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Detection and Relationship among Begomoviruses from Five Different Host Plants, Based on ELISA and Western Blot Analysis

MUHAMMAD SALEEM HAIDER, SIJUN LIU, W. AHMED, ADRIAN A. F. EVANS AND PETER G. MARKHAM

School of Biological Sciences, University of the Punjab, New Campus, Lahore, Pakistan (MSH), John Innes Centre, Norwich Research Park, Colney Lane, Norwich. UK. NR4 7UH (SL, PGM), Plant Virology Section, Ayub Agricultural Research Institute, Faisalabad. Pakistan (WA) and Imperial College at Silwood Park, Ascot . Berks. UK. SL5 7PY (AAFE)

Abstract.- Five different species of plants were found infected with begomoviruses in and around the cotton fields in Punjab, Pakistan. Only Solanum yellow leaf curl virus (SYLCV) infecting *Solanum nigrum* was mechanically transmissible to *Nicotiana benthamiana*. However, all the five were graft transmissible. All the virus isolates were found to be serologically related to african cassava mosaic virus (ACMV) when analyzed using western blot. The use of indirect ELISA was necessary for accurate serotyping. The serological reactivities of particles of all the virus isolates were reliable and consistent regardless of the host factor, from which they were extracted. On the basis of the serological differentiation indexes (SDI) using polyclonal antisera, three isolates have been delineated as distinct begomoviruses namely Ageratum yellow vein virus-Pakistan (AgYVV-PK), Solanum yellow leaf curl virus (SYLCV) and *Eclipta prostrata* yellow vein virus (EPYVV). An isolate from *Zinnia elegans* was confirmed as a strain of AgYVV-PK and an isolate from *Capsicum annuum* was identified as strain of SYLCV. On the basis of the present study it was found that AgYVV and SYLCV were more closely related to cotton leaf curl virus (CLCuV) than were either ACMV or EPYVV.

Key words: Begomoviruses, Serology, ELISA, Western blot.

INTRODUCTION

In Pakistan, cotton leaf curl viral (CLCuV) disease has become a serious cause of crop loss during the last decade. The area of cotton (*Gossypium hirsutum*) affected by the disease increased from about 60 hectares in 1988-1989 to almost 900,000 hectares in 1993-1994, when yield losses in many fields exceeded 80% (Ali *et al.*, 1995) and the country's economy was seriously affected. The disease is caused by a whitefly-transmitted geminivirus (Mansoor *et al.*, 1993).

Begomoviruses have geminate virus particles, a circular, single stranded DNA genome with coat protein amino acid similarities of 80-95% and all are transmitted by *Bemisia tabaci*. It has been suggested that a single epitope, detectable using a monoclonal antibody, may be the structure that determines whitefly transmissibility (Thomas *et al.*, 1986). Tests with polyclonal antisera have shown that the particles of several begomoviruses are antigenically related to one another (Roberts *et al.*, 1984).

The present study compares the serological relationships of the Pakistani isolates of begomoviruses infecting different host plant species in and around the cotton fields in Punjab, Pakistan.

Enzyme linked immunosorbant assay (ELISA) has been used extensively for the detection and differentiation of begomoviruses (Swanson *et al.*, 1992; Muniyappa *et al.*, 1991; Harrison *et al.*, 1991a,b; Aiton and Harrison, 1989; Thomas *et al.*, 1986; Roberts *et al.*, 1984). Polyclonal antisera raised against most whitefly-transmitted geminiviruses are probably suitable for virus detection by serologically specific electron microscopy, ELISA, and western blot analysis. Indirect antigen coated plate (ACP) ELISA (Pinner *et al.*, 1988) was used in the present studies.

MATERIALS AND METHODS

Virus source and maintenance

Plants showing symptoms of virus infection

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were collected from vicinity of the Districts, Multan and Bahawalpur in Punjab. Samples of these plants were brought to the United Kingdom and studies conducted at John Innes Centre, Norwich. The viruses were maintained on the original/wild host plants or suitable alternate host plants (Table I).

Virus purification

In the present studies some established procedures for the purification of begomoviruses were used with minor modifications, because the newly introduced viruses were suspected to be infected with begomoviruses (Haider *et al*., 2002).

Each virus was tested by using ACP-ELISA, using virus preparations as the antigen. Healthy plant sap or 1 X PBS was used as negative control.

Production of antisera

For the production of antisera for all five virus isolates 300 µL of each virus preparation was thoroughly mixed with an equal volume of Friend's complete adjuvant (Sigma) and injected subcutaneously into a rabbit New Zealand White. A second injection with 150 µL of the virus preparation mixed with an equal volume of Friend's incomplete adjuvant was given two weeks later using the same method. Samples of 5-10 mL of blood were taken from the rabbits a week after the second injection. Antisera were prepared using the method of Harlow and Lane (1988). The injections and test bleeding continued at 2 week intervals until the titers were high enough, the rabbits were bled finally. The titers of the bleeds of the antisera were tested using ACP-ELISA against purified virus preparations. Polyclonal antisera of ACMV and CLCuV were very kindly provided by Sijun Liu and Marrion Pinner (John Innes Centre) respectively.

Antigen coated plate (ACP)-ELISA

ACP-ELISA was performed as described by Pinner *et al.* (1988). Purified virus diluted 1:100 in phosphate-buffered saline (PBS) pH 7.4 was directly absorbed to the wells of microtitre plates (Falcon microtitre III) followed by blocking of remaining reaction sites by incubation with 10g/L bovine serum albumin (BSA) in PBS+0.5 ml/L Tween-20. Twofold serial dilutions of the antisera were made and bound antibody was detected with goat anti-rabbit serum conjugated to alkaline phosphatase (Sigma) at the recommended dilution. The bound conjugate was detected by addition of p-nitrophenyl phosphate at 1 mg/mL in diethanolamine buffer pH 9.8. A Titertek Multiscan Photometer (Flow laboratories) was used to measure the absorbance at 405 nm (A_{405nm}).

Determination of serological differentiation index (SDI) values

The serological relatedness of each virus was expressed by an SDI (Jaegle and van Regenmortal, 1985). The SDI value for each virus was calculated by comparing the number of twofold dilution steps between samples of homologous and heterologous isolates that gave an absorbance value of 1.0 (Jaegle and van Regenmortal, 1985). These chosen values were a minimum of three times the background value (Sutual *et al*., 1986).

Virus preparations were diluted 1:50, 1:100, and 1:200 in PBS, pH 7.0 and their reactions against homologous and heterologous antisera assessed by ACP-ELISA. All the polyclonal antisera (titre $2x10^5$) were diluted as follows: (1) 1/256000; (2) 1/128000; (3) 1/64000; (4) 1/32000 (5) 1/16000; (6) 1/8000; (7) 1/4000; (8) 1/2000; (9) 1/1000; (10) 1/500.

Protein analysis techniques

SDS-PAGE

The SDS-polyacrylamide gel electrophoresis (PAGE) gels were prepared using a discontinuous system described by Laemmli (1970). The gels were prepared using Bio-Rad Miniprotein II gel apparatus.

Western blot analyses

Western blot analyses were based on the method described by Blake *et al.* (1984). After transfer, the nitrocellulose membranes were immediately blocked by washing for 60 minutes in 2% (w/v) Marvel (Instant dried skimmed milk, The Boots Company) in 1x PBS (consisting of 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4 pH 7.2).

Table I.- **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 II.- **SDI values in vertical direction, as calculated in response to the reaction from seven virus isolates against each individual antiserum.**

		Antisera						
	VIRUS Isolates	**ACMV -SDI-**	**ZiLCV -SDI-**	**EPYVV -SDI-**	**SYLCV -SDI-**	**PeLCV -SDI-**	**AgYVV -SDI-**	**CLCuV -SDI-**
Antigen	**ACMV**	0.0	2.2(2)	3.9(4.2)	4.1(4.2)	2.6(2.5)	2.8(2.7)	3.1(3.2)
	ZiLCV	1.8(2)	0.0	1.8(2.1)	3.2(3.3)	1.2(1.4)	1.0(.9)	1.2(1.2)
	EPYVV	4.6(4.2)	2.4(2.1)	0.0	2.6(2.5)	3.2(3.0)	2.0(1.9)	3.6(3.4)
	SYLCV	4.3(4.2)	3.5(3.3)	2.5(2.5)	0.0	0.8(.9)	3.6(3.7)	2.0(1.8)
	PeLCV	2.4(2.5)	1.7(1.4)	2.9(3.0)	1.0(.9)	0.0	2.4(2.4)	1.6*
	AgYVV	2.7(2.7)	0.8(.9)	1.8(1.9)	3.8(3.7)	2.4*	0.0	1.4(1.3)
	CLCuV	3.4(3.2)	1.2(1.2)	3.3(3.4)	1.6(1.8)	1.6(1.6)	1.2(1.3)	0.0

SDI values for each homologous antigen/antiserum was zero. Values given in brackets are the average of two reciprocal reactions.
* Value taken from one way reaction i.e. no reciprocal reaction.

RESULTS

Serological variability of virus preparations

Each virus preparation was tested at a saturating concentration. Figure 1 shows virus preparations subjected to PAGE followed by western blot. The preparations that reacted most strongly in ELISA, however, did not contain the great amount of coat protein as indicated by the intensity of the reaction with the protein bands lane 4 and 10 (coat proteins, Fig. 1). These tests suggested that although individual virus preparations varied in concentration, this variability could be reduced by careful standardization of the procedure.

There was only weak reaction to healthy plant sap (very low back ground), which confirmed the quality (specificity) of the antisera. All the reactions that reached or exceeded the standard A^o value of 1.0 and three times the back ground level were considered to be positive. SDIs of two were considered to be significant (Jaegle and van Regenmortal 1985).

The mean absorbance values (A°) of negative controls ranged from 0.086 (1x PBS) to 0.389 (Healthy plant sap). Table II summarizes the results. The ZiLCV antiserum strongly reacted with AgYVV (SDI of 0.8), this data coincides with the results from coat protein sequence analysis (Haider, 1996). The reaction of AgYVV antiserum to CLCuV and ZiLCV (SDI of 1.2 and 1.0, respectively) was homologous (as compared to other viruses tested) and shared more than one epitope(s) however, AgYVV antiserum reacted relatively distantly with EPYVV (SDI of 2.0). SYLCV showed a close relationship with PeLCV and then with CLCuV (SDI of 1.0 and 1.6, respectively), all the rest of the viruses indicated a distant relationship. The reaction of the PeLCV antiserum showed that ACMV and EPYVV differed significantly from PeLCV, suggested that different epitopes might be recognized and that these were minor, as the SDIs were relatively high (2.6 and 3.2, respectively).

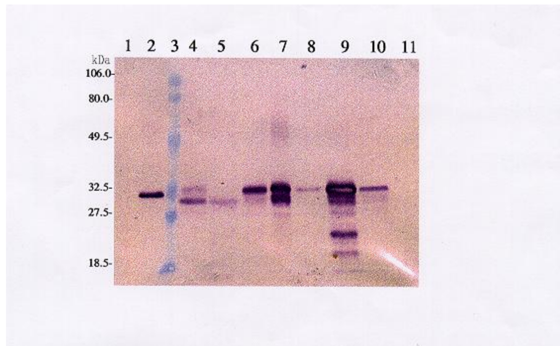

Fig. 1. Western blot analysis of the begomoviruses from Pakistan. ACMV polyclonal antiserm was used as labelling antibody, samples of the purified virus preparations were used in the lanes except otherwise mentioned. Healthy *D. stramonium* plant sap (lane 1), ACMV infected *N. benthamiana* (lane 2), Marker (lane 3), ZiLCV infected tomato (lane 4) EPYVV infected *E. prostrata* (lane 5), SYLCV infected *N. benthamiana* (lane 6) SYLCV infected tomato (lane 7), PeLCV infected *D. stramonium* plant sap and virus preparation (lane 8 & 9) respectively, AgYVV infected *A. conyzoides* (lane 10), Healthy plant preparation from tomato (lane 11). Dublets in lane 7 and 9 are due the degraded proteins because the same virus sample gave single band in lane 6 and 8 respectively, in 1st case the difference lies with the host plant and change of buffer, however, in the 2nd case coat protein degraded during purification procedure (lane 9) i.e. sample from plat sap produces singe band although the intensity was light (lane 8).

AgYVV antiserum produced a significantly stronger reaction to ZiLCV and CLCuV (SDIs of 1.0 and 1.2, respectively) when compared to ACMV and PeLCV (SDIs of 2.8 and 2.4, respectively), data from nucleic acid hybridization test also suggests similar relationships among these viruses (Haider *et al*., 2003b). CLCuV antiserum also showed a distant relationship to ACMV and EPYVV (SDIs of 3.1 and 3.6, respectively) [a common characteristic to most of the antisera from Pakistan viruses] and close relationships with ZiLCV and AgYVV (SDIs of 1.2 and 1.4, respectively.

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*., 2003a; Haider *et al*., 2002) respectively, that support our present findings.

The results showed that serological differences amongst the viruses tested could be distinguished. Most of the Pakistan viruses reacted with the ACMV antiserum in a heterologous manner, which suggested that a common epitope was being recognized and this epitope was a major one masking any specific reaction to ACMV.

Virus preparations reacted well using the indirect ACP method by binding directly to the plate without interference from non-specific proteins. The indirect ACP-ELISA procedures reported to be the most useful methods for serotyping the MSV isolates (Jaegle and van Regenmortal, 1985; Pinner *et al*., 1988).

Data obtained from the reciprocal tests, while using very sensitive ELISA techniques clearly showed that all the viruses react with different antisera to varying degrees. Furthermore, the ACMV and EPYVV antisera revealed distant relationships with most of the viruses tested.

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, Chilies 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.

Different begomoviruses infecting the same host plant species in different countries have different epitope profiles, whereas distinct begomoviruses which have different host ranges but occur in the same geographical area show a general similarity in epitope profile (Harrison *et al*., 1991a, b). All the begomoviruses from Pakistan gave better response to the antisera from Pakistan viruses in

comparison to ACMV indicated the geographical impact in the structural integrity of the coat protein of these viruses. A similar type of result has been reported for other begomoviruses in different countries, even among those infecting the same host plant species. Isolates of okra leaf curl virus from West Africa have very different epitope profiles from those of 'Bhindi' (okra) yellow vein mosaic virus (BYVMV) from India (Harrison *et al.*, 1991a, b). Similarly, the epitope profile of AYVV infecting ageratum and tomato in Singapore differed from the profiles of ageratum and tomato infecting begomoviruses from India. Indeed, the epitope profile of Indian *A. conyzoides* virus closely resembles that of Indian tomato leaf curl begomovirus, and provides evidence that *A. conyzoides* is a field host of this virus (Muniyappa *et al.*, 1991).

All the begomoviruses for which vectors are known are transmitted by *B. tabaci.* Moreover the vector specificity of different genera of the family geminiviridae seems to be determined by the specificity of their coat protein (Briddon *et al.*, 1990; Roberts *et al.*, 1984). This raises the question that if *B. tabaci* may occur as different biotype in different geographical regions (Bedford *et al.*, 1994), could each biotype be acting to select virus isolates in the same area to give a coat protein that has structural features particularly suited for transmission by that biotype (Haider *et al.*, 2003a). If this situation exists, both the differences in epitope profile of viruses causing the same diseases in different regions, and the epitope similarity of different viruses occurring in the same region, could readily be explained.

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