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Recent advances in geminivirus detection and future perspectives

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A B S T R A C T

The detection and identification of viruses has been a challenge since the advent of the discipline of plant virology over a century ago. A great variety of methods have been developed that permit differentiation of viral pathogens. These methods, initially based solely on identifying the distinct biological characteristics of different viruses, were soon supplemented with methods based on light or electron microscopy and serology and subsequently by Enzyme Linked Immunosorbant Assay (ELISA) and finally the use of molecular (nucleic acid-based) techniques have revolutionized the diagnosis of plant viruses. While the technologies available to virologists have obviously become more diverse and improved, the challenges have also changed greatly. Detection of plant viruses is becoming more critical as globalization of trade, particularly in horticultural commodities increase. The potential effects of climate change have further aggravated the movement of viruses and their vectors, transforming the diagnostic landscape. Geminiviruses are a group of plant viruses characterized by circular, single-stranded (ss) DNA genomes (~2.7 kb size) and twined icosahedral virions (18 x 30 nm). Based on genome organization, host range and insect-vector, geminiviruses are divided into four genera: *Mastrevirus*, *Curtovirus*, *Topocuvirus* and *Begomovirus*. Begomoviruses are transmitted by whiteflies (*Bemisia tabaci* Gennadius) and usually possess a bipartite genome of two DNA components approximately 2.7 kb in size, designated as DNA-A and DNA-B. Monopartite begomoviruses are also known to occur. Recently, a novel class of molecule, known as satellite DNA-β of ~1.35 kb size are also found to occur in India. Whitefly transmitted geminiviruses categorized as begomoviruses are reported since long. During the last two decades, whitefly-transmitted geminiviruses have emerged as serious pathogens of several agricultural and horticultural crops (like tomato, chillies, cotton, pulses, papaya, cucurbits, okra etc.) causing enormous losses in the tropics, which provide ideal conditions for the perpetuation of viruses and the insect-vector. Despite concerted efforts to contain begomoviruses and their vectors, menacing disease epidemics caused by newly emerging or re-emerging begomoviruses are becoming frequent and appearing even in new regions, previously free from such diseases. Techniques for geminivirus detection include biological indexing, electron microscopy, antibody-based detection, including ELISA, nucleic acid hybridizations like dot blot, squash blot and Southern blot techniques, polymerase chain reaction and other DNA polymerase-mediated assays, and microarray detection. Of these, microarray detection provides the greatest capability for parallel yet specific testing and can be used to detect individual or combinations of viruses with sensitivity comparable to ELISA. Methods based on PCR provide the greatest sensitivity

Keywords: ELISA, Geminivirus, Polymerase chain reaction, Rolling circle amplification, microarray, detection

among the listed techniques but are limited in parallel detection capability even in "multiplexed" applications. Better, easier and cheaper than polymerase chain reaction or antibody detection, rolling circle amplification using the bacteriophage φ29 DNA polymerase allows for a reliable diagnosis of geminiviruses and presumably all viruses with small single-stranded circular DNA genomes. The results show the efficiency of this technique in characterizing viral DNA components of several geminiviruses from experimental and natural host plant sources. Nucleotide sequence data offers identification of viral molecule to strain/species level for accurate diagnosis.

Introduction

The ever increasing number of ailments of man, animals and plants are assigned to viruses and any form of life including algae, fungi, bacteria and mycoplasmas seems liable to virus infection. Since, there is no cure to virus infected plants under natural conditions, management options emphasizes on exclusion of the viruses, minimizing virus spread. This important task critically depends on the comprehensive diagnosis of the virus/viruses involved in the disease aetiology. This is a difficult task, considering that several hundred diseases have been reported to affect crop plants around the world and the scientific literature concerning virus occurrence, characterization, diagnosis, detection and control of these viruses is growing at a tremendous pace.

Geminiviruses (family *Geminiviridae*) are one of the most devastating viruses of several crops in tropical and subtropical regions of the world. They are large and diverse group of plant viruses characterized by their unique paired icosahedral capsids (Lazarowitz, 1992). They have circular, single-stranded DNA genomes that replicate via double stranded DNA intermediates in the nuclei of infected cells (Hanley-Bowdoin *et al.* 1999; Gutierrez 1999). Geminiviruses are classified into four genera: *Begomovirus, Curtovirus, Mastrevirus,* and Topo-cuvirus based on their genome organization, insect vector and host range (Fig. 1). (Hull 2002).

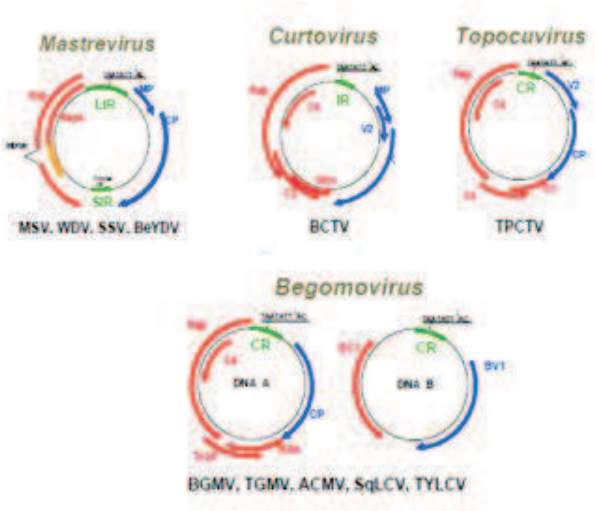

Fig 1. Genetic organization of the four genera of the Geminiviridae family (*Gutierrez 1999)*

Members of the genus *Begomovirus,* family *Geminiviridae* were formerly termed subgroup III geminiviruses, and are transmitted by the whitefly, *Bemisia tabaci* (Gennadius) [Polston and Anderson, 1997; Chakraborty *et al.* 2008]. Begomovirus genomes described to date can be

categorized into a number of genome categories, the main division being into monopartite or bipartite depending on whether they have one or two circular ssDNA components. Majority of the described begomoviruses are bipartite containing DNA-A and DNA-B molecules, each of these being approximately 2600-2800 nt in size. However, some Old World tomato viruses like *Tomato yellow leaf curl virus* (TYLCV) and *Tomato leaf curl virus* (ToLCV)-[AU] lack a DNA-B component, requiring only their DNA-A component to systemically and symptomatically infect plants (Dry *et al.* 1993; Kheyr-Pour *et al.* 1991; Navot et al. 1991) Others have been found to require in addition to DNA-A, ssDNA component termed DNA-β, to develop characteristic disease symptoms (Briddon et *al.* 2004; Jose & Usha 2003; Saunders *et al.* 2000) Some monopartite begomoviruses have also been found to be associated with a different type of ssDNA component, approximately half the size of Begomovirus DNA-A and termed DNA1. Although the function of the latter is unknown, the majority of monopartite viruses containing a DNA-β, have also been found to contain a DNA1 component (Briddon *et al.* 2004).

Irrespective of genome organization, both these type of begomoviruses possess ability to undergo recombination and pseudo-recombination processes resulting into changed sequence or a new viral strain which may be more virulent or resistant-breaking strain. Therefore, there have always been demands for very sensitive, efficient and reliable methods of detection for geminivirus infection in crop plants. Here, we have attempted to summarize the information available on detection and diagnosis of geminiviruses.

Enzyme - Linked Immunosorbant Assay (ELISA)

Since the development of the microplate method of enzyme-linked immunosorbant (ELISA) (Clark and Adams 1977), ELISA has become a routine method for detection and quantification of virus level in the plant (Stein *et al.* 1983; Duan *et al.* 1995). ELISA is simple to perform, sensitive, and easily used in large scale analysis. Several modifications and refinements have also been done in ELISA techniques since its inception (Cooper *et al.* 1986; Tsutsumi *et al.* 1986; Gow and Williams 1989; Al-Bitar and Lusioni 1995). They mostly differ in the quality and specificity of the reagents. Examples include the availability of a range of virus-specific monoclonal antibodies (MAbs), the use of conjugated and affinity-purified second antibodies or protein-G to detect virus-specific mouse or rabbit antibodies in indirect ELISA; application of alternative enzymes or substrates; use of streptavidin in place of avidin; colloidal gold-conjugates; chemiluminescent substrates (Crespi *et al.*1991) and the development of an amplification technique to increase the sensitivity of alkaline phosphatase conjugates in microtitre plate assays (Torrance 1992).

ELISA methods have been used for the detection of several geminiviruses (Table1). Monoclonal antibody prepared using coat protein of an isolate of *Bean golden mosaic virus* (BGMV) from Brazil (BZ), expressed in *Escherichia coli* were used as serological

probes for detection of whitefly-transmitted geminiviruses (Cancino *et al.* 1995). Monoclonal antibodies (MAbs) developed to particles of *African cassava mosaic virus* (Thomas *et al.*1986) or *Indian cassava mosaic virus*, have been used for the detection of three geminiviruses that occur in Europe: *Abutilon mosaic virus* in *Abutilon pictum 'Thompsonii', Tobacco leaf curl virus* in *Lonicera japonica* var. *aureo-reticulata* and *Tomato yellow leaf curl* virus in *Lycopersicon esculentum* (Konate *et al.* 1995). Double monoclonal antibody ELISA using antibodies to African cassava mosaic virus have been used to detect geminiviruses (Givord *et al.*1994). In India, Double Antibody Sandwich-ELISA (DAS-ELISA) has been used for the detection of Potato apical leaf curl virus (PALCV) from the parts of north India (Venkatasalam *et al.* 2005). Devaraja *et al.* (2003) successfully produced monoclonal antibodies to ToLCV and detected begomovirus infections in tomato samples, and other crop species. DAS-ELISA (Double antibody sandwich ELISA) and TAS–ELISA (Triple antibody sandwich ELISA) were also used and standardised for the detection of *Tomato leaf curl Bangalore virus* (ToLCBV) (Devaraja *et al.* 2005) (Table 1).

However, detection of geminiviruses through ELISA has certain limitations. Although it is a sensitive method for virus detection it has a low specificity. Development of specific antibodies and low antigenicity of virus increase the limitation boundary of ELISA method for geminiviruse detection. Moreover, one major problem is the cross-reaction of the antibodies produced against one geminivirus coat protein with other geminiviruses. This cross-reaction is due to high level of homology between different geminiviruses which result in the presence of many shared epitope. Therefore, it becomes difficult to distinguish between two closely related strains/species. It has also been observed that ELISA is effective in detecting TYLCV in purified preparations but not in crude extract of infected plants. Therefore, new modifications were introduced in ELISA to enhance its detection sensitivity over a period of time.

Dot Immunobinding Assay

The principle of Dot Immunobinding Assay (DIBA) is same as ELISA except that antigen and antibodies are bound to nitrocellulose membrane instead of microplate and also the product of the enzyme reaction is insoluble. DIBA generally exhibit higher sensitivity by avoiding non specific reaction and require fewer antibodies than ELISA. DIBA has been used in the detection of TYLCV in a number of studies with satisfactory result (Noris *et al.* 1994; Hajimorad *et al.* 1996) (Table 1). Moreover, DIBA has been used to screen for *Tomato yellow leaf curl virus* (TYLCV) resistance tomato genotypes (Lapidot *et al.* 1997; Lapidot 2002). Unlike ELISA, DIBA was sensitive enough to allow a differentiation among genotypes with varying degrees of resistance to TYLCV.

Lateral Flow Kits

This technique utilizes the specific monoclonal and polyclonal antibodies in an immuno-chromatographic, format-incorporating antibody-coated latex particle. These systems

Table 1.
Different techniques used for geminivirus detection

Sl. No.	Method of virus detection	Example of detected virus	Host	References
1.	ELISA	African Cassava mosaic virus (ACMV),Tomato leaf curl virus (ToLCV), Indian cassava mosaic virus (ICMV), Squash leaf curl virus (SqLCV), Okra leaf curl virus (OLCV).Tobacco leaf curl virus (TbLCV).	Cassava Tomato Cassava Squash	Givord *et al.*1994; Devaraja *et al.*2005 Farag *et al*. 2005; Konate *et al.*1995
2.	DIBA	Tomato Leaf Curl Virus (ToLCV), Squash leaf curl virus (SqLCV)	Tomato	Noris *et al*. 1994; Hajimorad *et al*. 1996
3.	Hybridization method -Squash/Dot Blot -Southern Blot	Tomato leaf curl virus (ToLCV)	Whiteflies, Tomato	Caciagli & Bosco 1997; Lapidot *et al*. 2001
4.	PCR	Maize streak virus (MSV), Tomato leaf curl virus (ToLCV), Chilli leaf curl virus (ChiLCV), Squash leaf curl virus (SqLCV)	Maize,Wheat Tomato *Capsicum* sp. Pumpkin	Rybicki *et al.*1990; Navot *et al.*1992; Chattopadhyay *et al* 2008; Singh *et al.* 2009
	Multiplex PCR	African cassava mosaic virus (ACMV), East African cassava mosaic Cameroon virus (EACMCV), Tomato leaf curl sedinia virus (ToLCV)	Cassava	Olufemi *et al.* 2008; Mason *et al.*2008
	RT PCR		Tomato whiteflies	
5.	RCA	Maize streak virus (MSV), Chilli leaf curl virus (ChiLCV), Tomato yellow vein streak virus (ToYVSV) etc.	Maize *Capsicum* sp. Tomato	Dionne *et al.* 2008; Chakaraborty *et al*. 2009; Inoue-Nagata. *et al.* 2004

were developed as a result of the need for rapid pathogen detection and identification in the field or green house conditions.

In this simple procedure, tissue taken from a suspected plant is macerated in a small plastic bottle containing buffer and small plastic beads in the field. Two to three drops of the extract are applied to small windows in the field. After a few mins, the appearance of two blue lines in the viewing window indicates viral infection. One blue line serve as a cassette internal control, the other serve as the test line and the appearance of the control blue line alone indicates no viral infection. Lateral flow kit for the detection of TYLCV has been developed and is available commercially (Danks & Barker 2008).

However, all of these serological techniques were sensitive enough to detect virus from infected sample but due to low specificity and

inability to detect very low level of virus titre in the sample, researchers attempted to develop techniques that can be more sensitive and specific over these serological methods.

Nucleic Acid Hybridization Method

There are two basic types of nucleic acid hybridization assays which are based on the means of application of nucleic acid on membrane, usually nylon membrane, followed by hybridization with labelled viral nucleic acid as probe. This method is simple, sensitive, specific and results can be readily quantified.

Dot-blot Hybridization (Nucleic acid spot hybridization)

In dot blot, crude extract of DNA from plant tissue, ground in water or 0.4 M NaOH, is dotted on to nylon membrane and hybridized with radioactively labelled viral DNA (either partial fragment or full-length). It is a very sensitive method and viral DNA can be detected in nanogram level and both qualitative and quantitative detection can be done (Polston *et al.* 1997; Caciagli *et al.* 2009). Using this technique geminivirus has been detected in ornamental crotons in India (Raj *et al.* 1996).

Squash - Blot Hybridization

In this method, infected plant tissue is squashed onto either nylon or nitrocellulose membrane. Squashing is a simple process which doesn't require extraction buffer, prior knowledge and specialized equipment. Squashed blot is hybridized with radiolabeled viral nucleic acid. It is a very sensitive method but this method cannot be used for quantitative determination of viral nucleic acid in infected host. Squash blot have been used for the detection of TYLCV DNA in different plant parts e.g. leaves, stems, flowers, roots and young fruits (Nakhla *et al.* 1993) (Table 1).

Southern Blot

A Southern blot is a method routinely used in molecular biology for detection of a specific DNA sequence in DNA samples. Southern blotting combines transfer of electrophoretically separated DNA fragments to a filter membrane and subsequent fragment detection by probe hybridization (Southern, 1975). In this method total DNA is isolated from infected plant and transferred on membrane followed by its hybridization with radiolabeled probe which is generally made from partial or full length viral nucleic acid. Southern blotting method has been used for quantitative determination of many begomoviruses from different parts of the world, e.g., Chilli Leaf Curl Varanasi Virus, Tomato Leaf Curl New Delhi Virus, Tomato Leaf Curl Varanasi Virus, Squash leaf curl virus (Chakraborty *et al.* 2008; Singh *et al.* 2009) (Table 1; Fig 5).

Polymerase Chain Reaction (PCR) based assay

Since the last few years, PCR is the routine assay for plant viral genome detection. It is many times more sensitive than ELISA and hybridization techniques. Rapid improvement and availability of different DNA polymerases for use in PCR, combined with a small closed circular ssDNA and published sequence, make it relatively easy to design overlapping or nearly overlapping primers to amplify the full-length

geminivirus genome in a single reaction.

PCR detection of geminivirus using degenerate primer

The PCR technique allows rapid, sensitive, and accurate detection of a diverse array of WTGs in cultivated and weed hosts, with minimal sample preparation. This assay also is highly effective with purified nucleic acid extracts or partially purified virion preparations, either treated or untreated with a ssDNA-specific nuclease (Ieamkhang *et al.* 2005; Ruhui *et al.* 2008).

All begomoviruses share region of high homology in their genomes. Degenerate universal primers have been designed to anneal to these highly conserved sequences in the genome of begomoviruses (Rojas *et al.* 1993, Briddon & Markham 1994, Deng *et al.* 1994, Wyatt & Brown 1996). The degenerate primers are mixtures of similar but not identical primers with base changes in one or more positions. They serve as universal primers that will amplify a DNA fragment from all begomoviruses (Table 1-2) (Fig 2-4).

Degenerate oligonucleotide primers, designed for amplification of an approx. 500 bp fragment of DNA-A of five well characterised whitefly-transmitted geminiviruses were used in the polymerase chain reaction (PCR) to detect known or putative geminiviruses infecting seven plant species and originally obtained from Africa, India, America or Europe (Atzmon *et al.* 1998; Deng *et al.* 1994). The versatility of this method makes it the simplest, most broad-spectrum PCR-based detection system available to date for detection of subgroup III WFT geminiviruses.

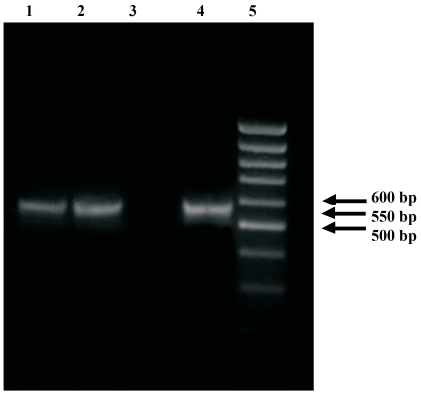

Fig 2. PCR based detection of geminiviruses using degenerate primers of coat protein gene of DNA-A from infected samples (Wyatt & Brown 1996). Samples are obtained from tomato, chilli and okra 3 has no virus DNA.

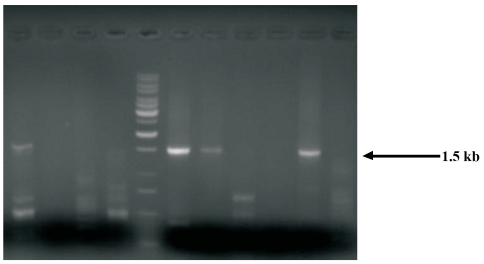

Fig 3. Detection of a mixed geminivirus infection in field collected samples of different vegetables crops by using the PCR and the primer pair PARc715 and PAL1v1978 (Rojas *et al*. 1993)

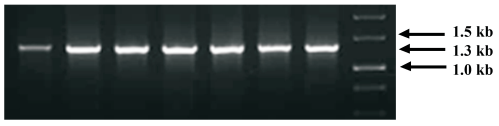

Fig 4. Detection and amplification of a DNA-β from field collected samples of different vegetables crops by using the PCR and the primer pair DNA-β1 and DNA-β2 (Briddon *et al*. 2002)

Multipex PCR

Multiplex PCR (using multiple pairs of PCR primers simultaneously) allows the specific detection of multiple viruses in the same

Table 2.
Primer sequences and amplification conditions of universal PCR primers used for geminivirus detection

Primers	Sequence data*	PCR cycle	Product size and reference
Rojas universal primers			
PARc715	5'GCATCTGCAGGCCCACATYGTCTTYCCN GT3'	94 ^{0}C-60 sec 56 ^{0}C-45 sec	~1.5kb
PAL1v1978	5'GATTTCTGCAGTTDATRTTYTCRTCCAA3'	72 ^{0}C-110 sec	Rojas *et al.* 1993
Rojas universal primers			
PALIc1960	5`ACNGGNAARACNATGTGG GC 3`	94 ^{0}C-50 sec 52 ^{0}C-45 sec	~1020bp
PARIv722	5`GGNAARATHTGGATGGA 3 `	72 ^{0}C-90 sec	Rojas *et al.*1993
Brown universal primers			
Brown1	5`GCCCATGTATAGAAAGCCAAG3`	92 ^{0}C-60 sec 60 ^{0}C-20 sec	550bp, S. D. Wyatt and
Brown2	5`GGATTAGAGGCATGTGTACATG3`	72 ^{0}C-30 sec	J. K. Brown 1996
Deng universal primers			
Deng A	5`TAATATTACCKGWKGVCCSC 3`	94 ^{0}C-50 sec 52 ^{0}C-45 sec	~530bp
Deng B	5`TGGACYTTRCAWGGBCCTTCACA 3 `	72 ^{0}C-90 sec	Deng *et al*.1994
Beta primers			
Beta1	5' GGTACCACTACGCTACGCAGCAGCC 3'	94 ^{0}C-60 sec 60 ^{0}C-45 sec	~1.35kb
Beta2	3'GGTACCTACCCTCCCAGGGGTA CAC 3'	72 ^{0}C-90 sec	Briddon *et al.*2002
Pumpkin DNA-A primers			
PYABFP	5`GTGGGGATCCATTATTGCACG3`	94 ^{0}C-60 sec 56 ^{0}C-60 sec	~2.7 kb
PYABRP	5`CCGGATCCCACATGTTTGTAGA3`	72 ^{0}C-180 sec	Singh *et al.*2009
Pumpkin DNA-B primers			
PYBHFP	5`GAAAGCTTACTGGTCTTACCATGTCC3`	94 ^{0}C-60 sec 60 ^{0}C-60 sec	~2.7 kb
PYBHRP	5`TGAAGCTTGATATATGAACGAACCCTG3`	72 ^{0}C-180 sec	Singh *et al.* 2009

*B=G+T+C, H=A+T+C, K=G+T, N=A+C+G+T, R=A+G,W=A+T and Y=C+T

reaction; usually this also allows identification of each virus to the species level. A multiplex PCR was developed for simultaneous detection of *African cassava mosaic virus* (ACMV) and *East African cassava mosaic Cameroon virus* (EACMCV) in cassava affected with cassava mosaic disease (CMD) (Olufemi *et al.* 2008). One set of three primers consisting of an upstream primer common for both viruses and two downstream virus-specific primers were designed for simultaneous amplification of 368 bp and 650 bp DNA fragments specific to the replicase gene of ACMV and EACMCV, respectively. Similarly, a second set of three primers were designed for simultaneous amplification of 540 bp and 655 bp fragments specific to the coat protein gene of EACMCV and ACMV, respectively (Table 1).

The problem with multiplex PCR, however, is that only a limited number of targets can effectively be detected simultaneously. As more primers are added to a PCR reaction, unexpected interactions begin to occur. The method used to resolve the products of multiplex PCR also adds further limitations. One approach is to resolve the products by size; however, smaller products tend to be preferentially amplified, thus preventing amplification of larger products and identification of other viruses in the mixture.

Reverse transcription polymerase chain reaction (RT-PCR)

It is also a sensitive method for geminivirus transcript level detection and its quantification in infected host. In this method, total RNA is isolated and viral mRNA is converted into viral cDNA using viral specific primer by reverse transcriptase enzyme followed by normal PCR. We used ChiLCV DNA-A AC1 region primer and AV1 region primer for reverse transcription of RNA from ChiLCV-infected N. *benthamiana*. We also used ChiLCV DNA-A AC1 region primer for the detection of transcript of ToLCV (Chakraborty *et al.* personal communication) (Fig 9; Table 1).

Real Time PCR

The use of real-time PCR to quantify the infection of a plant by a pathogen has increased during the last few years (Winton *et al.* 2002) . Several studies targeting plant viruses have concluded that real-time PCR offers greater sensitivity than conventional PCR, molecular hybridisation or serological methods (Mumford *et al.* 2000; Korimbocus *et al.* 2002; Boonham *et al.* 2004). For its higher sensitivity, it has also been applied to detect RNA or DNA viruses. The TaqMan technology (Holland *et al.* 1991; Higuchi *et al.* 1993; Heid *et al.* 1996) provides accurate and sensitive methods to quantify nucleic acids by measuring fluorescence intensity during the exponential phase of DNA amplification. Fluorescence is generated by the cleavage of a TaqMan probe by the 5'exonuclease activity of Taq DNA polymerase. The fluorescence measured can be directly related to the amount of DNA by analysing samples containing known amounts of reference DNA, in parallel reactions. Standard curves generated by plotting the threshold cycle (Ct) versus the logarithm (log) of the amount of the

starting quantities (SQ) are used to calculate the amount of DNA present in the experimental samples and evaluate the reaction efficiency. Besides, real-time PCR has the advantage that no post-reaction processing is required to detect the reaction products.

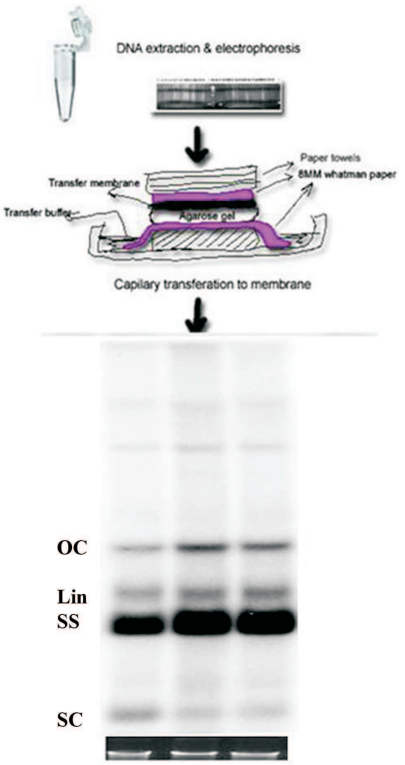

Fig 5. Hybridization membrane and autoradiography : Southern blot of total DNA isolated from tomato plants showing symptom of ToLCV. The blots were hybridized with coat protein (DNA-A) ToLCGV-IN[IN:Var:01] probe The positions of single-stranded (SS), super-coiled (SC), and open-circular (OC) DNA forms are indicated. Each lane was loaded with 10 µg DNA. Ethidium bromide-stained DNA is shown below each lane as loading control. (Chakraborty *et al*. 2008)

Real-time PCR methods are used for quantification of TYLCSV in both tomato plant and whitefly extracts (Mason *et al.* 2008) (Table 1). They found the real-time PCR was 2200-fold more sensitive than membrane hybridisation, allowing detection of as few as 10 viral copies in a sample.

Rolling Circle Amplification

Rolling Circle Amplification RCA) is better, easier and cheaper than polymerase chain reaction (PCR) or antibody-based detection of geminiviruses. RCA utilizes bacteriophage φ29 DNA polymerase to exponentially amplify single or double-stranded circular DNA templates by rolling circle amplification (Dean *et al.* 2001; Lizardi *et al.* 1998). This isothermal amplification method produces microgram quantities of DNA from picogram amounts of starting material in a few hours. The proofreading activity of φ29DNA polymerase ensures high fidelity DNA replication (Johne *et al.* 2009; Estaban *et al.* 1993). The starting material for amplification can be a small amount of bacterial cells containing a plasmid, an isolated plasmid, intact M13 phage, or any circular DNA sample.

RCA using the bacteriophage φ29 DNA polymerase allows for a reliable diagnosis of geminiviruses and presumably all viruses with small single-stranded circular DNA genomes. The results show the efficiency of this technique in characterizing viral DNA components of several geminiviruses from experimental and natural host plant sources. The advantages are: (a) that no expensive devices are necessary, (b) simple handling, (c) detection of all infecting circular DNA components without any knowledge of sequence information in a single step, and (d) low costs per reaction. In addition, RCA-amplified viral DNA can be characterized by

restriction fragment length polymorphism (RFLP) analysis and directly sequenced up to 900 bases in a single run, circumventing cloning and plasmid purification (Haible *et al.* 2006) (Fig.7). RCA was first applied for detection and cloning of bipartite geminivirus DF-BR2 infecting tomato plants in Brazil to amplify its DNA B component (Inoue-Nagata *et al.* 2004) (Table 1).

Microarray/DNA chip

DNA microarray technique is one of the latest advances in the field of molecular biology and medicine. This multiplex technique with the combination of bioinformatics and statistical data analysis has revolutionized virus detection methods. Microarray consists of an arrayed series of thousands of microscopic spots of DNA oligonucleotides, called features, each containing picomoles of a specific DNA sequence which is either a short section of a gene or other DNA segments used as probes to hybridize a cDNA or cRNA sample under high-stringency conditions. Probe-target hybridization is usually detected and quantified by fluorescence-based detection of fluorophore-labelled targets to determine relative abundance of nucleic acid sequences in the target. By high specific hybridization of probe nucleic acids with target nucleic acids from infected samples it allows effective detection of various viruses with high speed, sensitivity and specificity (Fig. 8).

Microarray techniques have also been used for detection of many viruses (David *et al.* 2002; Manoj 2009). Plum Pox Virus (PPV) is the most damaging viral pathogen of stone fruits. Pasquini and co-researchers (2008) developed a long 70-mer oligonucleotide DNA microarray capable of simultaneously detecting and genotyping PPV strain and this PPV microarray is capable of detecting and identifying all the strains of PPV in infected peach, apricot and *Nicotiana benthamiana* leaves. A microchip

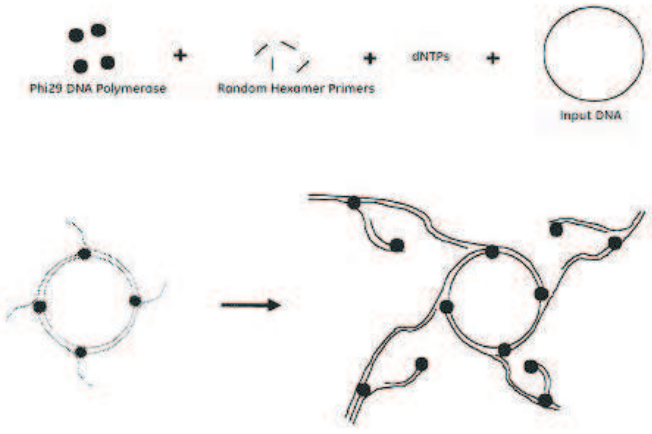

Fig 6. Schematic diagram of the RCA process. Random hexamer primers anneal to the circular template DNA at multiple sites. φ29 DNA polymerase extends each of these primers. When the DNA polymerase reaches a downstream extended primer, strand displacement synthesis occurs. The displaced strand is rendered single-stranded and available to be primed by more hexamer primer. The process continues, resulting in exponential, isothermal amplification.

detecting potato viruses PVA, PVS, PVM, PVX, PVY and PLRV, in both single and mixed infections was developed and tested and the chip was also designed to distinguish between the main strains of PVY and PVS, (Lee *et al.* 2003). Boonham and colleagues (2007) constructed a microarray which is capable of detecting several common potato viruses (PVY, PVX, PVA and PVS) in single and mixed infections. The cDNA chip was designed for detection and differentiation of the four species of selected cucurbit-infecting tobamoviruses [target viruses: Cucumber green mottle mosaic virus (CGMMV), Cucumber fruit Mottle mosaic virus (CFMMV), Kyuri green mottle mosaic virus (KGMMV) and Zucchini green mottle mosaic virus (ZGMMV) (Gung *et al.* 2003). Initiatives on microarray-based diagnostics of geminiviruses are being undertaken in many laboratories.

Conclusion

Diagnosis remains difficult for plant virus diseases. It has long been far from simple to demonstrate the presence of viruses. Viruses cannot be seen with the using the tools commonly used by plant pathologists for examining diseases in plants.

During recent years, geminiviruses have emerged as one of the most devastating plant virus groups declining the production and improvement of several important crops worldwide. Their detection is necessary to enable the identification of virus in reservoir hosts to be identified, virus resistant plant varieties to be evaluated and consequently control measures to be devised or improved. Their emerging speed have catalysed urgent development of very efficient, ultrasensitive and more specific techniques for geminivirus detection. ELISA is a very common technique which has been used for detection of several geminivirus in different parts of the world. However, it is a sensitive method but due to cross reaction and low antigenicity of geminivirus protein, it is difficult to identify and characterise a particular strain of geminivirus with ELISA.

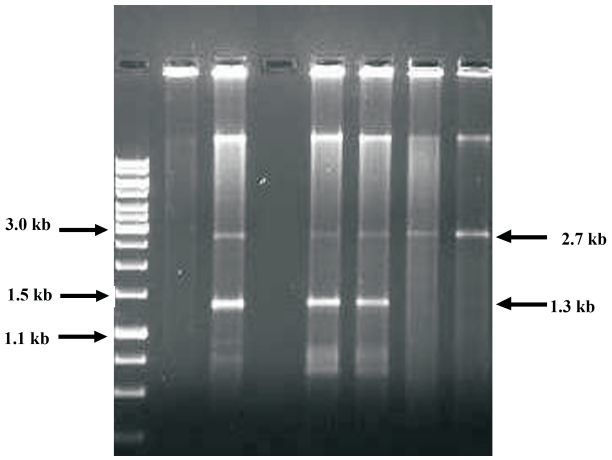

Fig 7. RCA/RFLP analysis of vegetable infecting gemini-viruses. Independent field samples from tomato, chilli, okra and radish collected and were subjected to RCA, products were digested with *Bam H*I, separated on a 2%Agrose gel, and stained with ethidium bromide.

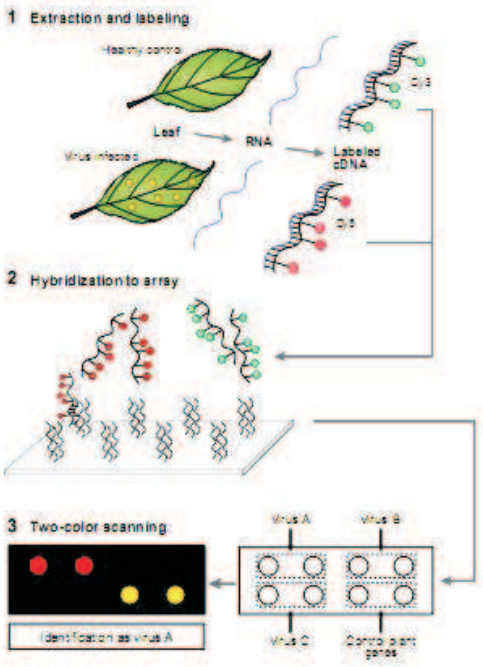

Fig 8. A schematic diagram detailing a simple approach to virus detection using a microarray. RNA is extracted from the test material and known healthy leaf material and reverse transcribed into cDNA incorporating fluorescently labelled nucleotides. Cy 3 is used for the healthy and Cy 5 for the test material (1). The cDNAs are pooled and hybridized to the array (2). Following washing and scanning of the slides, the results reveal specific hybridization of Cy5-labeled (red) target to the probe spots belonging to virus A. As an internal positive control, probes homologous to plant genes are also present on the array, these hybridize to cDNA from both the test (labeled with Cy5) and healthy (labeled with Cy3) plants; thus these spots appear yellow (Boonham *et al.* 2007)

Nucleic acid hybridization method fulfilled some of the requirements of geminivirus detection method. Different kinds of hybridization methods have been developed like squash blot, dot blot and southern blot for the geminivirus detection. All of them are more sensitive and specific than ELISA techniques and both qualitative and quantitative assays can be performed with this single technique. Several geminiviruses which were not detected by ELISA were identified and characterized by nucleic acid hybridization methods. Apart from showing good sensitivity and specificity nucleic acid hybridization techniques has also some limitations. Since many geminivirus genomes share common conserved nucleotide sequences; hence during hybridization non specific binding may occur and also hybridization techniques is not sensitive for very low level (10^{-15} g) viral genome present in the

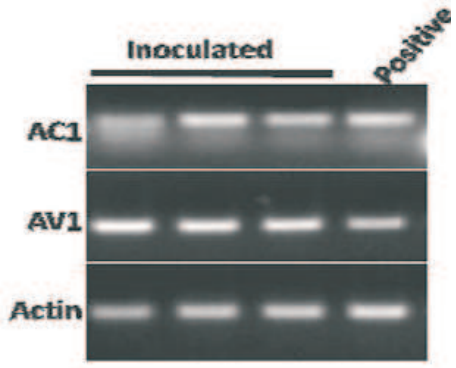

Fig 9. Detection of ChiLCV transcript by RT-PCR using AC1 and AV1 specific primer: Lane 1,2,3 from symptomatic N. benthamiana after 20 dpi and lane 4th is positive control. All three symptomatic plants are showing amplification of AC1 and AV1 transcript (Chakraborty *et al.* 2009).

In order to detect such an ultra low level viral genome, PCR came in to the trend which revolutionised the geminivirus detection method. Since many geminiviruses have conserved region, universal primers designed from that region can be used to detect any geminivirus infection in the sample. Primers designed from non-conserved regions have been used for identification and characterization of several geminiviruses. Along with this different kinds of PCR like multiplex PCR,

nested PCR, reverse transcription PCR, real time PCR, have been used for the detection, quantification and characterization of geminiviruses. But a need of full-length amplification and cloning of geminivirus with low error in nucleotide sequence also emerged. In this case PCR efficiency can not be considered up to the requirement.

The most recent technique rolling circle amplification again strengthens the detection techniques of geminivirus. RCA is very sensitive and has been used for detection, full length amplification, cloning and characterization of many geminiviruses with low error in virus genome sequence. Current detection methods are either specific to a particular pathogen, being unable to detect uncharacterized pathogens, or are restricted to members of a single taxonomic group. Microarrays based on oligonucleotides capture probes representing nucleic acid sequences conserved between members of a taxonomic group offer the possibility of a universal detection system for plant viruses, and for identification of uncharacterized viruses to the genus level; the technology has been proven for animal viruses. While the use of microarray in the detection of causal organism and investigation of infectious diseases is still in its infancy, new innovations in this emerging technology will throw more light on our understanding of the molecular basis of infectious agents and the diseases. Microarray-based diagnostics is being developed in many countries and new strategies need to be incorporated into the assays to increase their versatility.

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