Pak.J.Bot., 31(1): 115-123, 1999.

RAPID MULTIPLEX PCR FOR THE SPECIFIC DETECTION OF TWO WHITEFLY-TRANSMITTED GEMINIVIRUS SPECIES ASSOCIATED WITH COTTON LEAF CURL DISEASE IN PAKISTAN

SHAHID MANSOOR, AFTAB BASHIR, SULTAN H. KHAN, MAZHAR HUSSAIN, M. SAEED, YUSUF ZAFAR, PETER G. MARKHAM*, AND KAUSER A. MALIK

National Institute for Biotechnology and Genetic Engineering (NIBGE), P.O. Box 577, Jhang Road, Faisalabad, Pakistan.

Abstract

Cotton leaf curl disease in Pakistan is associated with two whitefly-transmitted geminivirus species named cotton leaf curl virus Pk1 (CLCuV-Pk1) and cotton leaf curl virus Pk2 (CLCuV-Pk2). PCR is a highly specific and reliable technique for the detection of geminiviruses. A protocol has been developed for rapid isolation of a suitable template for PCR. The method is based either on the adsorption of DNA template from cleared lysate on PCR tubes or a rapid minipreparation of total DNA by CTAB method. Similarly, a simplified protocol is used for the isolation of total DNA from individual whitefly which is suitable for PCR amplification. Primers have been designed in such a way that the two geminivirus species are amplified in a single tube by multiplex PCR. In this PCR virus sense primer is common to both viruses in the rep gene whereas the complementary sense primer is specific for either of the two viruses. For CLCuV-Pk1, the reverse primer is designed at the start of C4 ORF gene whereas for CLCuV-Pk2 the primer is designed at the start of rep gene. The two PCR products of about 360 bp (CLCuV-Pk1) and 510 bp (CLCuV-Pk2) are resolved on an agarose gel. A rapid profile for multiplex PCR was used and completed in 2 hr. The whole process of template preparation, PCR and the detection of PCR product by agarose gel electrophoresis is completed in a single day. The protocol has been used reliably for the detection of cotton geminiviruses in plant and whitefly samples collected from the field.

Introduction

Leaf curl disease of cotton is causing heavy losses to the cotton crop in Pakistan. It is estimated that during the last five years the disease has resulted in a loss of 7.4 million bales of cotton with an estimated value of US$ 4.98 billion. Recently, the disease has spread to Sindh which was previously free of the disease (Mansoor *et al*, 1998). The disease is associated with a whitefly-transmitted geminivirus (Mansoor *et al*, 1993; Hameed *et al.*, 1994). It has been found that the leaf curl disease of cotton is associated with variable geminiviruses (Zafar *et al.*, 1997; Zhou *et al.*, 1998). Two of these viruses which are most diverse have been named as cotton leaf curl virus Pk1 (CLCuV-Pk1) and cotton leaf curl virus Pk2 (CLCuV-Pk2) (Zafar *et al.*, 1997) and correspond to CLCuV-Pk type 26 and CLCuV-Pk type 72b, respectively as described by Zhou *et al.* (1998). This is indeed a unique example where four variable

*Department of Virus Researches, John Innes Center, Norwich, NR4 7UH, U.K.

geminiviruses are associated with the same disease in the same geographical area. It is essential to know the distribution of these viruses in different cotton growing areas as well as their presence in different cotton genotypes, alternate hosts and whiteflies. Such studies require rapid and sensitive method for the detection of these geminiviruses.

Methods used for the detection of plant viruses are based either on the detection of viral proteins or viral nucleic acids. Although ELISA has been reported to detect geminiviruses (Pinner & Markham, 1990) but due to highly conserved nature of coat protein among whitefly-transmitted geminiviruses, polyclonal antisera raised against the virus are often cross-reactive and cannot differentiate whitefly-transmitted geminiviruses. Methods based on the detection of viral nucleic acids such as nucleic acid hybridization, PCR and DNA sequence analysis provide precise information about the identity of plant viruses (Maule *et al.*, 1983; Padidam et *al.*, 1995; Rybicki *et al.*, 1990). PCR is a highly sensitive and reliable technique which has been widely used for the detection of geminiviruses. The specificity of PCR depends on the design of PCR primers. Degenerate primers based on highly conserved sequences of DNA-A or DNA-B can be used to amplify all whitefly transmitted geminiviruses (Rojas *et al.*, 1993; Briddon *et al.*, 1994; Wyatt & Brown, 1996). On the other hand primers based on non-conserved sequences can be used to amplify a particular virus species or strain (Rybicki *et al.*, 1990; Briddon *et al.*, 1994, McGovern *et al.*, 1994). Ideally, the size of PCR product should range from 300-500 bases for routine diagnostic purposes. However, routine application of PCR is limited because of the requirement of suitably pure DNA for PCR and the time required for PCR as well as analysis of PCR product. The other factor is the cost of PCR-based tests.

In this study we have used a simple protocol for the isolation of template suitable for PCR and the design of multiplex PCR such that the whole procedure of template preparation, PCR and agarose gel electrophoresis is completed in a single day. The procedure requires minimum manipulation and is suitable for less sophisticated laboratories.

Materials and Methods

Plant and whitefly samples: Total DNA isolated from tobacco plant infected with Nigerian strain of African cassava mosaic virus (ACMV), and tomato plant infected with tomato yellow leaf curl virus (TYLCV) from Israel was kindly provided by Marion Pinner, John Innes Centre, Norwich, UK. Total DNA was isolated from cotton plant infected with cotton leaf curl virus (CLCuV) from Pakistan by CTAB method (Doyle & Doyle, 1987). Infected cotton samples were collected from fields in 1996 and 1997 from cotton growing districts of Punjab. White flies (*Bemisia tabaci*) were collected either from a Pakistani colony maintained on CLCuV infected cotton plant or infected cotton plants growing in the field.

Design of PCR primers sequences: Conserved sequences in the two geminiviruses were identified in rep gene and a primer CLCuV-V2091 (5'-CTGTCTAGATTTG CATTTAAATTATGAAATTG-3') in the viral sense was designed. This corresponds to nucleotide no. 2091 to 2123 both in CLCuV-Pk1 and CLCuV PK2 (Zafar *et al.*, 1997).

The underlined sequence is the *Xba*I restriction site. A primer specific for CLCuV-PK1 was designed at the start of C4 ORF in the complementary sense and is called CLCuVPk1-C2442 (5-CGAccATGGGAGCCCTCATCTCCATGTGC-3') and correspond to nucleotide no. 2442 to 2413 of CLCuV-Pk1 (Zafar *et al.*, 1997). The lower case letters show nucleotide changes to create a *Nco*I site. Similarly, a primer specific for CLCuV-PK2 was designed at the start of rep gene in the complementary sense and is called PCL2 (5'-CATGCCTCCAAAGCGGAACGGTATTTATT-3') and corresponds to nucleotide no. 2601 to 2572 (Zafar *et al.*, 1997). Primers specific for CLCuV-PK1 were expected to amplify a product of 360 bp whereas primers specific for CLCuV-Pk2 were expected to amplify a product of 510 bp. The primers were custom synthesized by Life Technologies, USA.

Direct PCR detection of cotton geminiviruses in leaf extract: Fresh or dried tissue was ground in the buffer used for particle purification of cotton leaf curl virus (Zeidan & Czosnek, 1991). The composition of buffer was; 100 mM sodium citrate pH 6.0, 18.5 mM ascorbic acid, 60 mM sodium sulfite, 1% 2- mercaptoethanol and 5 mM EDTA. A small leaf (0.2 - 0.3 g) was ground in a pestle and mortar with 5 ml of extraction buffer. The extract was centrifuged in a microfuge tube for 10 min., at 10,000 g and 50 μl of the extract was transferred to an autoclaved polypropylene PCR tube. The tube was incubated on ice for 20-30 min. and then washed three time with 200 μl of the same buffer and finally with distilled water. The PCR solution mixture was added to the dried tube and was directly used for PCR amplification.

Minipreparation of total nucleic acid: A small leaf (0.2 - 0.3 g) was ground in CTAB buffer used for plant DNA extraction (4) and the volume of buffer was adjusted to 750 μl. The extract incubated at 60°C for 30 min., was extracted with chloroform, isoamyl alcohol (24:1). Supernatant fluid was removed by centrifugation at 14,000 g for 5 min, in a microcentrifuge. Total DNA was precipitated by adding 2/3 volume of isopropanol to the supernatant which was kept on ice for 30 min. Microfuge tubes were centrifuged at 14000 g for 10 min., and was washed with 70% ethanol. The pellet was dissolved in 200 μl distilled water.

DNA extraction from a single whitefly: Total DNA was isolated from individual whiteflies as described by Zeidan & Czosnek (1991). In the modified simple procedure individual whiteflies were ground in 50 μl of DNA extraction buffer (50 mM Tris. HCl (pH 8.0), 0.5 % Tween 20, 0.25 mg/ml Proteinase K) and were incubated at 65°C for 2 h. After incubation, 5 μl of the extract was used directly in a 50 μl PCR reaction or was denatured for 5 min., at 94 °C before adding to the PCR mixture. To check that DNA template suitable for PCR had been extracted, DNA isolated from individual whiteflies was also amplified by random primer no. OPJ-10 (Operon Technologies, USA) as described previously (Iqbal *et al.*, 1997).

PCR Amplification:. The PCR mixture contained 200 μM of each nucleotide dATP, dCTP, dGTP, and dTTP, 5 μM of forward primer, 5 μM of two reverse primers and 2 mM $MgCl_2$. The total volume of PCR reaction was 20 μl. Amplification was performed in a Perkin Elemer thermal cycler using a profile of 30 sec at 94°C, 30 sec at 55°C and 30 sec at 72°C. Total DNA isolated from ACMV, TYLCV and TLCV (PK) infected plants were used to check the specificity of primers whereas total DNA isolated from

CLCuV infected cotton was used as a positive control. The conditions for PCR with random primers were those described previously (Iqbal *et al.*, 1997). PCR products were analysed on 1% agarose gel in TBE buffer.

Results

Specificity of PCR primers: The primers were tested for their specificity. There was no amplification of viral DNA extracts from ACMV, TYLCV and TLCV-Pk. The expected size PCR product was obtained from their respective clones only, indicating that PCR primers are specific for the the two viruses (Fig.1).

Amplification of two geminivirus species from field collected cotton samples: The primers were tested for the presence of two whitefly-transmitted geminivirus species in cotton samples collected from the field. Both viral species were detected either as single infection or double infection (Fig. 2). The intensity of the two PCR product bands was similar, indicating that primers can readily amplify both viruses with the PCR conditions used.

PCR amplification from leaf extract: DNA extracted from infected leaves by grinding leaves in virus extraction buffer was tested for the amplification of two geminivirus

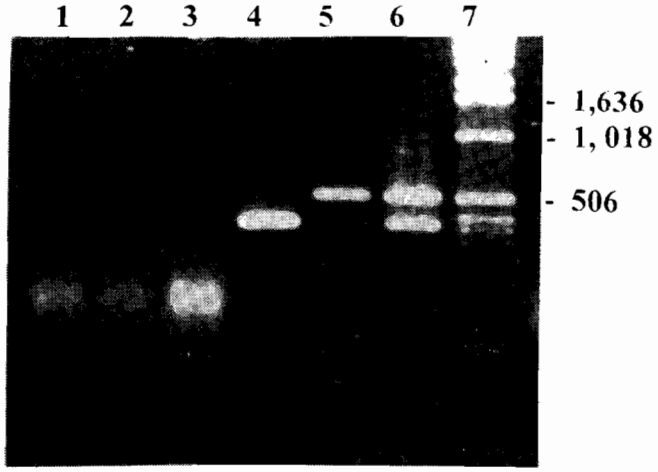

Fig.1. Analysis of specificity of PCR primers for the amplification of CLCuV-Pk1 and CLCuV-Pk2.
The lower band is the PCR product from CLCuV-Pk1 (360 bp) with primers CLCuV-V2091 and CLCu-VPk1-C2442 while the upper band is PCR product from CLCuV-Pk2 (510 bp) with primers CLCuV-V2091 and PCL2. No amplification in the first three lanes by multiplex PCR : Lane 1, ACMV (Nigerian isolate; Lane 2, TYLCV from Isreal; Lane 3, TLCV-PK; Lane 4, CLCuV-Pk1 amplification from a full-length clone; Lane 5 CLCuV-Pk2 amplification from CLCuV-Pk2 clone, Lane 6 amplification of two geminiviruses from a plant known to be infected with the two cotton geminiviruses Lane 7, Kb Lane 8 non-infected cotton plant.

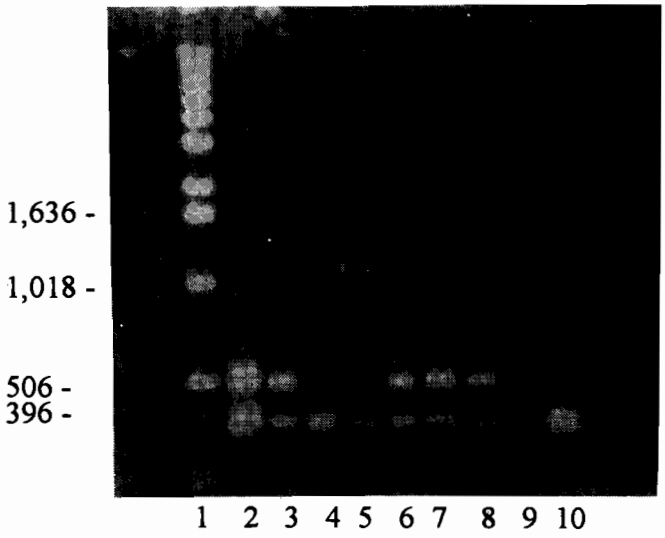

Fig.2. Amplification of two cotton geminivirus species CLCuV-Pk1 and CLCuV-Pk2 from leaf extract adsorbed on PCR tubes by multiplex PCR. Lane 1, kb ladder; lane 2-10, Various cotton samples collected from the field. The lower band corresponds to CLCuV-Pk1 while upper band represents CLCuV-Pk2.

species. Both viruses were detected by PCR using immobilized PCR template on the walls of the microfuge tubes. Incubation on ice of leaf extract for 20-30 min., was enough for DNA immobalization and both geminivirus species could be detected by PCR (Fig. 3).

PCR amplification from single whiteflies: The modified protocol was used successfully for the extraction of DNA from single whiteflies. The extract was used directly for virus amplification and PCR products of expected sizes were obtained from whiteflies collected from the field (Fig.4). These results showed that the simplified procedure gave a template suitable for PCR.

Discussion

The present report describes the design of multiplex PCR such that the two cotton geminivirus species are specifically amplified in the same tube and are distinguished in the agarose gel on the basis of size. Use of this PCR protocol with a simple template preparation method provides a rapid tool for the detection of two geminivirus species found associated with cotton leaf curl disease in Pakistan. We have tried to overcome some of the problems which are normally encountered while doing PCR on a routine basis such as inhibition of PCR by interfering compounds, time and cost of PCR. Inhibitory compounds present in plant extracts such as polysaccharides and phenolic compounds especially in samples collected from the field often cause PCR inhibition.

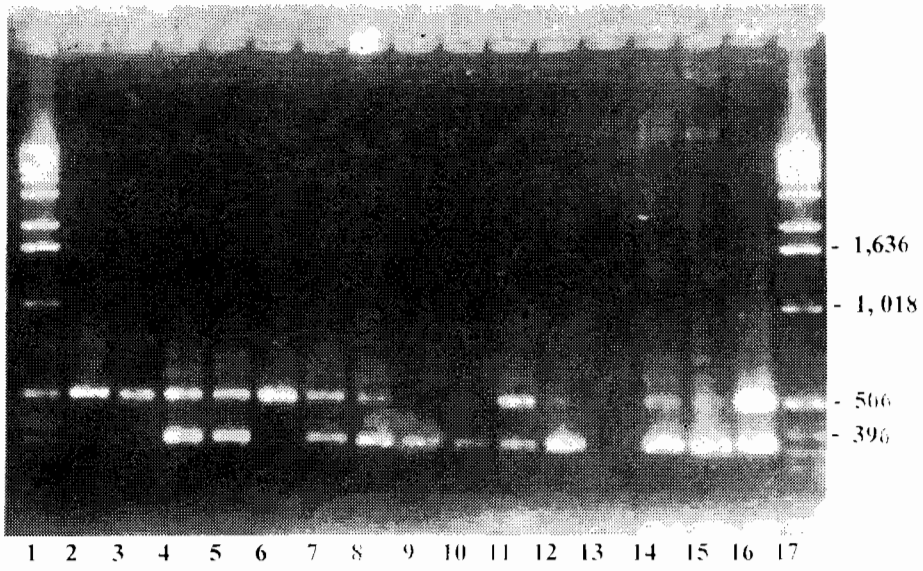

Fig.3. Amplification of CLCuV-Pk1 and CLCuV-Pk2 by multiplex PCR from DNA isolated by modified CTAB method. Lane 1 and 17 kb ladder; Lane 2 to 16 various diseased cotton samples collected from cotton growing areas of Pakistan.

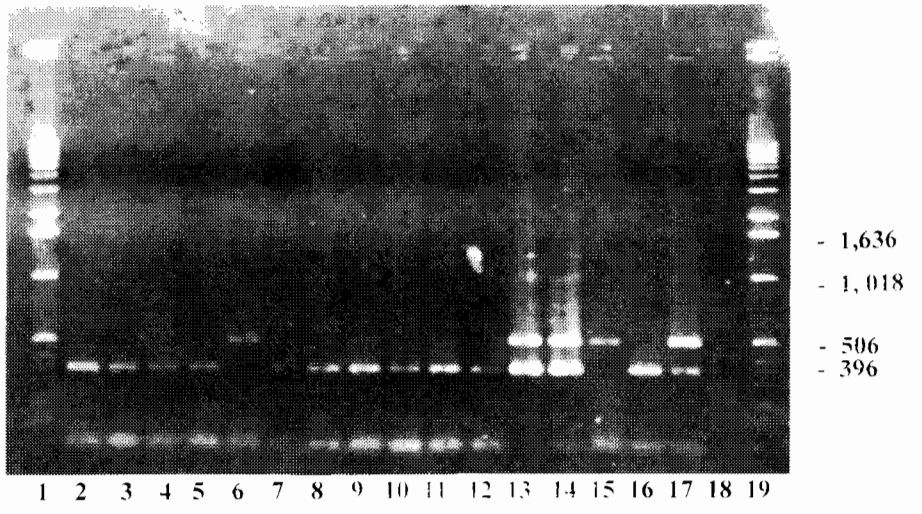

Fig.4. Amplification of two cotton geminivirus species by multiplex PCR. Lane 1 Kb ladder; Lane 2 -5, DNA isolated from individual whiteflies from a colony maintained on diseased cotton plant; lane 6 to 17, individual whiteflies collected from the field; lane 19 kb ladder.

For plant samples tissue was ground in a buffer which is used for the isolation of virus particles. This buffer uses antioxidants which prevent oxidation of phenolic compounds and thus avoid inhibition of PCR. Using this protocol both virus particles and viral DNA is released which are probably adsorbed on the PCR tube. The protocol is simple and is suitable for field studies or laboratories with less sophisticated equipment. Wyatt & Brown (1996) have used a similar protocol for the detection of geminiviruses where tissue is ground in TE buffer. The use of virus purification buffer was more reliable for cotton than TE buffer (data not shown), probably because of prevention of oxidation of phenolic compounds present in cotton plants. We have also used DNA minipreparation by CTAB method for the isolation of DNA suitable for PCR. This method requires more time than DNA adsorption on tubes but is more desirable as DNA can also be used for some other techniques such as dot-blot hybridization or stored for future use.

Whiteflies are important sample source of virus in epidemiological studies as well as virus transmission studies. The protocol is available for the isolation of DNA from single whiteflies used SDS (Zeidan & Czosnek, 1991). We have used Tween 20, a non-ionic detergent instead of SDS in DNA extraction buffer. This detergent is compatible with Taq polymerase and thus does not require DNA precipitation or phenol-chloroform extraction as has been done previously (Zeidan & Czosnek, 1991). The inactivation of Proteinase K by heat was not necessary when the sample is used directly for PCR. Proteinase K is inactivated by heating for 5 min., at 94°C before the start of PCR cycles. We have also checked the suitability of DNA for PCR by random primers which indicated that DNA isolated by this method is equally suitable for PCR.

We have used a simple PCR profile such that the PCR is completed in 2 hrs. This is achieved by designing primers which amplify part of viral genome while maintaining specificity and sensitivity of PCR. This protocol will be particularly useful where large number of samples are required to be tested for the presence of cotton viruses. Moreover, amplification of products of smaller size increase the efficiency of PCR. We have tried to reduce the cost of PCR by designing PCR primers which can amplify both viruses in a single tube. The cost is further reduced by PCR amplification in a total volume of 20 μl. The method has been used successfully for the detection of cotton geminiviruses from field collected cotton and whitefly samples. Since there are many uncharacterized geminiviruses found in the cotton-based ecosystem, the possibility of amplification of some very closely related virus species with these primers cannot be ruled out. The use of this technique for the detection of cotton geminiviruses in alternate hosts plants should be used carefully and may be confirmed by some other techniques. The method will be useful in epidemiological studies and screening of alternate hosts as well as evaluation of breeding lines for the presence of the two geminivirus species.

Acknowledgements

Financial assistance provided by Ministry of Food, Agriculture and Livestock (MINFAL), Government of Pakistan under a loan by Asian Development Bank for cotton research and management project is gratefully acknowledged. Part of the funding

was also provided by Common Fund for Commodities (CFC), Holland through International Cotton Advisory Committee (ICAC), Washington, USA. We also thank Dr. M. I. Rajoka for critical reading of the manuscript.

References

Briddon, R. W., P. Lunness, L. C. L. Chamberlin and P. G. Markham. 1994. Analysis of the genetic variability of maize streak virus. *Virus Genes*, 9: 93-100.

Briddon, R. W., and P. G. Markham. 1994. Universal primers for the amplification of dicot-infecting geminiviruses. *Mol. Biotechnol.*, 1:202-205.

Doyle, J. J. and J. L. Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bulletin*, 19:11-15.

Hameed, S., S. Khalid, E. Ulhaq and A. A. Hashmi. 1994. Cotton leaf curl disease in Pakistan caused by a whitefly-transmitted geminivirus. *Plant Dis.*, 78:529.

Harrison, B.D.,Y. L. Liu, S. Khalid, S. Hameed, G. W. Otim-nape and D. J. Robinson. 1997. Detection and relationships of cotton leaf curl virus and allied whitefly-transmitted geminiviruses occurring in Pakistan. *Ann. Appl. Biol.*, 130: 61-75.

Iqbal, M. J., N. Aziz, N. A. Saeed, Y. Zafar and K. A. Malik. 1997. Genetic diversity evaluation of some elite cotton varieties by RAPD analysis. *Theor. Appl. Genet.*, 94:139-144.

Lazarowitz, S. G. 1992. Geminiviruses: Genome structure and gene function. *Crit. Rev. Plant Sci.*, 11: 327-349.

Mansoor, S., I. D. Bedford, M. S. Pinner, J. Stanley and P. G. Markham. 1993. A whitefly-transmitted geminivirus associated with cotton leaf curl disease in Pakistan. *Pak. J. Botany*, 25: 105-107.

Mansoor S., J. Stanley, K. A. Malik and P. G. Markham. 1995. Molecular characterization of a geminivirus associated with cotton leaf curl disease in Pakistan. *Proc. Int. Symposium on Biotechnology for Sustainable Development. Faisalabad*, pp. 123-128.

Mansoor, S., M. Hussain, S. H. Khan, A. Bashir, A. B. Leghari, G. A. Panwar, W. A. Siddiqui, Y. Zafar and K. A. Malik. 1998. Ploymerase chain reaction-based detection of cotton leaf curl and other whitefly-transmitted geminiviruses from Sindh. *Pak. J. Biol. Sci.*, 1:39-43.

Maule, A J., R. Hull and J. Donson. 1983. The application of spot hybridization to the detection of DNA and RNA viruses in plant tissues. *J.Virol. Method*, 6: 215-224.

McGovern, R.J., J. E. Polston, G. M. Danyluk, E. Hiebert, A. M. Abouzid and P. A. Stansly. 1994. Identification of a natural weed host of tomato mottle geminivirus in Florida. *Plant Dis.*, 78: 1102-1106.

Padidam, M.R., N. Beachy and C.M. Fauquet. Classification and identification of geminiviruses using sequence comparison. *J. Gen. Virol.*, 76: 249-263.

Pinner, M.S. and P. G. Markham. 1990. Serotyping and strain identification of maize streak virus isolates. *J. Gen. Virol.*, 71:1635-1640.

Rojas, J.R., R.L. Gilbertson, D. R. Russell and D. P. Maxwell. 1993. Use of degenerate primers in the polymerase chain reaction to detect whitefly-transmitted geminiviruses. *Plant Dis.*, 77: 340-347.

Rybicki, E.P. and F. L. Hughes. 1990. Detection and typing of maize streak virus and other distantly related geminiviruses of grasses by polymerase chain reaction amplification of a conserved viral sequence. *J. Gen. Virol.*, 71:2519-2526.

Swanson, M.M., J. K. Brown and B. T. Poulos, B. D. Harrison. 1992. Genome affinities and epitope profiles of whitefly-transmitted geminiviruses from the americas. *Ann. Appl. Biol.*, 121: 285-296.

Wyatt, S.D. and J. K. Brown. 1996. Detection of subgroup III geminivirus isolates in leaf extracts by degenerate primers and polymerase chain reaction. *Phytopathology*, 86: 1288-1293.

Zafar, Y., A. Bashir, S. Mansoor, M. Saeed, S. Asad, N. A. Saeed, R.W. Briddon, P. G. Markham, C.M. Fauquet and K.A. Malik. 1975. Cotton leaf curl virus epidemic in Pakistan: Virus characterization, diagnosis and development of virus resistant cotton through genetic engineering. Proc. 56th Plenary Meeting of Int. Cotton Advisory Committee Asuncion, Paraguary. pp: 33-39, October, 1997.

Zeidan, M. and H. Czosnek. 1991. Acquisition of tomato yellow leaf curl virus by the whitefly *Bemisia tabaci. J. Gen. Virol.*, 72: 2607-2614.

Zhou, X., Y. Liu, D. J. Robinson and B. D. Harrison. 1998. Four DNA-A variants among Pakistani isolates of cotton leaf curl virus and their affinities to DNA-A of geminivirus isolates from okra. *J. Gen. Virol.*, 79:915-923.

(Received for publication 31 August 1998)