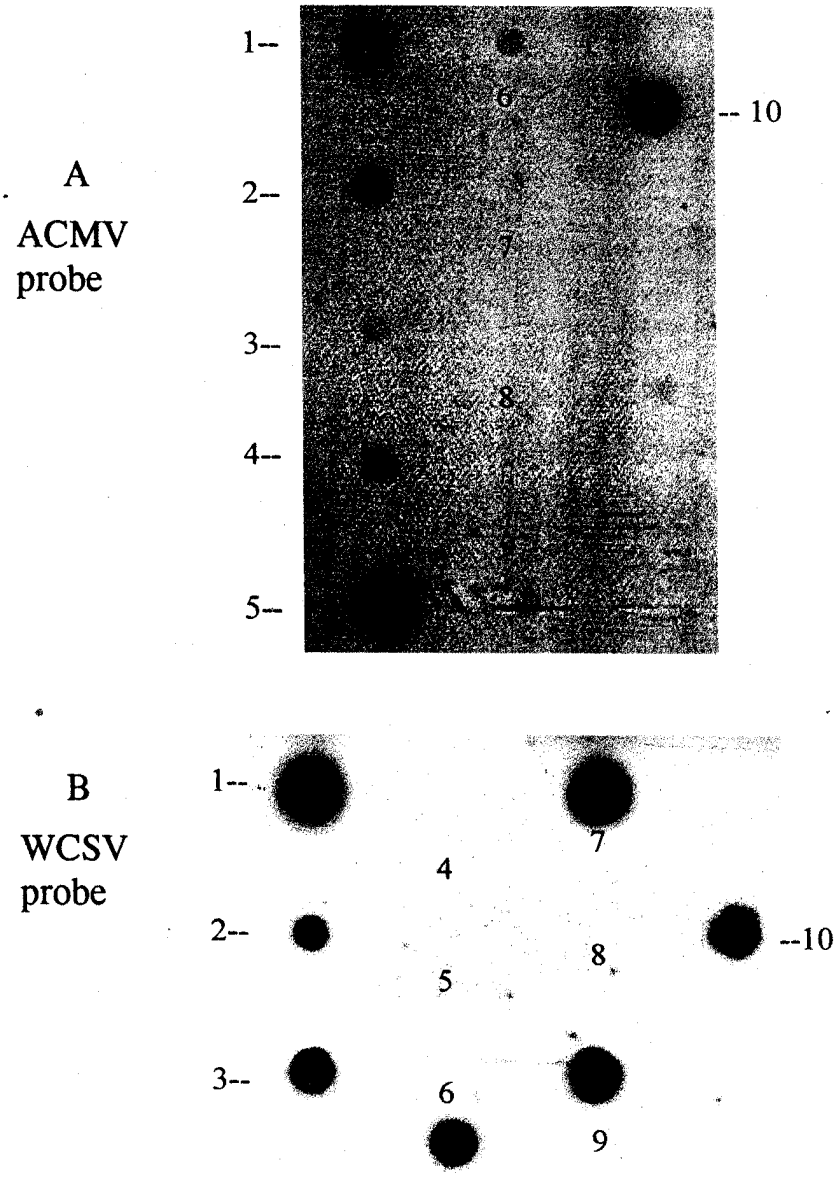

Fig. 2: Dot blot hybridization tests, radioactively labeled DNA A of ACMV (A) & WCSV (B) was used as a probe. In put samples were the total DNA (10 µg/sample) extracted from (A), PLCV infected *N. benthamiana* (1), AgYVV infected *D. stramonium* (2), EPYVV infected *E. prostata* (3), SYLCV, ACMV & EPYVV infected *N. benthamiana* (4, 5 & 6) respectively, healthy *N. benthamiana*, tomato and *Ageratum* (7, 8 & 9) respectively, ZLCV infected tomato (10). (B), WCSV infected watermelon (1), CLCuV infected *N. tabaccum* (2), SYLCV infected and healthy *N. benthamiana* (3 & 4) respectively, EPYVV infected *E. prostata* (5), PLCV infected *N. benthamiana* (6), ZLCV infected and healthy tomato (7 & 8) respectively, AgYVV infected *D. stramonium* (9) ACMV infected *N. benthamiana* (10). Washing was done under medium stringent conditions.

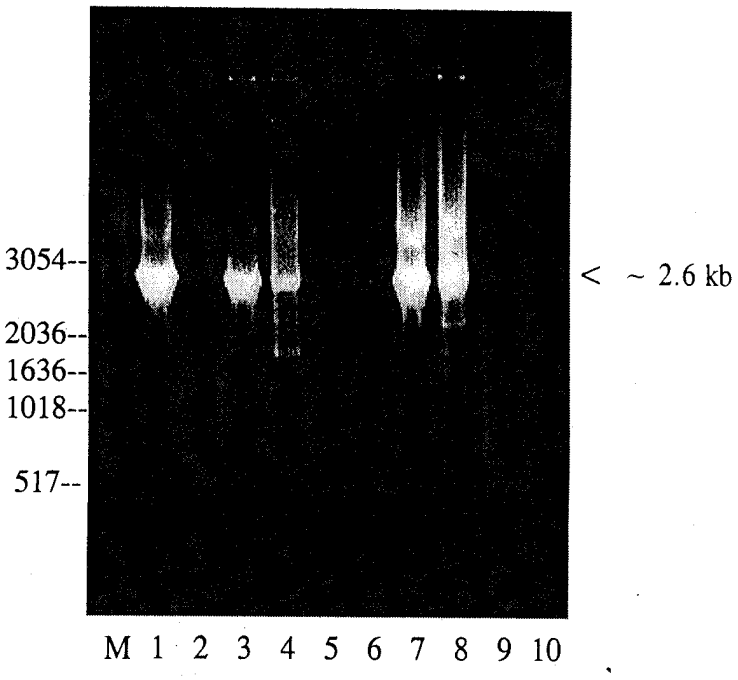

Fig. 3: Ethidium bromide stained agarose 1% gel, samples were the amplification products resulting from PCR reactions containing nucleic acids extracted from ACMV infected *Nicotiana benthamiana* (lane 1), healthy *N. benthamiana* (lane 2), ZLCV infected *Lycopersicon esculentum* (lane 3), AgYVV infected *Ageratum conyzoides* (lane 4), healthy tomato and *Ageratum* (lane 5 & 6), SYLCV and PLCV infected *N. benthamiana* (lane 7 & 8) respectively, EPYVV infected and healthy *N. benthamiana* (lane 9 & 10) respectively. The sizes (bp) of co-electrophoresed markers are given. PCR reaction conditions and sample preparation have been described in materials and methods.

Much of the information on begomoviruses has come from time-consuming studies using virus-vector-host complexes to define pathogen-insect relationships and host ranges. Such information is crucial to developing strategies for disease control. Biochemical characterization and investigations of fundamental aspects of begomoviruses require the establishment and maintenance of homogenous virus cultures. The need for routine serial transfer of viruses from plant to plant using the whitefly vector has complicated and delayed the investigation of many whitefly-transmitted viruses. Many begomoviruses are not mechanically transmissible and thus must be manipulated exclusively through the use of the whitefly vector. On the other hand several viruses used routinely in molecular laboratories (eg ACMV, TGMV, BGMV, AbMV) are no longer insect transmissible because of continuous laboratory culture.

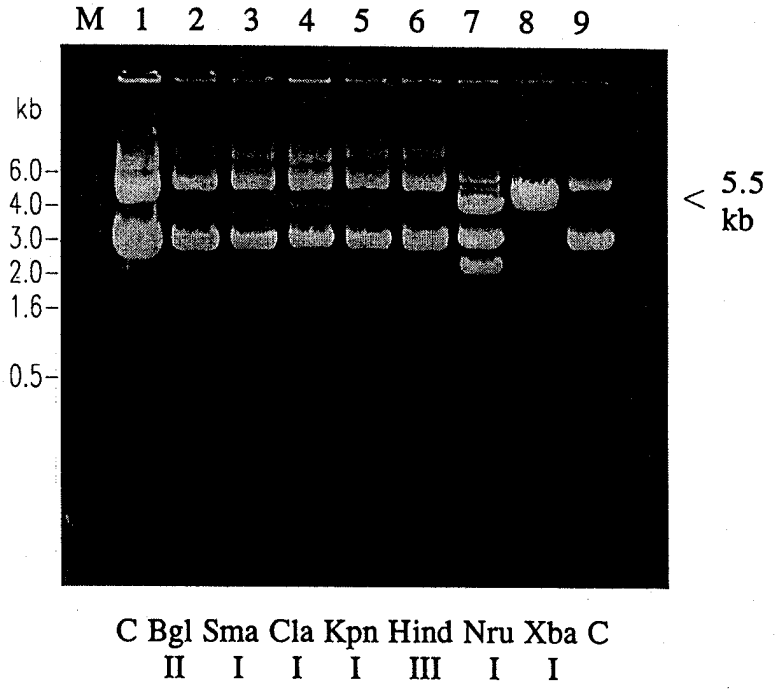

Fig. 4: Ethidium bromide stained agarose gel, samples were the products of pGEM-T vector cloned DNA A genome of SYLCV, digested with Bg1 II (lane 2), Sma I (lane 3), Cla I (lane 4), Kpn I (lane 5), Hind III (lane 6), Nru I (lane 7), Xba I (lane 8), undigested controls (lane 1 & 9). In all the lanes 10 µl of the sample was loaded, except in lane 1, where 20 µl was loaded. Arrow marks the position of band ~5.5 kb in size i.e., a product of a single cut by restriction enzyme Xba I (lane 8). The sizes (bp) of co-electrophoresed markers are given.

Such detailed assessment of the biology and molecular biology may help in designing control measures. For example, in geminiviruses, interference with replication by defective-interfering viral genomes may be a likely method for controlling them (Stanley *et al.*, 1990) and since subgenomics occur naturally they may account for the appearance of milder than normal symptoms or attenuation of symptoms in certain hosts.

Diagnosis of begomoviruses requires a laboratory assay whose results must be corroborated by tedious biological characterization. A time-consuming, multifaceted diagnostic approach is essential, particularly with previously unrecognized begomoviruses. Improved diagnostic technologies based on fundamental knowledge of the composition and organization of viral genomes and on virus-encoded polypeptides are needed.

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