Plant Virus Detection and Diagnosis: Progress and Challenges

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Abstract

The production of healthy planting material requires robust diagnostic procedures. The advancements of the molecular virology and biotechnology have witnessed major breakthroughs in the recent years resulting in sensitive and effective technologies/methods.

PCR has been the major a breakthrough leading successful detection RNA/DNA viruses.

The development of qPCR assays helped to avoid the risk of post-PCR contamination and has benefits of high reproducibility and accuracy. The nonthermal methods, like HDA, RPA, NASBA, etc., have proved suitable for poorly resourced laboratories and on-site testing.

NGS technology involving massive parallel sequencing approach followed by bioinformatics analysis has revolutionized discovery of novel viruses. With the increasing competition for machinery and analytical reagents among companies, high-throughput multiplexing and virus detection techniques are becoming cheaper. However, the challenge remains in their use at the site ensuring to maintain highest crop health standards at affordable price.

Keywords

Detection of virus • Diagnostic techniques • ELISA • Immunological diagnosis • Isothermal amplification • Molecular diagnosis • Nucleic acid hybridization • PCR • RFLP

7.1 Introduction

Viruses are catastrophic despite being miniscule acellular parasites. The simplest viruses comprise small stretch of nucleic acid carrying the genetic information encapsidated by coat protein.

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Being obligate parasite, viruses exclusively utilize the host metabolism for its reproduction. Viruses are known to infect wide range of living organism, viz. animals, plants, fungi and bacteria. Most of our crops are under the threat of various viroids and plant viruses causing plants diseases. Symptoms by these pathogens ranges from mild to catastrophe resulting in loss of crops over large area. The plant viruses cause many economically significant plant diseases worldwide affecting crop yield and quality. Thus, virus infections result in altered physiology of plant that includes stunted growth, inferior quality, and reduced yield leading to severe economic losses. Plant viruses attribute to significant losses in crop production of cash crops, ornamentals, etc. (Table 7.1).

Table 7.1 List of economically significant plant viruses infecting food and horticultural crops

Crop plant	Important viruses reported
Fruits	
Apple	*Apple mosaic virus* (ApMV) *Apple stem grooving virus* (ASGV) *Apple stem pitting virus* (ASPV) *Apple chlorotic leaf spot virus* (ACLSV)
Banana	*Banana bunchy top virus* (BBTV) *Banana streak badnavirus* (BSV) *Banana bract mosaic virus* (BBrMV)
Citrus	*Citrus psorosis virus* (CPsV) *Citrus tristeza virus* (CTV) *Citrus ringspot virus* (CRSV)
Papaya	*Papaya ringspot virus* (PRSV) *Papaya leaf distortion mosaic virus* (PLDMV) *Papaya mosaic virus* (PMV) *Papaya leaf curl virus* (PLCV)
Watermelon	*Watermelon mosaic virus* (WMV) *Watermelon silver mottle virus* (WSMV) *Squash vein yellowing virus* (SqVYV)
Vegetables	
Okra	*Bhendi yellow vein mosaic virus* (BYVMV) *Okra mosaic virus* (OMV)
Brinjal	*Eggplant mottled crinkle tombusvirus*(EMCV) *Tomato bushy stunt virus* (TBSV) *Eggplant mottled dwarf virus* (EMDV)
Tomato	*Tomato spotted wilt virus* (TSWV) *Tomato leaf curl virus* (ToLCV) *Tomato yellow leaf curl virus* (TYLCV)
Potato	*Potato leaf roll virus* (PLRV) *Potato virus X* (PVX) *Potato potyvirus* (PVY)
Cucumber	*Cucumber mosaic virus* (CMV)
Cucurbits	*Cucurbit mild mosaic virus* (CuMMV)
Pumpkin/squash	Squash mosaic virus (SqMV)
Zucchini	*Zucchini yellow mosaic virus* (ZYMV) *Zucchini yellow fleck potyvirus* (ZYFV)
Cereals	
Barley	*Barley stripe mosaic virus* (BSMV) *Barley yellow dwarf virus* (BYDV)
Maize	*Maize dwarf mosaic virus* (MDMV) *Maize streak virus* (MSV)

(continued)

Table 7.1 (continued)

Crop plant	Important viruses reported
Rice	*Rice ragged stunt virus* (RRSV) *Rice tungro bacilliform virus* (RTBV) *Rice tungro spherical virus* (RTSV) *Rice yellow stunt virus* (RYSV)
Oat	*Oat blue dwarf virus* (OBDV) *Oat chlorotic stunt virus* (OCSV)
Wheat	*Wheat American striate mosaic virus* (WASMV) *Wheat* streak mosaic *virus* (WSMV) *Triticum mosaic virus* (TriMV)
Legumes	
Bean	*Bean common mosaic virus* (BCMV) *Bean common mosaic necrosis virus* (BCMNV) *Bean yellow mosaic virus* (BYMV)
Mungbean	*Mungbean yellow mosaic virus* (MYMV)
Soybean	*Soybean mosaic virus* (SMV) *Soybean dwarf virus* (SbDV) *Soybean vein necrotic virus* (SVNV)
Pea	*Pea green mottle virus* (PGMV) *Pea enation mosaic virus* (PEMV) *Pea streak virus* (PSV) *Pea seed-borne mosaic virus* (PSbMV)
Tubers	
Cassava	*Cassava common mosaic virus* (CsCMV) *Cassava virus X* (CsVX) *Cassava green mottle virus* (CGMV) *Sri Lankan cassava mosaic virus* (SLCMV) *African cassava* mosaic *virus* (ACMV)
Cacao/cocoa	*Cacao swollen shoot virus* (CSSV) *Cacao mottle leaf virus* (CMLV) *Cacao necrosis virus* (CNV) *Cacao yellow mosaic virus* (CYMV)
Oil crops	
Groundnut/*peanut*	*Peanut clump virus* (PCV) *Peanut yellow mosaic virus* (PeYMV) *Groundnut bud necrosis virus* (GBNV)
Sunflower	*Sunflower chlorotic mottle virus* (SuCMoV) *Sunflower mosaic virus* (SuMV) *Sunflower necrosis virus* (SFNV)
Sugar yielding crops	
Sugarcane	*Sugarcane mosaic virus* (SCMV) *Sugarcane streak mosaic virus* (SCSMV)
Sweet potato	*Sweet potato feathery mottle virus* (SPFMV) *Sweet potato latent virus* (SPLV) *Sweet potato mild speckling virus* (SPMSV)
Beetroot	*Beet ringspot virus* (BRV) *Beet* necrotic yellow vein virus (BNYVV)

Few examples to show the estimated annual economic losses include *Tomato spotted wilt virus* infecting various plant species such as tomato, tobacco, groundnuts, etc. (Sherwood et al. 2003), which led to loss of ~$1 billion worldwide (Pappu 1997; Hull 2002); *Cacao swollen shoot virus* causing $28 million losses to cocoa beans in Africa (Bowers et al. 2001); and *Rice tungro virus* causing $1.5 billion losses to rice in southeast Asia (Hull 2002).

Due to variability in populations in genotypes of phytopathogens including viruses, it is hard to manage the infection and control the disease. Hence, prevention of viral diseases and subsequent economic damage warrants the fast and accurate methods for detection and diagnosis of the causal organisms, severity of disease and mechanism of virulence. Such information can then be significantly used to minimize the disease by inhibiting its virulence mechanisms.

Precise identification of viral diseases is essential because environmental stresses or other plant pathogens also produce symptoms similar to viruses. The development of diagnostic methods and their application is influenced by multiple factors. Plant viruses constitute highly diverse group of life lacking rRNA sequences unlike other cellular pathogens. Due to increasing globalization of trades including major ornamentals as well as the change in climate, it has significantly contributed to increase in the viruses and their vector movement, thus making the diagnostics more challenging and warranting the urgent needs for the transformations in diagnostic tools and techniques.

The most widely used techniques for screening the propagation materials, seeds and other plant samples for the specific virus infection or its latent presence include electron microscopy and immunological detection like ELISA, PCR and microarray. The viral diseases result in the spread of viruses under favourable conditions by causing either global damages or severe local losses. The conventional agronomic practices for virus control include the use of virus free stock/propagule, roughing of diseased plants, control of alternative vector or hosts and implementation of quarantines. Molecular diagnosis is of significance for the diseases that are not easily diagnosed by symptoms or are symptomless or similar symptoms are produced by other viruses as well. The several techniques known for viral disease diagnosis have advantages and limitations.

7.2 Serological/Immunological Techniques

Immunological tests are used to test the viral antigens by cross-reaction with antisera containing antigen-specific antibodies. These antigen-specific antibodies are raised in a model animal by the introduction of foreign/viral proteins into the bloodstream of animal. These antibodies can be polyclonal or monoclonal antibodies which are available for economically significant viruses both at laboratories and commercially. Monoclonal antibodies have been extensively used for the detection of plant viruses such as potyviruses (Karyeija et al. 2000; Balamurlikrishnan et al. 2002; Desbiez et al. 2002; Ounouna et al. 2002; Villmor et al. 2003; Crosslin et al. 2005).

7.2.1 Production of Antibodies

There are three major ways for the production of antibodies for diagnostics.

Polyclonal antibodies are raised by introducing the pathogen extracts into an animal (usually rabbit) followed by the collection of blood after some time. The blood sample collected is allowed to clot and serum thus produced contains antibodies against the protein(s) in the extract injected. Such antibodies are directly used from isolated serum or can be further used for purification of IgGs (Dijkstra and DeJager 1998). The genus- and strain-specific antibodies enable to differentiate the virus at generic level as well as can differentiate the viruses of the same species at the strain level as used for *Potato virus Y* (Bhat et al. 1997), *Bean common mosaic virus* and *Blackeye cowpea mosaic virus* (Khan et al. 1990).

Plant viruses have been successfully detected using polyclonal antibodies, but their utility is compromised as sometimes they are cross-reactive, specificity is variable across batches, and they are produced in limited quantities. Viral proteins are also expressed in bacteria to raise the polyclonal antibodies (Bragard et al. 2000; Hema et al. 2003; Fajardo et al. 2007; Yadav and Khan 2009). Unlike conventional method of raising antibodies against structural protein, bacterial expression system provides a chance to raise the antibodies against nonstructural proteins (replicase, HC-Pro, etc.) instead of structural protein (capsid or coat protein) (Riedel et al. 1998; Joseph and Savithri 1999; Rodoni et al. 1999).

Monoclonal antibodies are produced by hybridoma technology. Antibody secreting plasma cells are isolated from the spleen of

primed animal and fused with cultured myeloma cells. It generates multiple hybrid cell lines called hybridomas. Every hybridoma produces antibody against an epitope of the antigen called monoclonal antibodies. The individual cell lines are propagated to harvest single monoclonal antibodies. Therefore, hybridoma technology provides antibody more specific to antigen and maintains its unlimited supply. Major limitations of monoclonal antibodies are slow production, cost ineffectiveness and maintenance of hybridomas that may undergo apoptosis or fail to secrete antibody. However, despite its limitations, monoclonal antibodies are extensively used in the diagnosis of plant pathogens including viruses (Gonsalves 1979; Karande et al. 1998; Torrance 1995).

7.2.2 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA is the most favourite immunodiagnostic method because of its simplicity, accuracy and low cost. It is based on the specificity of antibodies to interact with proteins, mostly the capsid protein of target virus. The microtitre plate is coated with the extract from virus-infected plant which is then detected with primary antibodies. The target virus if present in the plant sap will interact with primary antibodies. Before adding secondary antibodies, any traces of the unbound primary antibody are washed off. The secondary antibodies are already conjugated with the reporter molecule, commonly an enzyme, to enable the detection of the virus by producing the chromogenic product which acts on substrate. The quantity of the chromogenic product is determined by the spectrophotometer corresponding indirectly to the viral load (Clark and Adams 1977). Therefore, ELISA enables both quantitative and qualitative analyses. On the basis of order of antigen and antibodies added in the detection reaction, the ELISA can be of following types: antigen-coated (Clark et al. 1988; Cooper and Edwards 1986; Van Regenmortel and Dubs 1993) and sandwich ELISA (Fig. 7.1).

The antigen-coated ELISA (Fig. 7.1a) uses viral antigens bound to the surface of well of microtitre plate which can be detected directly using enzyme-conjugated primary antibodies

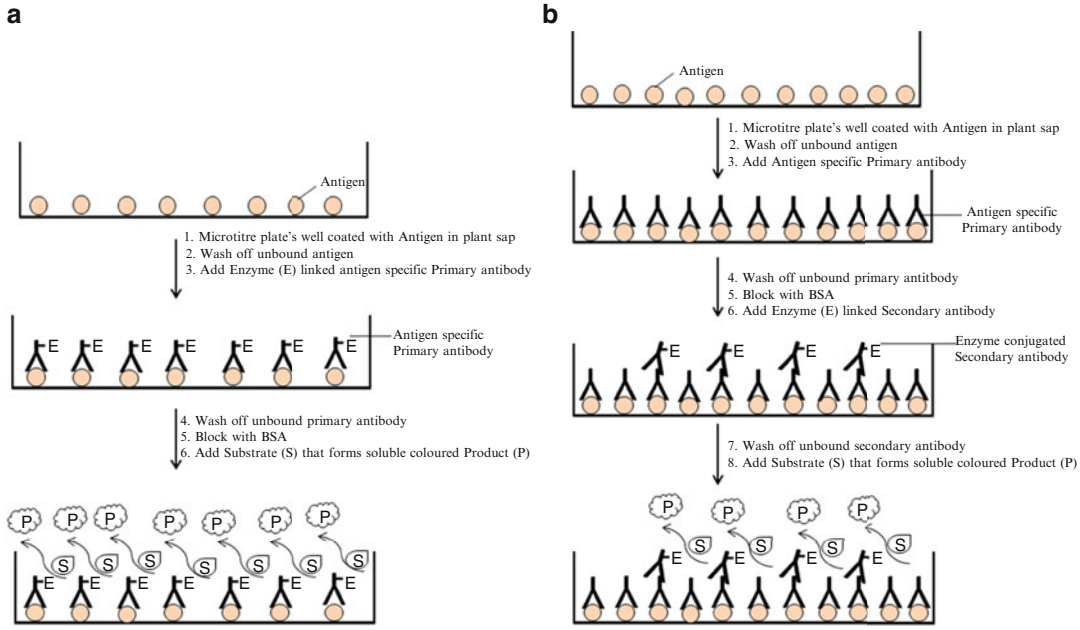

Fig. 7.1 Antigen-coated ELISA: (**a**) Direct ELISA and (**b**) indirect ELISA

followed by colour development by the addition of substrate; this is direct ELISA. It can also be done using indirect ELISA, where viral antigens coated on the surface of well of microtitre plate are initially detected by primary antibodies against target antigen, which is subsequently detected by enzyme-linked secondary antibodies followed by colour development by the addition of substrate. Secondary antibodies used are against the constant region of primary antibodies; e.g. for primary antibodies against viral antigen produced in rabbits, it will use enzyme-linked anti-rabbit IgGs raised in other mammals like bovine, horse and goat.

Another approach is sandwich or double-antibody sandwich (DAS)-ELISA where the viral antigen is sandwiched between two antibodies raised against uncommon epitopes (Fig. 7.2a). DAS-ELISA is highly strain-specific procedure that needs enzyme-conjugated antigen-specific antibody for detection. Another popular method is triple-antibody sandwich (TAS)-ELISA similar to DAS-ELISA, except that an additional step is involved before adding enzyme-conjugated secondary antibody specific to constant region of primary antibodies (Fig. 7.2b). Alkaline phosphatase and horseradish peroxidase are most common enzymes used as the reporter molecules in enzyme-conjugated antibody.

Utilizing multiple wells of the microtitre plate ELISA can enable testing different plant samples for the presence of a virus or single plant for multiple virus infections (Van den Heuvel and Peters 1989; Smith et al. 1991). ELISA tests are highly virus specific (Banttari and Khurana 1998). The accuracy of the test can be improved by performing the same reaction in duplicate or triplicate in the same microtitre plate. Such reactions can be performed with higher precision only if the polyclonal/monoclonal antibody is raised against the target viral protein under consideration and does not cross-react with host plant proteins. The presence of contaminant host protein(s) in purified viral protein to be used for antiserum generation results in antibodies that are cross-reactive as polyclonal antiserum is able to identify the host as well as viral pathogen epitopes. So in such cross-reactions, the antibodies should be cross adsorbed with healthy plant sap so that antibodies against the plant proteins are removed before using such polyclonal antisera for ELISA.

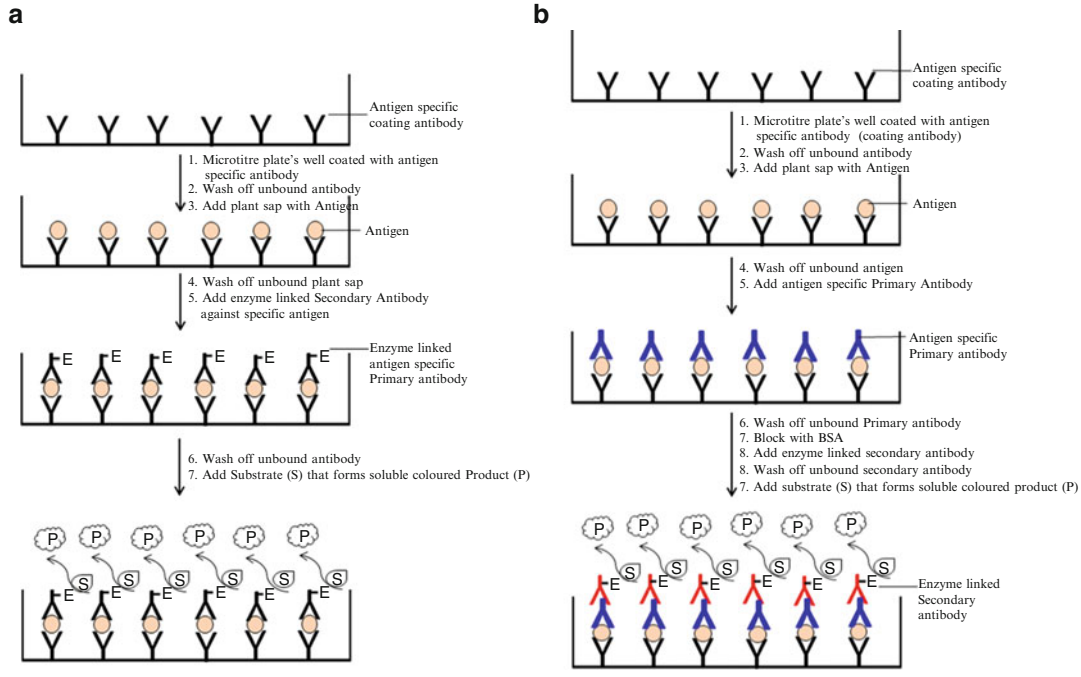

Fig. 7.2 Sandwich ELISA: (**a**) DAS ELISA and (**b**) TAS ELISA

Variant of ELISA, such as voltammetric enzyme immunoassay, is based on the detection of modulation in electrical conductivity of the substrate, instead of chromogenic detection. It is more sensitive than the conventional ELISA and has been used for the detection of CMV (Sun et al. 2001).

7.2.3 Phage Display

Phage display is a powerful technique to capture proteins/viral antigens that interact with phage surface-displayed antibody fragments. Mostly plant virus diagnostic assays make use of PAbs or MAbs. MAbs recognize single epitope of viral antigen and thus give less cross-reactions to discriminate between viral strains (Torrance 1995). MAbs generated against conserved epitopes can be used to group virus isolates for epidemiological and taxonomical studies.

Antibody fragments are made up of heavy and light chains. The antigen binding domains contain hyper-variable sequences that provide the specificity to the antibodies. Phage display is another method of producing antibodies for immunodiagnostic assays of plant pathogen. It uses libraries that express PCR-amplified functional single-chain variable fragment (scFv) or antigen binding fragment (Fab) of antibody molecules obtained from animals including human (Hoogenboom et al. 1991; McCafferty et al. 1990). Thus bacteriophage displaying functional antibody fragments provides a selection system with high fidelity to obtain specific MAbs from combinatorial phage-antibody libraries without the use of laboratory animals (Clackson et al. 1991).

This technique have been utilized for optimization of immunodetection assays for phytopathogens including plant viruses, such as *Blackcurrant reversion associated virus* (Susi et al. 1998), *Potato virus Y* (Boonham and Barker 1998), *Beet necrotic yellow vein virus* (Griep et al. 1999; Uhde et al. 2000), *Tomato spotted wilt virus* (Griep et al. 2000), *Rice black-streaked dwarf virus* (Bai et al. 2002) and *Banana streak virus* (Heng et al. 2007). Several antibodies raised against plant pathogens are used as capture and detection reagent in DAS-ELISA or in immunofluorescence (Fig. 7.3).

This technique enables to mimic the immune system by display of antibody fragments on filamentous bacteriophage on the minor coat protein to detect/isolate low concentrations of peptides/proteins through sequential cycles of phage growth and selection via binding to antibody (Kushwaha et al. 2013). For isolation of species-specific antibodies, phage display has advantages over PAbs/MAbs as it is a fast, cost-effective procedure with continued supply of antibodies (Ziegler and Torrance 2002).

7.2.4 Tissue Blot Immunoassay (TBIA)

TBIA is similar to ELISA as it requires antibodies raised against virus or viral antigens. Plant tissue is pressed onto the nitrocellulose or nylon membranes to make an impression blotting paper which is used to detect the virus using labelled probes usually chemiluminescent. The main advantages associated with the procedure over ELISA include less labour intensive. Also it is a simple (requiring no virus extraction), rapid, sensitive and inexpensive method. This enables the survey of thousands of plant samples in a day providing an option to collect the sample on blotting paper in field that can be processed later (Webster et al. 2004).

Commercial detection kits are available from the International Centre for Agriculture Research in the Dry Areas (ICARDA) for the detection of a variety of viruses infecting legume crops based on polyclonal antisera including *Chickpea chlorotic stunt virus* (CpCDV), *Cucumber mosaic virus* (CMV), *Alfalfa mosaic virus* (AMV), *Bean yellow mosaic virus* (BYMV) and *Pea seed-borne mosaic virus* (PSbMV) and few monoclonal antibody-based kits such as for *Beet western yellows virus* (BWYV), *Bean leafroll virus* (BLRV) and *Soybean dwarf virus* (SbDV) (Katul 1992; Kumari et al. 2006, 2008; Makkouk and Kumari 1996).

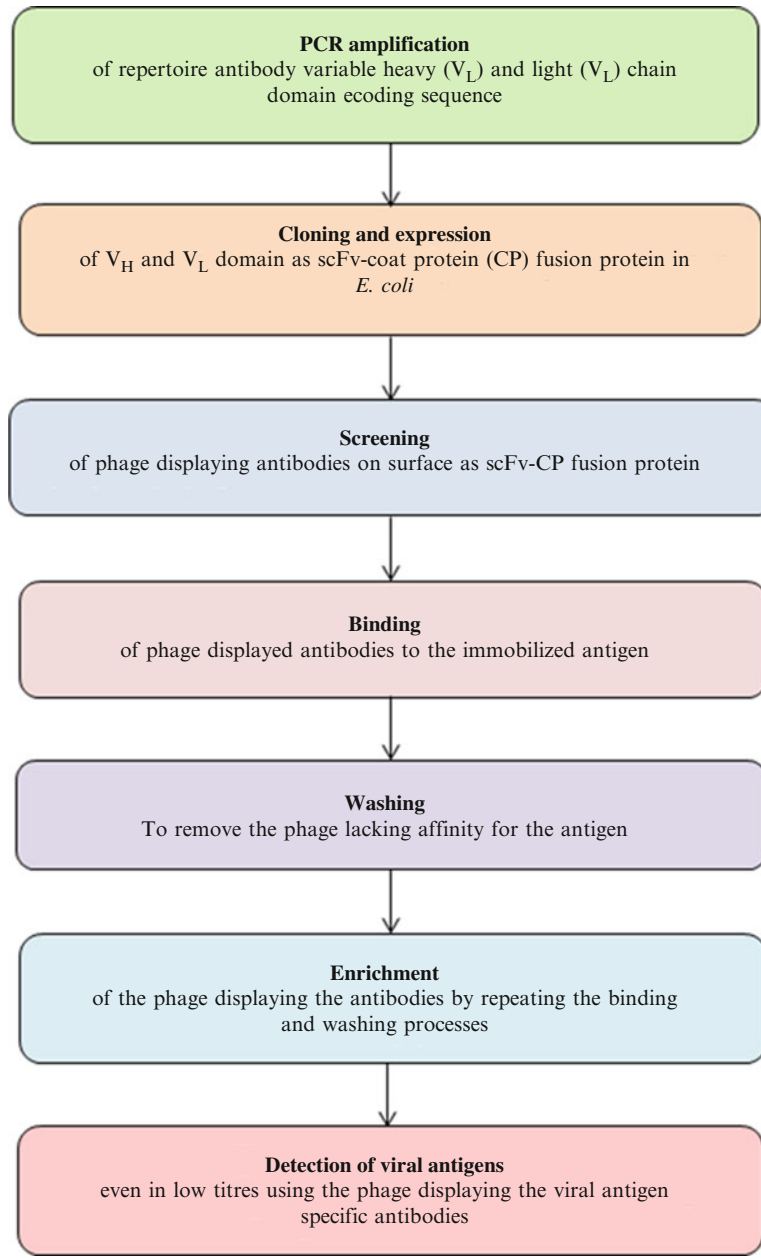

Fig. 7.3 Workflow of the phage display for the plant virus diagnosis

7.2.5 Quartz Crystal Microbalance (QCM) Immunosensors

The principle of QCM is that the adsorption of any substance on a quartz crystal surface modulates its resonance oscillation frequency (Cooper and Singleton 2007; Vashisht and Vashisht 2011). This technique utilizes virus-specific antibodies coated on quartz crystal disc (0.5 in. in diameter) for plant virus diagnostics. Purified virus or the crude sap from infected plant is allowed to bind on the immobilized antibodies. A voltage is applied across the disc that warps the disc slightly under a piezoelectric effect. Interaction of specific viral antigen with coated antibody leads to the increase in mass at surface of QCM which reduces the frequency of resonance oscillation. Change in frequency of resonance oscillation is

directly proportional to the amount of virus interacted with immobilized antibody on the quartz crystal disc (Fig. 7.4).

Therefore, it is a rapid, economic and sensitive method suitable for qualitative and quantitative detection. The first report describing QCM method detected as little as 1 ng of *Cymbidium mosaic virus* and *Odontoglossum ringspot virus* particles in crude extracts (Eun et al. 2002). *Tobacco mosaic virus* has also been detected successfully using QCM from sap of infected plants (Dickert et al. 2004).

7.2.6 Lateral Flow Immune Assay (LFIA)

LFIA is a rapid immunochromatographic technique applied to plant virus detection mainly for viruses of vegetable and floriculture crops (Danks and Barker 2000; Salomone and Roggero 2002; Salomone et al. 2002, 2004).

The major attractions associated with LFIA are easy to perform even by inexperienced person by the use of very simple device and allow infield detection of viruses generating quick results.

Thus, it has been used reliably to develop kits for plant virus diagnostics.

Lateral flow enables the mass express diagnosis of viral infections for virus-free plant culture industry or individual use requires inexpensive, rapid and simple technologies that make possible analysis without special skill and equipment even under field conditions. The principle of LFIA is based on immunoreaction between virus in question with antibodies against viral proteins and chromogenic colloidal substances applied on lateral flow devices that are marketed as test strips. When lateral flow device/strip is spotted with the infected/symptomatic plant tissue extract/sap, the liquid sample flows through it and initiates antigen-antibody interaction leading to the chemical reaction that is visible as chromogenic bar/line for test and control samples. Byzova et al. (2009)

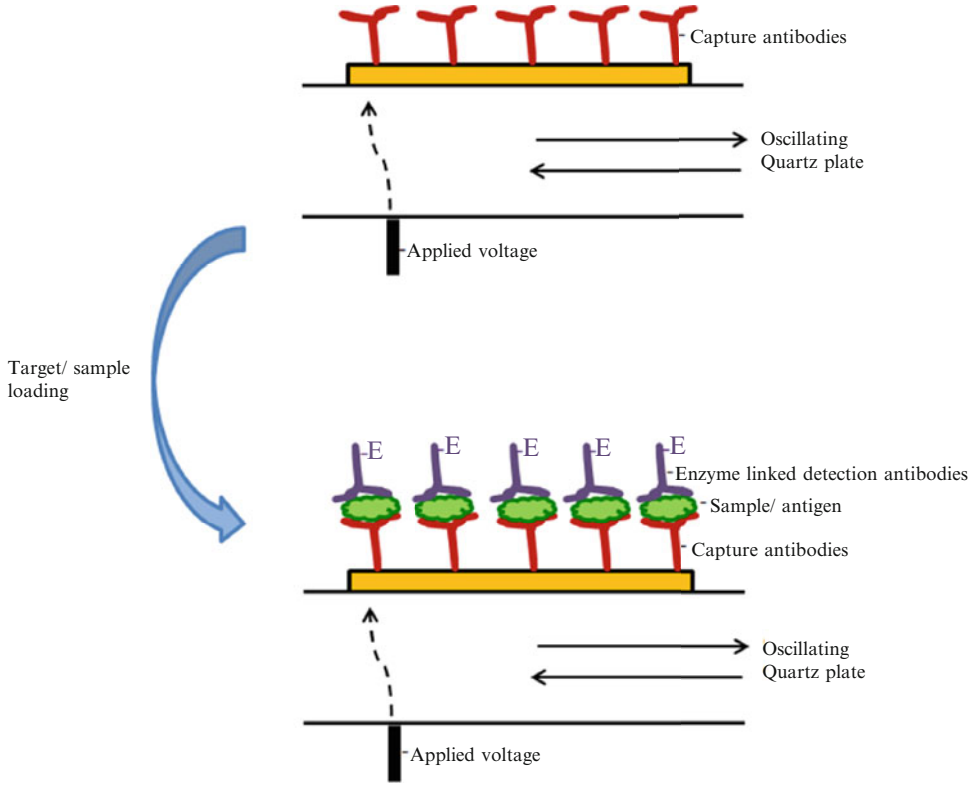

Fig. 7.4 QCM workflow showing binding of the target antigen/virus on immobilized antibodies causes a frequency change in the oscillation of the quartz crystal (Adapted from Grimme and Cropek 2013)

developed a test strip to detect five plant viruses simultaneously which are different in shape and size, namely, spherical (*Carnation mottle virus*, *Bean mild mosaic virus*), rod shaped (*Tobacco mosaic virus*: TMV) and filamentous (PVY, PVX) viruses. In India, CPRI has developed LFIA devices for the detection of five potato viruses, viz. PVX, PVA, PVS, PVM and PVY, either individually or in combination of two viruses, viz. PVA and PVX, PVA and PVS and PVY and PVM. Few recent reports on plant viruses using LFIA include the detection of PVX (Drygin et al. 2011; Safenkova et al. 2012), TMV and CMV (Lamptey et al. 2013).

7.2.7 Immunocapture Transmission Electron Microscopy (ICTEM)

ICTEM is a technique that combines the serological specificity and electron microscopy (Derrick 1973). It has been used for a wide variety of polyhedral and rod-shaped plant viruses for their detection by combining serological specificity for morphological visualization of virus particles (Lima and Purcifull 1980; Banttari et al. 1991; Milne 1991; Naidu and Hughes 2001). ICTEM has been used for the detection of low titres of viruses in potato nucleus stocks/mericlones, etc. (Khurana 1990). The ultrathin sections in electron microscopy add high sensitivity and specificity to morphological determination of virus (Khurana and Garg 1993). The process is based on the selective entrapment of virus particles from samples almost/completely devoid of host material, on grids coated with virus-specific antibodies (Lima and Purcifull 1980).

The electron microscopy uses copper grids with thin-layer Parlodion that are coated with 1000–6000-fold diluted virus-specific antibodies. The excess of antibody is washed off and treated with diseased plant tissue extract for 3–4 h at 25 °C. The grid is then washed thrice, stained with 1.0 % uranyl acetate in 50 % ethanol and dried followed by final scanning in electron microscope (Fig. 7.5).

The main advantage with the process is the requirement of small quantities of antibodies and viruses; moreover, labelling of antibodies is not required. It is a highly sensitive method for the detection and diagnosis of virions in leaf extracts in comparison with leaf dip method (Lima and Purcifull 1980). The additional advantages of using polyclonal antiserum has no interference with the virus particle visualization and immobilization of virus particle on grids that can be used even later for electron microscope analysis. The use of an electron microscope for the analysis of sample makes it suitable for small-scale samples for confirmation of the virus. Immunoelectron microscopy was found to detect twice as many banana streak-causing viruses from Nigeria and Ghana than detected through ELISA or IC-PCR (Agindotan et al. 2006).

7.3 Restriction Fragment Length Polymorphism (RFLP)

Combinatorial use of RFLP and PCR has enabled the identification of differences among the amplified virus genomes/genes by the presence or absence of restriction site(s) of a particular restriction enzyme within the amplified sequence. Initially PCR amplification is performed to yield amplified DNA followed by digestion with restriction enzyme(s) and analyses of the size of the fragments obtained by gel electrophoresis. Thus, RFLP enables to differentiate the virus isolates even without cloning and sequencing. This method accurately and effectively demonstrates the polymorphisms among the virus genomes/genes. The subgroup 2 of CMV in Western Australian lupin crops were detected and diagnosed using RFLP (Wylie et al. 1993).

The RFLP has been used to distinguish the related viruses and viroids including diversity among *African streak mastreviruses* (Willment et al. 2001) and *Citrus tristeza virus* isolates using coat protein nucleotide sequences (Corazza et al. 2012), for the screening of potato seeds for *Potato virus* M (Xu et al. 2010) and for the detection and discrimination of *Pospiviroids* (Luigi et al. 2014).

RFLP followed by PCR has been used to distinguish the 18 isolates of PVY (Glais et al. 1996), for large-scale screening of potato plants and tubers for *Alfalfa mosaic virus* (Xu and Nie 2006), to distinguish different strains of *Soybean*

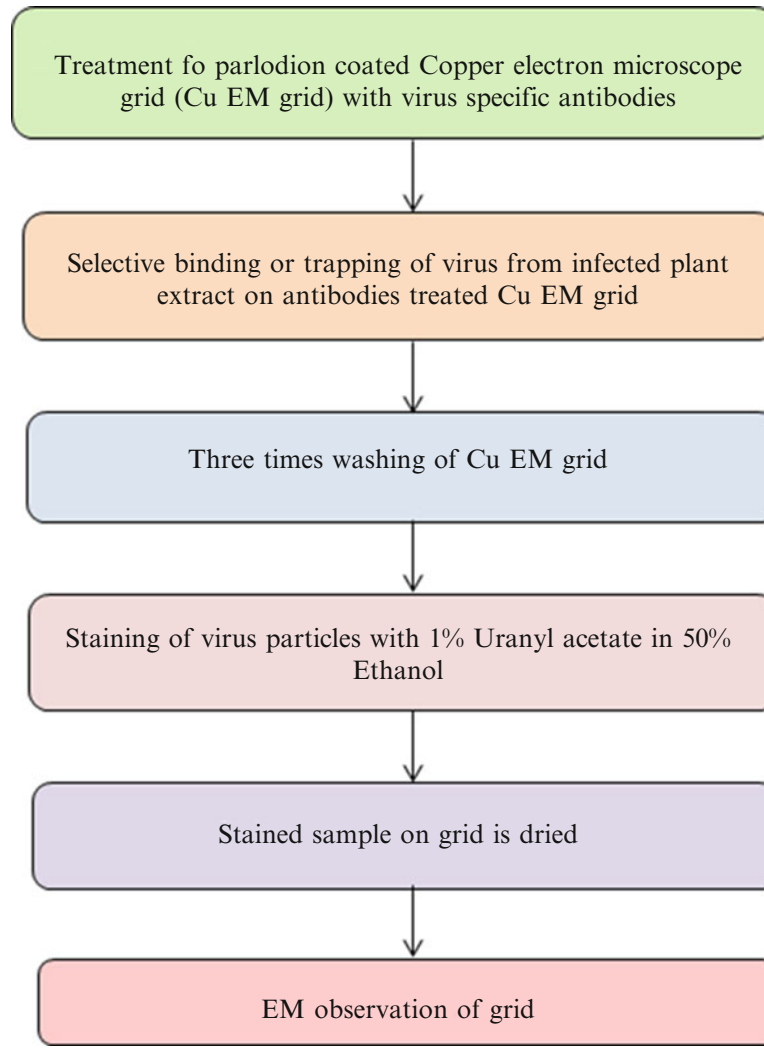

Fig. 7.5 Workflow of ICTEM

mosaic virus (Kim et al. 2004), for the detection and discrimination of *Melon necrotic spot virus* (MNSV) (Kubo et al. 2005) and for the identification and distinction of two strains of *Grape fan leaf virus* (GFLV) in Tunisia based on coat protein region (Fattouch et al. 2005) and viroids infecting pome fruits (Hadidi and Yang 1990).

7.4 Thermostable Amplification-Based Methods

PCRs and reverse transcriptase polymerase chain reactions (RT-PCRs) are the most popular thermostable amplification-based techniques for the detection and diagnosis of DNA and RNA plant viruses, respectively (Fig. 7.6).

These procedures are highly sensitive in comparison with serological methods and suitable for routine detection of plant viruses and hence have been widely applied for molecular detection. These methods are efficient in specific detection of the virus through sequence-specific primers, so availability of sequence information is a prerequisite. Also, with the growing number of sequence information available in the database, access to the viral genome sequences is now easy and can be utilized for designing the viral species or strain-specific primers (Chen and Adams 2001; Gibbs and Mackenzie 1997; Langeveld et al.

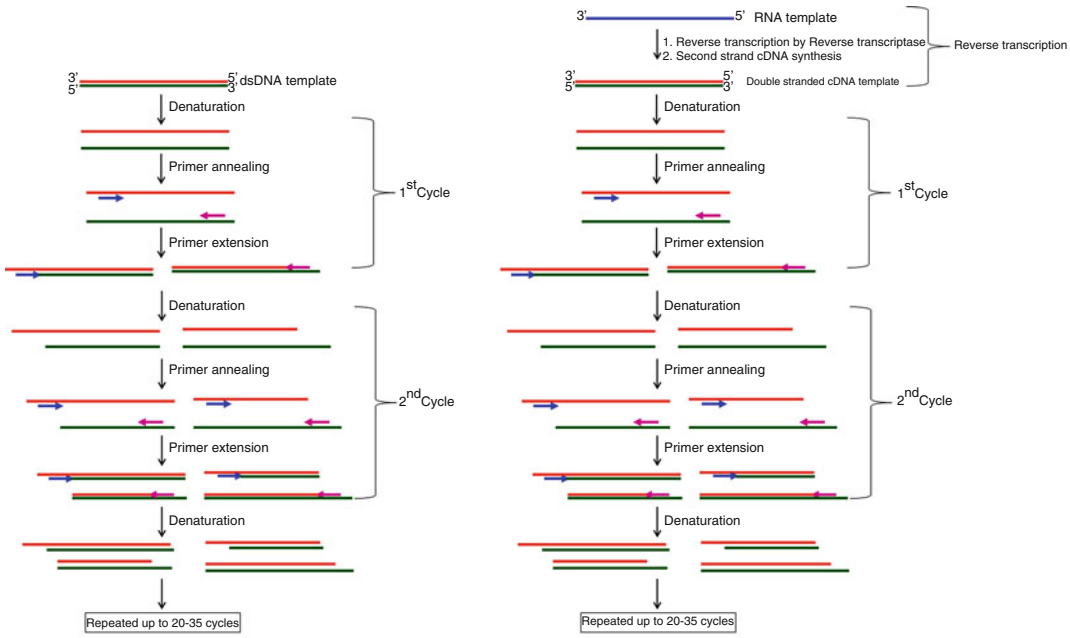

Fig. 7.6 Diagrammatic representation of (**a**) PCR and (**b**) RT-PCR

1991; Zheng et al. 2010). Being a highly sensitive technique, it essentially requires positive or negative controls to avoid false negatives or positives. PCRs are simpler and effective than ELISA as it is easy to design and synthesize new primers than the production of novel antibodies. Moreover PCR products if required can be cloned into suitable cloning vector and sequenced. Certainly these methods have stringent polymerization conditions and are performed at higher temperatures which utilize thermostable polymerase like *Taq* DNA polymerase from *Thermus aquaticus*. A variety of alternatives to *Taq* DNA polymerases are now available; the advantages related to them include *Thermococcus litoralis* (*Til*) polymerase with high fidelity than *Taq* polymerase, *Thermus thermophilus* (*Tth*) polymerase having DNA-/RNA-dependent DNA polymerization activity and *Sulfolobus acidocaldarius* (*Sac*) DNA polymerase have being identified to be highly thermostable than *Taq*.

The prerequisite for the optimization and development of any PCR-based diagnostic assay is to identify the DNA sequences for designing of primers enabling efficient and specific detection of the target pathogen. Virus genomes are relatively small; therefore a number of viral genomes have been completely sequenced and submitted to nucleotide databases. Coat protein gene is a highly preferred target sequence for virus diagnostics. However, other regions of virus genome have also been used for diagnostics providing significant sequence data from related viruses on the same region available on the database. RNA virus assay involves additional step to convert genomic RNA to cDNA before PCR for detection. Nucleic acid-based diagnosis uses two approaches: the first approach is based on targeting of known gene sequences in target and non-target viruses followed by the identification of unique regions to design primers, while the second approach is based on the use of randomly selected DNA fragments. PCR-based diagnostic methods have certain limitations. They require prior sequence information of target viruses to design primers and a thermocycler. Intelligent primer designing is critical for the successful detection of single or multiple strains of a genus. Table 7.2 summarizes the list of plant viruses and viroids identified by PCR.

Table 7.2 Short list of common viruses and viroids detected by PCR

Organism/agent	Hosts	Target sequence/sample treatment	Comments
Viroids			
Hop stunt	Hops	Viroid sequence; total nucleic acid extracted from infected host tissue	Amplification and sequencing of viroid cDNA
Apple scar skin Dapple apple Pear rusty skin	Pome fruits	Viroid sequence; total nucleic acid extracted from infected host tissue	Amplification of viroid cDNA
Citrus exocortis Cachexia Citrus viroid IIa	Citrus	Viroid sequence; total nucleic acid extracted from infected host tissue	Amplification of viroid cDNA
Viruses			
Cauliflower mosaic virus (CaMV)	Cabbage	CaMV sequence; DNA extracted from single aphids fed on infected plant tissue	Detection of viral DNA in a single aphid; *Caulimovirus*
Bean golden mosaic virus (BGMV)	Bean	BGMV sequence; DNA extracted from infected bean	Detection and sequencing of viral DNAs; *Geminivirus*
Cucumber mosaic virus (CMV)	Cucumber	CMV sequence; total nucleic acid from infected cucumber	Sequence amplified with *Taq* polymerase; *Cucumovirus*
Tomato ringspot virus (ToRSV)	Cucumber	ToRSV sequence; total nucleic acid from infected cucumber	Sequence amplified with *Taq* polymerase; *Nepovirus*
Tobacco mosaic virus (TMV)	Tobacco	Transport protein gene sequence; RNA extracted from infected leaf tissue	Sequence amplified with *Tth* polymerase; *Tobamovirus*
Luteoviruses	Cereals, sugar beet, potato	Conserved luteovirus sequences; total plant nucleic acid extracts	Detection of viral cDNA of barley yellow dwarf, beet western yellows and potato leaf roll luteoviruses Differentiation by RFLP analysis
Bean yellow mosaic virus (BYMV)	Gladiolus	BYMV-specific sequences; total plant nucleic acid extracts	Detection of viral cDNA in leaves and corns; *Potyvirus*
Pea seed-borne mosaic virus (PSbMV)	Pea	PSbMV-specific sequences; total plant nucleic acid extracts	Detection of viral cDNA; *Potyvirus*
Plum pox virus (PPV)	Pome fruit	PPV-specific sequences; total plant nucleic acid extracts	Detection of viral cDNA using biotin label in PCR reaction; *Potyvirus*
Potyviruses	Various	Conserved potyvirus sequences; extracts from purified virus or total plant nucleic acid extracts	Detection of viral cDNA, used degenerated primers
Cherry leafroll virus (CLRV)	Walnut	CLRV-specific sequences; crude tissue extract	Detection of viral cDNA; sample not treated with phenol or protease; *Nepovirus*

Modified after Henson and Roy (1993)

7.4.1 PCR and RT-PCR

The major breakthrough in detection, discovery and characterization of viruses was with the discovery and use of PCR (Mullis et al. 1986; Mullis and Faloona 1987). It has been successfully used to characterize viruses, utilizing sequence information available for the viruses of related genera or families (Chen and Adams 2001; Yadav and Khan 2008). It is a highly sensitive and specific diagnostic procedure for the detection of virus that relies on the presence of unique sequences in the virus genome (Bartlett 2003; Waterhouse and Chu 1995). In viruses containing RNA as the genome, a first-strand cDNA is synthesized with the help of reverse transcriptase (RT) enzyme, which in turn can be used a template for in vitro amplification using heat-stable polymerase enzyme. The PCR and RT-PCR have been widely employed successfully for the discovery and characterization of plant viruses. For the broad detection of virus infections in the plant samples, group-specific degenerate primers are utilized (Martin et al. 2000; Zheng et al. 2010). The degenerate primers are designed to target the conserved sites in the known sequences of viral genera. The conserved blocks or sites in the sequences are found by comparing the available sequences of related genera or families through the bioinformatics. Degenerate primers targeting the conserved sites in the genome of potyviruses, geminiviruses, closteroviruses, badnaviruses, etc., have been widely used for the initial screening and characterization of new variants or species infecting diverse crop species (Anderson et al. 2004; Baranwal et al. 2010; Yang et al. 2003). The amplification of specific portion of the viral genome obtained in PCR or RT-PCR needs to be sequenced to confirm its exact identity. The most commonly used sequencing strategy has been the capillary electrophoresis-based Sanger's dideoxy chemistry.

Multiple variations of the basic technique have been designed to improve the sensitivity and specificity with facilitated automation for the detection of plant viruses (Waterhouse and Chu 1995).

7.4.2 Multiplex PCR/RT-PCR

Multiplex PCR procedures, invented by Chamberlain et al. (1988), had revolutionized the PCR-based molecular detection of plant viruses. Such procedures were developed for convenient and sensitive detection of several DNA/RNA viruses, in one reaction/tube simultaneously (Rosenfield and Jaykus 1999; Nassuth et al. 2000; James 1999). The technique is very useful where plant viruses exist as multiple or mixed infection in the same host. Multiplex PCR involves the use of many sets of oligonucleotides targeting the genomic regions of multiple viruses (within the same genus, strains of the same species or viruses from taxonomically different groups) in one PCR amplification reaction mixture. Primers are designed in such a way that amplification can be carried out at the same annealing temperature and do not have complementarity with other primers. The PCR products of different sizes from various targets ensure easy analysis of amplicons by agarose gel electrophoresis. The multiplex PCR thus has advantages of saving reagents against individual PCR, convenient, rapid and cost-effective. This technique has been applied for the detection of a varied range of plant viruses (Deb and Anderson 2008; Nie and Singh 2000; Roy et al. 2005).

Thus, multiple species or strains of multiple viruses from different plant samples are detected in a single tube by adding the specific primers. Multiplex PCR is of great utility in plant virology as different RNA viruses are known to infect a single host and thus need sensitive detection methods of such viruses to raise virus-free propagules.

While performing the multiplex PCR, one must keep in mind that the amplified targets should be of different lengths/sizes also different primer pairs used should neither share any complementarity nor cross-reactivity/specificity.

Ito et al. (2002) have reported the multiplex RT-PCR assay for screening of diseased citrus for the detection of six citrus viroids and *Apple stem grooving virus* (ASGV) or *Citrus tatter leaf virus* (CTLV). Also it has been used for simultaneous

detection of five different viruses from diseased tobacco plants (Dai et al. 2012). It has also been successfully used for the one-step-one-tube detection of four viroid genera, *viz. Apscaviroid*, *Pelamoviroid*, *Pospiviroid* and *Hostuviroid* (Ragozzino et al. 2004), as well as for ascertaining the presence of eight viruses in suspected stone fruit trees (Sanchez-Navarro et al. 2005) and their effective amplification through the multiplex PCR has been reported. Recently, Kwon et al. (2014) using dual priming primer sets developed two multiplex PCR systems for the simultaneous detection of seven different viruses in cucurbits. The first system efficiently enabled to detect *Papaya ringspot virus*, *Watermelon mosaic virus* and *Zucchini yellow mosaic virus* whereas the other one was to detect *Cucumber green mottle mosaic virus*, *Cucumber fruit mottle mosaic virus*, *Kyuri green mottle mosaic virus* and *Zucchini green mottle mosaic virus*. The use of multiple primer sets designed against various target sequences in the same reaction saves time and reduces running cost of the optimized multiplex PCR assays (Bertolini et al. 2001; Walsh et al. 2001).

7.4.3 Immunocapture PCR (IC-PCR)

This variant of PCR combines advantages of serology to capture virus particles by antibodies and PCR to target a molecule using specific primers (Fig. 7.7). The process begins with the adsorption of the virus on antibody-coated microtitre plate or microfuge tube which is then removed by heating in the presence of Triton X-100, a non-ionic surfactant, followed by amplification of nucleic acid using RT-PCR. Capturing of viruses by antibody helps to purify the virions from the plant sample even when the titre of virus is low or when nucleic acids extracted have certain inhibitor compounds that hamper PCR.

This procedure is of significance for the diagnosis of plant viruses with very low concentration utilizing the sensitivity and accuracy of both immunological and molecular approaches. The virion particles trapped in tubes using the specific antisera are used for PCR amplification as template, providing a sensitive tool for the detection of plant viruses having low titre in diseased host or even the viral genomes integrated with genome of the host (LeProvost et al. 2006).

IC-PCR has been used for its high sensitivity and specific detection of PPV in plum tree saps (Wetzel et al. 1992) and *Sugarcane streak mosaic virus* from sugarcane sap (Hema et al. 2003). Harper et al. (1999a) reported the efficient detection of the episomal *Banana streak virus*, known to have its genome present naturally within the genome of banana through IC-PCR, which otherwise results in higher false positives upon conventional PCR. The IC-PCR has been

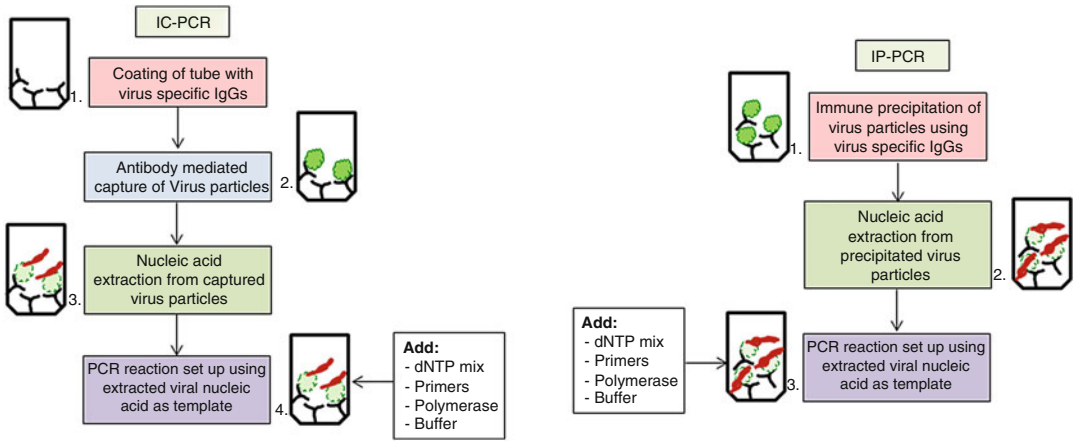

Fig. 7.7 Schematic representation of IC-PCR and IP-PCR

successfully used to validate the presence of many viruses, viz. *Pepper mild mottle virus* (PMMV), BYMV, CLRV, CMV, CTV, GFLV, PLRV and TSWV along with CMV satellite RNA (Narayanasamy 2011).

Thus, IC-PCR involving virus immobilization by antibody has been used for the identification and molecular characterization of plant viruses of various families and genera to assign appropriate taxonomic position. The major advantage of this highly specific technique includes no encounter with problems associated with RNA extraction through the combination of serological efficacy and nucleic acid amplification of many viruses, viz. PVY, tobamoviruses, etc. (Jacobi et al. 1998; Mulholland 2009; Gawande et al. 2011).

7.4.4 Immuno-precipitation PCR (IP-PCR)

IP-PCR is the modification of PCR technology involving the precipitation of virus particles via antibodies (Fig. 7.7). Like IC-PCR, it has been used for the identification and molecular characterization of plant viruses of various families and genera enabling to taxonomic positioning (Lima et al. 2011). The major advantage of this technique is its specificity and no requirement of RNA extraction. So it possesses the advantage of serological specificity and PCR amplification, making it a quick and specific detection method (Fig. 7.8).

The process begins with the precipitation of the virus particles by polyclonal antibodies followed by RNA extraction, and first-strand cDNA is synthesized and amplified by PCR. IP-PCR reduces the co-contamination of plant RNAs. This technique is based on partial virus purification by immuno-precipitation; thus it is especially significant for efficient detection of viruses present in low or variable titres in plant species containing PCR amplification inhibitors. It has remarkably contributed in the detection of various plant viruses. IP-PCR has been used to detect *Papaya lethal yellowing virus* (PLYV) in 10,000-fold diluted tissue extracts of the diseased plants without amplifying host cDNA from healthy plant extracts (Lima et al. 2012). IP-PCR because of its sensitivity and specificity has been used efficiently to detect the five virus species of different families, viz. Bromoviridae, Comoviridae, Potyviridae and Sobemoviridae (Lima et al. 2011).

7.4.5 Nested PCR

It is a modification of PCR that reduces non-specific amplification of products due to the additional primer binding sites and is completed by performing two PCR reactions using two sets of primer pairs. The first reaction is usually carried out with external primers pair to increase the concentration of target sequence which in turn acts as a template in the second PCR reaction. The external primers are mostly degenerate primers with low specificity. The second step PCR reaction is set up using an aliquot of the amplification product from the first round and the internal primers which necessarily anneal at a target sequence internal/within the first-round amplicon (Fig. 7.9). Hence, it has been reported for the successful detection of species belonging to *Vitivirus* and *Foveavirus* in grapevines using nested PCR (Dovas and Katis 2003).

Therefore, this approach is profound particularly for the infections where the viral load/titre is very low; also it is capable of increasing the target DNA molecule as well as results in dilution of inhibitors of DNA polymerase that are hard to remove from plant extract with nucleic acid purification (Fig. 7.9).

7.4.6 Multiplex Nested PCR

It is a single-tube reaction optimized for the sensitive and parallel detection of few viruses (such as CMV, CLRV, SLRSV, ArMV) and bacterium from diseased olive plant samples using 20 sequence-specific primers compatible with the target sequence (Bertolini et al. 2003). This method combines in a tube the sensitivity and accuracy of nested PCR with the advantage of simultaneous detection of multiple targets of

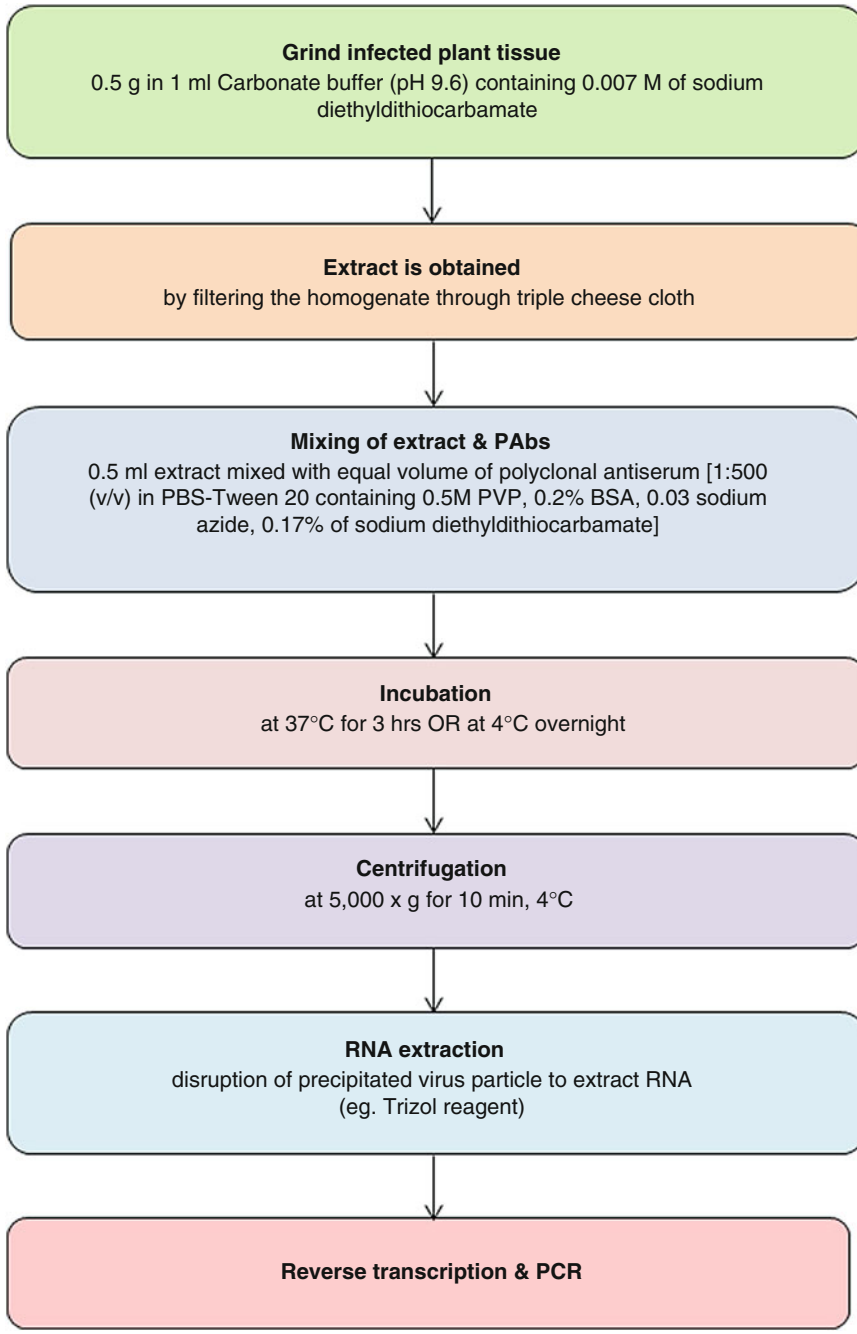

Fig. 7.8 Schematic representation of IP-PCR method

multiplex RT-PCR, thus making it a powerful tool to detect multiple viruses, bacteria and phytoplasmas (Biswas et al. 2013) simultaneously from angiosperms in one tube.

This is a cost-effective and time-saving method which works in a single tube but requires critically designed accurate internal and external primers. These external and internal primers used in multiplex PCR should not interfere with the

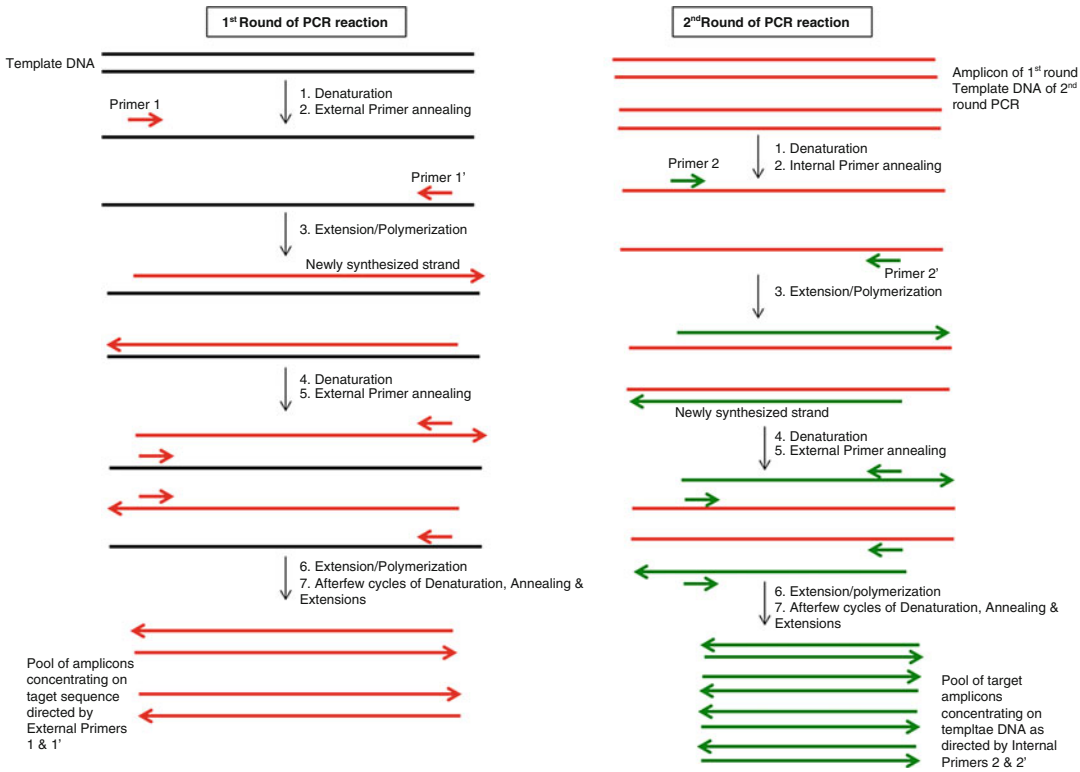

Fig. 7.9 Schematic representation of nested PCR

amplification process. Once the first round of amplification is completed with external sets of primers, the second round of the PCR is performed using internal sets of primers and amplicon of the first round of PCR as template to have nested multiplex amplification. Relatively higher concentrations of internal primers in the second round of PCR performed with an aliquot of the first round amplicon as template have nearly negligible concentrations of external primers, hence enabling the minimum interference in progress of nested multiplex PCR. The nested multiplex impart about 100 times more sensitivity and accuracy than routine multiplex-PCR/-RT-PCR used for the diagnosis of plant viruses. This approach enables the discrimination of different amplicons of even the same size representing various targets, when integrated with colorimetric detection.

7.4.7 Real-Time PCR (qPCR)

Quantitative PCR (qPCR) is an improved and highly sensitive protocol over standard PCR which is used to detect as well as quantify specific targeted DNA or RNA or cDNA molecule.

The principle underlying qPCR is that the time taken by the reaction to enter exponential phase of amplification is directly proportional to the quantity target DNA in question. It avoids the need for agarose gel electrophoresis after completion of reaction as amplified products produced at each cycle are detected and measured by a built-in fluorimeter. It involves the use of fluorescent dyes like SYBR Green that binds to any dsDNA, thus a simple and cheap way to quantify amplification. The major drawback with the SYBR Green is its incapability to discriminate between specific and non-specific dsDNA;

therefore non-specific amplifications and primer-dimers also produce signals. This can be overcome by another approach based on the use of sequence-specific fluorescent DNA probes like TaqMan DNA probes (Livak et al. 1995). The use of TaqMan DNA probes (Fig. 7.10b) reduces signals due to non-specific primer binding at target DNA or primer-dimer formation. It helps to detect only the specific amplifications in the reaction mixture even at early stage without requiring completion of reaction for observing and confirming amplification of precise region (Wittwer et al. 1997).

This approach uses two primers flanking target sequence and a fluorescent-tagged probe annealing within the target sequence being amplified. These probes contain fluorescent dyes, viz. a reporter dye and a quencher dye at 5′-end and 3′-end, respectively, reducing net fluorescence produced. While annealing of PCR cycle when fluorescent probes bind within the target sequence being amplified, the reporter dye is removed by 5′-exonuclease action of DNA polymerase. Hence, quencher and reporter dyes are separated, resulting in increased fluorescent signals proportional to quantity of product amplified. Additionally, few other fluorescent-tagged probes and primers that utilize reporter and quencher dyes include Molecular Beacons (Fig. 7.10a) (Mackay et al. 2002; Tyagi and Kramer 1996) and Scorpions (Thelwell et al. 2000).

The cycle threshold (C_t) is determined for all test samples, showing cycle number where statistically notable raised fluorescence was recorded. C_t value is inversely proportional to the target DNA amplified. The standard curve is generated for the C_t values of amplified reference DNA to compare with the concentration of unknown amplified sample DNA that allows its quantification. The qPCR machines have inbuilt software that enable quick analyses of data generated.

Real-time PCR is increasing its applications in plant virus diagnostics with many reported assays to detect viruses and viroids (Schaad et al.

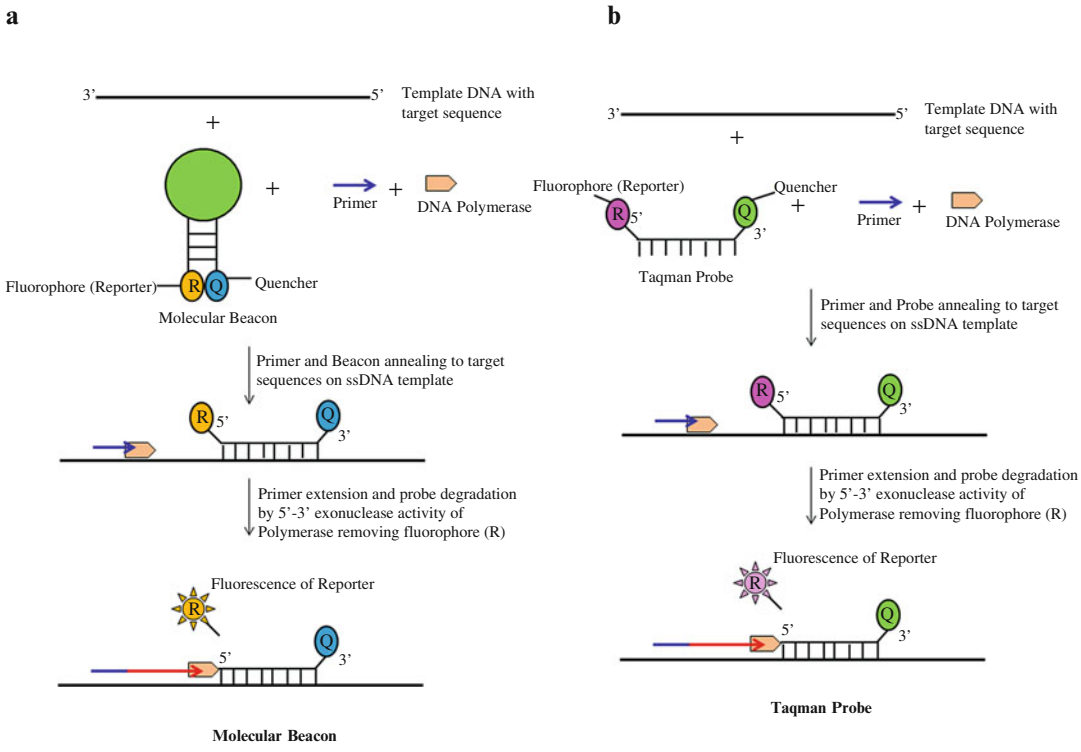

Fig. 7.10 Diagrammatic representation of (**a**) molecular beacons and (**b**) TaqMan probes in qPCR

2002; Boonham et al. 2004; Mumford et al. 2000; Schaad and Frederick 2002). TaqMan assay has been used to detect *Potato spindle tuber viroid* with ~1000 times more sensitivity than chemiluminescent assays (Boonham et al. 2004). This technique has been utilized for the successful screening of the virulent vectors and thrips for the presence of TSWV (Boonham et al. 2002) and has been efficiently contributed in the detection, quantitation and discrimination of *Wheat yellow mosaic virus* (WYMV) in wheat plants (Liu et al. 2013). The qPCR is finding its wide employability for quantitative detection of virus load and transcript level in diagnostics and gene expression studies, respectively. Other than diagnostics, it has also been used to analyse the consequences of viral infection on host gene expression by quantifying mRNAs and miRNAs in host plants.

7.4.8 Multiplex Real-Time PCR

This technique allows the simultaneous detection of multiple target sequences by the use of labelled probes designed for one target region in addition to the primer pair. The important feature of the labelling of probe is that the excitation/emission wavelength should not overlap with other fluorophores used in the same multiplex reaction as reporter dye. The major advantage of multiplex real-time PCR is that it can precisely detect and quantify multiple putative viruses in a sample. It is extensively used to determine the viral load(s) and single-nucleotide polymorphism (SNP) in plant viruses. It has been reported for the successful detection and differentiation of the immunologically cross-reactive *Chrysanthemum stem necrosis virus* and the closely related TSWV. Multiplex PCRs have been developed for the identification of multiple organisms and for identifying resistance- and virulence-specific sequences simultaneously. Simultaneous detection of multiple infections in plants is mainly possible by the use of degenerate primers having broad-spectrum specificity for the group or family of viruses. It has been used for the detection of *Tobacco rattle virus* and *Potato mop top virus* (Mumford et al. 2000). The major limitation of conventional multiplex PCR is the resolution of amplification products which can be dealt with unique fluorescent tagging of different probes (Mumford et al. 2000). This procedure has been used for the detection of two orchid viruses (Eun et al. 2000) and six viruses from olive trees (Bertolini et al. 2001). It has the ability to discriminate between severe and mild strains of CTV using unique oligonucleotide probes each for severe and mild isolates (Ruiz-Ruiz et al. 2009). Saponaria et al. (2013) have reported the use of multiplex qPCR assays for high-throughput and simultaneous detection of few important invasive citrus pathogens, namely, *Candidatus* Liberibacter asiaticus (*C*Las), CTV genotypes (VT and T3), *Hop stunt viroid* (HSVd) and *Citrus exocortis viroid* (CEVd).

7.5 Isothermal Amplification Methods

Isothermal amplification methods depend on the nonthermal separation or melting of the double strands of DNA. Such methods are similar to the conventional PCRs which also aim to amplify the target DNA using sequence-specific primers to carry out extensions with the DNA polymerase or in limited cases by RNA polymerase. Thus isothermal amplifications have primer binding to the target DNA without following thermal cycling or repeated denaturation and annealing steps.

Different procedures for nonthermal denaturation and annealing of template sequences and primer include transcription, strand displacement of circular DNA, nicking or partial degradation of primer extension products to allow extension or further priming and formation of secondary structure in single-stranded primer binding sites. These methods are being carried out at single temperature instead of thermal cycling and hence are cost-effective and require less power-consuming equipment. Being cheaper and simpler to perform, they can be easily used for field detection of viruses (Boonham et al. 2014). Table 7.3 summarizes the comparison of different isothermal methods.

Table 7.3 Comparison of several methods of isothermal amplification

	HDA	RPA	NASBA	LAMP	RCA
Reaction temperature	65 °C	37–42 °C	41 °C	60–65 °C	30 °C
Enzymatic denaturation	Yes; helicase	Yes; recombinase	No	No	No
Thermal denaturation	No	No	Initial thermal denaturation required	No	No
Primers used	Sequence specific	Sequence specific	A pair of target specific primer with T7 promoter sequence	3–4 pairs; including external and internal loop primers	Exo-resistant random hexamers
DNA polymerase used/source	Exo$^-$ Klenow Fragment of DNA polymerase I; Bst DNA polymerase	Bsu DNA polymerase I (large fragment)/*Bacillus subtilis*	By simultaneous action of Avian myeloblastosis virus reverse transcriptase (AMV-RT), T7 DNA-dependent RNA polymerase (DdRp), RNaseH	Bst DNA pol./*Bacillus stearothermophilus*	*phi29* polymerase/ Bacteriophage φ
Reaction time	30–60 min	30 min or less	90 min	16–60 min	1 h
True isothermal process	Yes	Yes	No	Yes	Yes

7.5.1 Helicase-Dependent Amplification (HDA)

HDA is the isothermal amplification method that relies on the enzymatic duplex strand denaturation via helicase (Fig. 7.11).

The helicase-dependent separation of the DNA strands allows binding of primers followed by amplification by DNA polymerase at ~65 °C. The HDA reaction completes in 30–90 min. This method is convenient for diagnostics as it is better used for the amplification of short target DNA amplicons of ca. 70–120 bp (Vincent et al. 2004). The whole process of HDA is carried out at a single temperature. Reverse transcriptase-helicase-dependent amplification (RT-HDA) has been used for accurate, sensitive and early detection of *High plains virus* (HPV) damaging wheat crop at early developmental stages (Arif et al. 2014).

7.5.2 Recombinase Polymerase Amplification (RPA)

RPA methodology is based on enzymatic or non-thermal denaturation by the use of recombinase that forms primer-recombinase complex for the initiation of amplification (Fig. 7.12).

This method amplifies the target DNA by the use of recombinase, DNA polymerase and SSB proteins. It works with the complex of nucleoprotein and primers where recombinase scans dsDNA to facilitate primer binding to target sequence and displaces non-template strand which is stabilized by SSB proteins. The recombinase then dissociates from 3′-end of the primer and makes it accessible to DNA polymerase. This process leads to exponential amplification of the target sequence by cyclic replication. Usually this does not require initial denaturation step. But for better results, manual tube agitation for at least for 5 min is

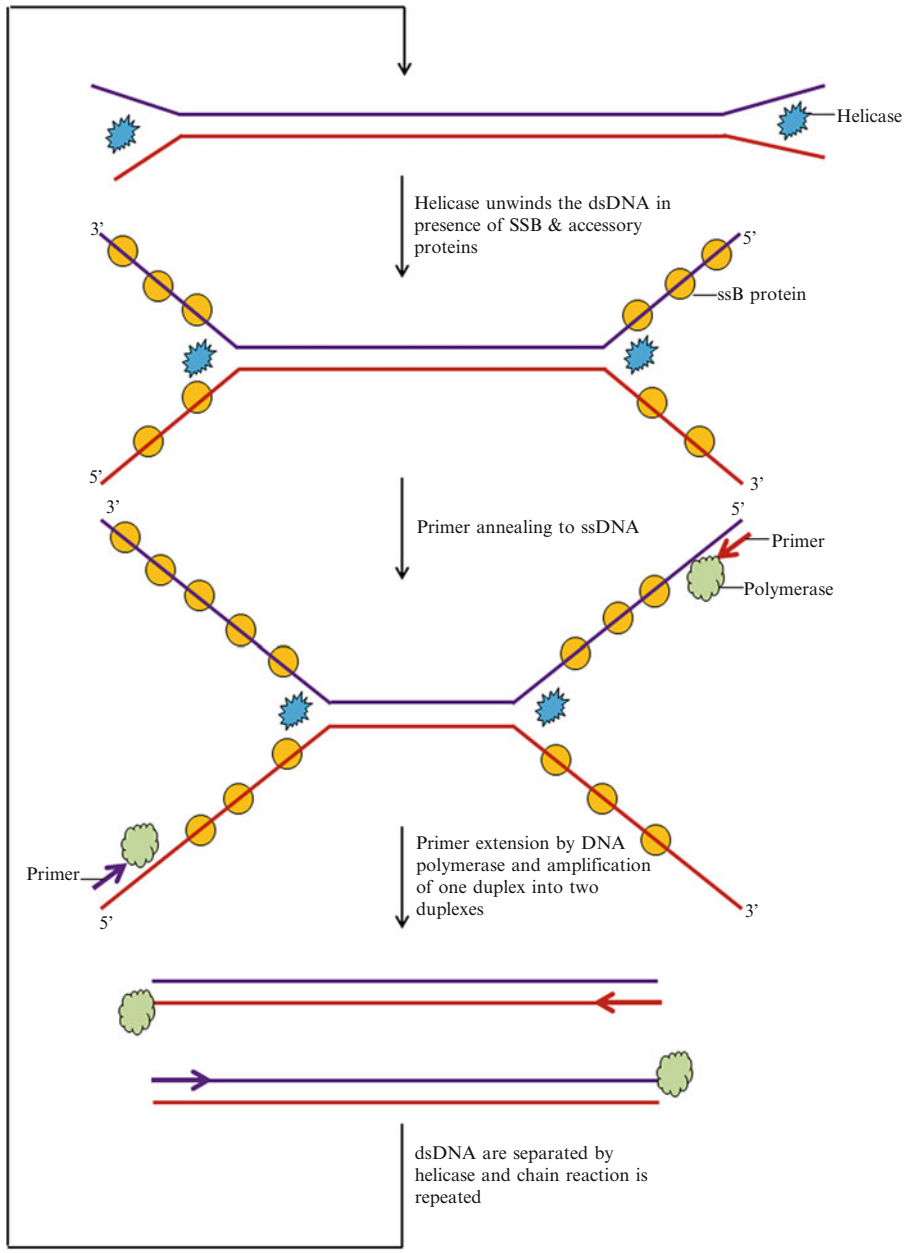

Fig. 7.11 Schematic representation of HDA (Adapted from Vincent et al. (2004))

preferred after amplification. The reaction temperature for the RPA usually ranges from 37°C to 42 °C and thus eliminates the need of high power-consuming instruments for thermal denaturation and incubation. At the same time, such low reaction temperatures may result in non-specific amplification in comparison with the isothermal amplification methods using relatively high reaction temperature. The major advantage associated with RPA is short reaction time of 30 min or less. Zhang et al. (2014) used RPA for the rapid detection of PPV in *Prunus* plants.

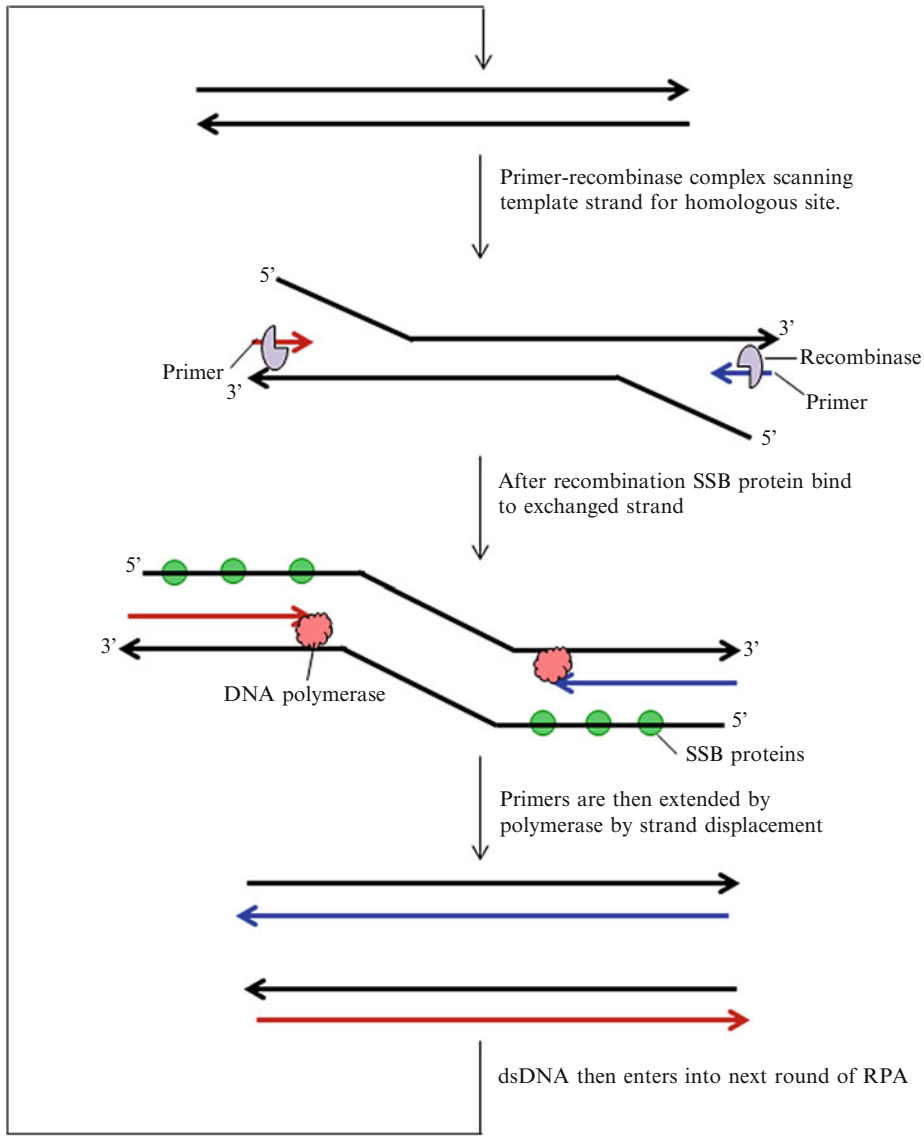

Fig. 7.12 Schematic representation of RPA (After Piepenburg et al. (2006))

7.5.3 Nucleic Acid Sequence-Based Amplification (NASBA)

In this method, RNA is isothermally amplified on the principles of transcription (Compton 1991). Primers are modified in a way that they can incorporate T7 RNA polymerase promoter sequence in the dsDNA intermediate. It makes the promoter functional and transcription reaction of the ssRNA product is performed at 41 °C (Fig. 7.13).

Olmos et al. (2007) used NASBA to detect *Plum pox virus*.

The modification of this approach using molecular beacons called AmpliDet is reported to detect various phytopathogens including ASPV (Klerks et al. 2001), PVY and *Arabis mosaic virus* (ArMV) (Szemes and Schoen 2003) and *Strawberry vein banding virus* (Vaskov et al. 2004). The AmpliDet is based on real-time measurement of fluorescence representing the

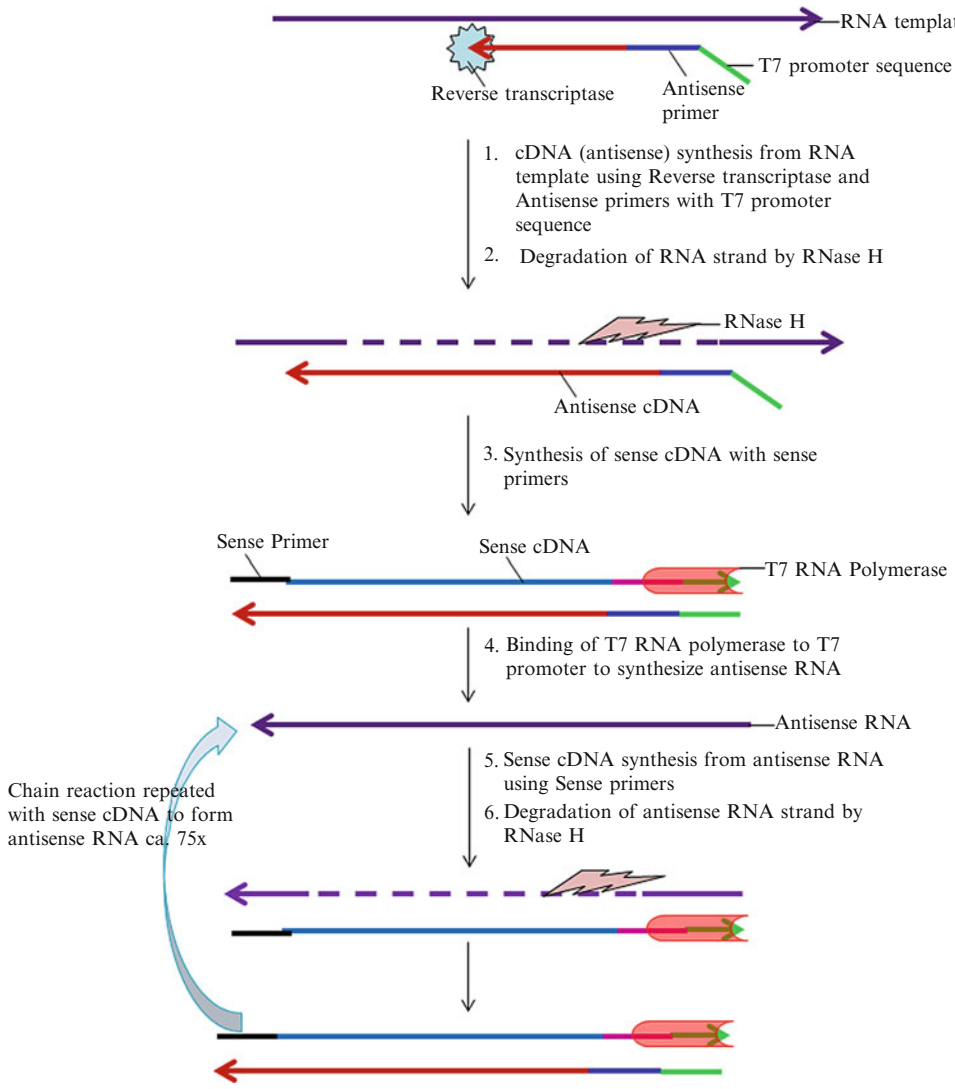

Fig. 7.13 Schematic representation of NASBA (Adapted from Compton (1991)

hybridization of the single-stranded target amplicon with the probes or beacon but requires specialized machine for monitoring. This method requires the initial denaturation of the template DNA for annealing of the primer followed by the addition of the non-thermostable polymerase and thus is not truly isothermal. The use of probe or molecular beacon makes it a very sensitive method for detection, but it has long reaction time of at least 90 min.

7.5.4 Loop-Mediated Isothermal Amplification (LAMP)

PCR and RT-PCR-based approaches have certain limitations, viz. the requirement of thermal cycler, low specificity and amplification efficiency in some instances. Therefore, LAMP is being developed that can amplify very low copies of target DNA in a very short time. It is rapid, highly sensitive and accurate. In a LAMP reac-

tion, four primer pairs complementary to six specific regions in template DNA are used along with *Bst* DNA polymerase possessing high strand-displacement activity (Notomi et al 2000). The reaction is carried out at 65 °C without requiring repeated denaturation and is completed in 16–60 min. LAMP uses three pair of primers, i.e. internal, external and loop primers, generating single-stranded loop regions enabling the annealing of primers without template denaturation at ~60–65 °C. Therefore, LAMP can be used for accurate detection of viral targets in a short time without expensive thermocyclers. The LAMP primers can be designed using software PrimerExplorer V4 (https://primerexplorer.jp/elamp4.0.0/). LAMP Assay Versatile Analysis (LAVA) is a program for signature designing for LAMP primers. It identifies six LAMP primer signature regions and eight LAMP primers including loop primers (http://lava-dna.google-code.com/).

Initially, LAMP or reverse transcriptase LAMP (RT-LAMP) has been also used in the diagnosis of infectious diseases. LAMP has been used to detect plant viruses and virus-like pathogens such as *Japanese yam mosaic virus* (Fukuta et al. 2003a), *Tomato yellow leaf curl virus* (Fukuta et al. 2003b), *Plum pox virus* (Varga and James 2006), *Banana bunchy top virus* (Peng et al 2012) and many more. RT-LAMP has been used for the diagnosis of *Zucchini yellow mosaic virus* (Kuan et al. 2014), *Potato leafroll virus* (Ahmadi et al. 2013) and *Wheat yellow mosaic virus* (Zhang et al. 2011). Nine rice viruses in Asia have been detected using RT-LAMP (Le et al. 2010). It has been used to detect and distinguish SRBSDV and RBSDV from host plants and insect vectors using RT-LAMP (Zhou et al. 2012). There is a great scope to use this technique for highly sensitive and specific detection of broad range of viral pathogens.

7.5.5 Rolling Circle Amplification (RCA)

The other major breakthrough which revolutionized the discovery and characterization of single-stranded circular DNA viruses especially begomoviruses is RCA (Haible et al. 2006).

It was used for the first time in cloning of a single-stranded circular DNA genome segment of a *Begomovirus* (Inoue-Nagata et al. 2004). RCA-based amplification protocol involves sequence-independent amplification at isothermal temperature using the *phi*29 polymerase. The technique has specific advantage of amplifying the novel sequence variants as it does not require the use of specific primers, but instead exo-resistant random hexamer primers are used (Johne et al. 2009; Jones 2009). RCA has been used to detect episomal badnavirus in banana (Baranwal et al. 2013). RCA has proved to be highly sensitive in comparison with PCR and serological methods for the detection and differentiation of integrated and episomal viral sequences of *Banana streak virus* (James et al. 2011; Wambulwa et al. 2012). Being isothermal, amplification method does not require expensive device such as thermocycler and thus the cost per reaction is low. The RCA-amplified fragment can be characterized by RFLP analysis and direct sequencing (Haible et al. 2006).

7.6 Nucleic Acid Sequence Hybridization Techniques

Nucleic acid hybridization facilitates the detection of plant viruses with single- or double-stranded genome using DNA/RNA probes. Polyprobe systems are designed for concurrent detection of several plant viruses.

7.6.1 In Situ Hybridization

This procedure is used for the detection of specific viral sequences or proteins. The combination of microscopy and hybridization has found significant application in plant virus diagnostics and in virus-host interactions. The experimental analysis gives an insight about the localization of target viral nucleic acid within a cell or tissue. Thus, it has been successfully used for the identification of replication-associated viral sequences

(Cillo et al. 2002). Also it has been used to track viral movement and detect any viral sequences integrated into the plant chromosome as in case of *Banana streak virus* infecting *Musa sp.* (Harper et al. 1999b).

7.6.2 Macroarray (Dot Blot/ Slot Blot)

DNA strands have very high affinity based on the sequence complementarity which gives high degree of specificity to interaction. Such specific interactions are the backbone of the assays based on nucleic acid hybridization where the two nucleic acid strands can be DNA-DNA/DNA-RNA/RNA-RNA. It uses a single-stranded labelled DNA or RNA to detect the hybridization. The label/tag can be radioisotope or nonradioactive tag so that target-probe hybrid molecules are detected depending on tag or reporter molecule attached to probe. Dot/spot and slot blot are the major techniques based on the hybridization of nucleic acids for plant virus detection and diagnosis (Maule et al. 1983; Garger et al. 1983; Owens and Diener 1984; Rosner et al. 1986; Baulcombe and Fernandez-Northcote 1988). Nucleic acid hybridization is a tool for the detection of single-/double-stranded viral nucleic acids using DNA or RNA probes (Fig. 7.14). The cDNA clones prepared from any segment of viral genome can be used as probes for the detection of viruses (Eweida et al. 1990) which maintain continuous supply of pure DNA and yield uniform results.

DNA probes can be labelled by PCR, random primer labelling or nick translation, while RNA probes are prepared by in vitro transcription. The cRNA probes with radioactive isotopes or nonradioactive label are preferred over cDNA probes, during the detection of RNA viruses due to the high stability of RNA-RNA duplex over DNA-RNA duplex on hybridization. But the major limitations of RNA probes are the risk of its degradation by RNAase contamination during hybridization and high cost. Thus, DNA probes become a more common choice for assays developed for plant virus detection. Macroarray has been used to detect 50 and 250 pg of *Cymbidium mosaic virus* and *Odontoglossum ringspot virus*, respectively, in the sap of diseased tissues used to immobilize viral nucleic acid on nylon membrane (Hu and Wong 1998).

Radiolabelled probes produce radioactive signals detected by autoradiography but do not have longevity due short half-life of ^{32}P and emit hazardous radioactive rays, thus requiring extensive and expensive procedures for safety maintenance. Hence, nonradioactive label probes such as biotin/streptavidin and digoxigenin (DIG) are popular (Dietzgen et al. 1994; Singh and Singh 1995; Wesley et al. 1996). The disadvantage of biotin/streptavidin probe is intense background produced by endogenous biotin in sap due to nonspecific binding with streptavidin. The digoxigenin (DIG)-labelled probes have been used widely for plant virus diagnostics, where hybridization is followed by exposure to anti-DIG antibodies conjugated with enzyme (ALP, HRP) to produce insoluble, chromogenic product from suitable substrate.

7.6.3 Microarray

DNA microarray chips are prepared by photolithography by etching a very large number of specific DNA/RNA sequences as the capture probes. Each nucleic acid fragment is specific for a DNA or RNA sequence in single assay. Glass, nylon or other polymers are used for the biochip preparation. A single microarray chip contains up to 70,000 short and unique DNA fragments. These arrayed probes can be short synthetic oligonucleotide of 30–50 bp or PCR products. The arrayed chips are allowed to hybridize with fluorescently tagged DNA/RNA in test sample using several fluorophores that reveal the presence/absence of target sequence in sample when read with laser technology.

Nanochips developed by Nanogen (San Diego, USA) contain streptavidin immobilized on the solid support for increased affinity of biotin-labelled DNA. The limitation of microarray is that concurrent detection of multiple targets such as bacteria and virus is ambiguous.

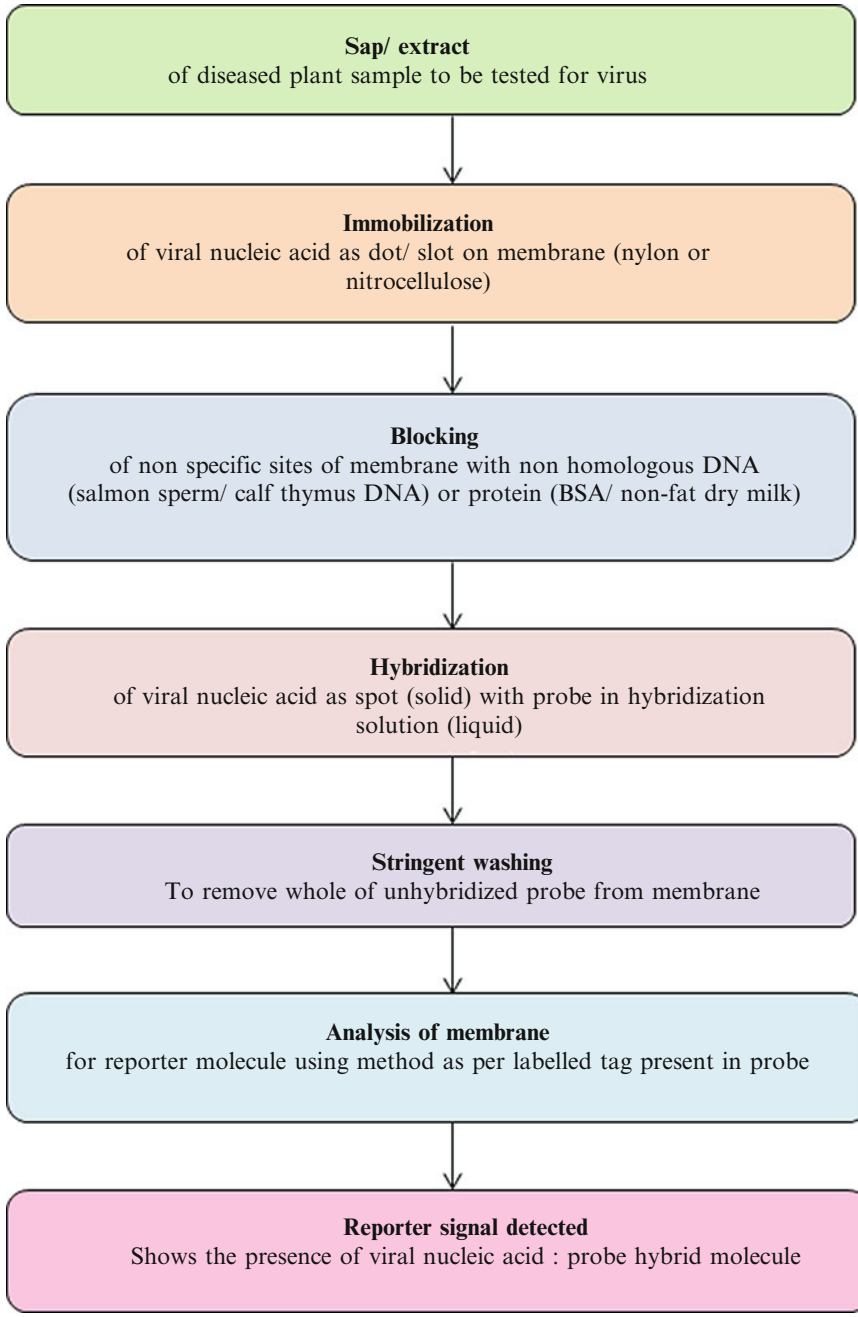

Fig. 7.14 Flow chart depicting steps of dot/slot blot hybridization assay

Different procedures used in various laboratories for microarray-based detection of plant pathogens demand the PCR-amplified fragments which reduces the sensitivity. Thus, utility of microarray is substantially reduced as a diagnostic tool as compared to its widespread use for functional genomics. Although this method is efficient in generating quick information about

several pathogens, experimentation cost is very high and demands high skill to interpret the huge data generated.

Microarray is an emerging method offering alternative to conventional serodiagnostic methods (Boonham et al. 2007). DNA microarray can detect large number of virus-specific serotypes using specific probes in single assay with high precision and facilitate a comprehensive and unbiased analyses of viral prevalence. This new approach uses single-stranded short synthetic oligomers encrypted on microchips. It has been used for the detection of various potato viruses such as PLRV, PVA, PVY, PVX, PVM and PVS (Boonham et al. 2003) from single and mixed infected potato plants. This method discriminates sequences that show <80 % identities but detects variants that differ in sequence with >90 % identities. Thus, microarray is useful for discriminating viruses at the species level, especially RNA viruses having high degree of genome variability. DNA microarray chip is developed to screen diseased tomato plants for the presence of ten different economically significant viruses known to infect and damage the tomato cultivation by using "CombiMatrix" that uses 40-mer oligoprobes for detection. This platform was efficient and specific and could concurrently detect, differentiate and genotype multiple RNA viruses, their strains and viral satellite infecting tomato, viz. CMV, CMV-satellite RNA, *Tomato mosaic virus*, *Tomato infectious chlorosis virus*, *Tomato spotted wilt virus*, *Pepino mosaic virus*, *Tomato chlorosis virus*, PVY and TMV (Tiberini et al. 2010). A single-nucleotide polymorphism (SNP) utilizing single-base primer extension generated genotype of *Wheat streak mosaic viru*s on DNA microarray chip. Among the commonly used techniques used for testing the plant materials including seeds and vegetative propagules, microarray gives the highest capabilities for specific and parallel screening. It provides a platform for that has much higher sensitivity than ELISA for testing samples for individual or mixture of viruses. DNA microarrays have potentially impartial ability to detect plant viruses using 30-mer and 50–70-mer oligoprobes. The assay uses random primers for the amplification of target sequences and its subsequent in vitro transcription to generate complementary RNA to be used for hybridization after labelling (Grover et al. 2010). The DNA virus chip provides a reliable useful tool for quarantine and certification purposes. It can also be used as unbiased tool for virus discovery on the basis of sequence similarities of new viral genome with the available microarray probes.

7.7 Next-Generation Sequencing (NGS)

NGS is a very recent, high-throughput comprehensive sequencing technology which has greatly reduced DNA sequencing cost than routine automated chain termination procedure. It generates millions or billions of DNA sequences which range from 25 to 400 nt. The sequences obtained are much smaller than those of conventional Sanger's sequencing reads which usually range from 300 to 750 nt. This technology has now been improved to generate sequences >750 nt in length.

Most recently, NGS platforms have been used in the discovery and characterization of novel viruses and their variants. It involves the rapid sequencing of hundreds of gigabases of sequences in single sequencing reaction using the highly sophisticated technology (Prabha et al. 2013). NGS platforms provide a high-throughput technology where the results can be obtained with high resolutions. NGS has been used as the most powerful tool for the discovery and characterization without the prior knowledge of macromolecular sequences (Barzon et al. 2011). The different NGS platforms used in the plant virus research are 454 FLX, Illumina, HeliScope, Ion Torrent, etc. (Schadt et al. 2010; Barzon et al. 2011; Prabha et al. 2013). This strategy includes either the extraction of total genome from the infected plant sample and its subsequent massive sequencing or the isolation of dsRNA (representing the viral RNA) followed by sequencing. The other strategies involved the isolation of siRNAs from plant samples having virus infection and its sequencing following NGS platforms; the reads

thus obtained are then mapped to the particular viral genome (Kreuze et al. 2009). The use of NGS and metagenomics has been successfully demonstrated in the discovery of novel viruses from the infected samples of sweet potato, tomato, grapevine, pepper, cucumber, cotton, etc. (Prabha et al. 2013).

NGS has been used as a powerful tool for studies on plant pathogens, especially for plant virus identification. NGS is sequence-independent and culture-independent approach, used for concurrent detection of RNA/DNA viruses and viroids having very low titre in plant samples. It is a revolutionary technology for plant virus diagnostics aiming for easy identification of novel unidentified viruses as compared to classical amplification and immunological diagnostic procedures which uniquely target a species/strain, whereas other alternatives like electron microscopy and sap inoculation of indicator species support identification only at genus level. Thus, NGS has successfully proved its utility for survey and identification of viruses in numerous plant species (Barba et al. 2014; YaJuan et al. 2014). Also, it is an alternate method for the identification of viruses using sequence generated in non-specific manner by similarity search against GenBank.

NGS and conventional techniques have been used for the identification of a new disease of maize in Kenya. ELISA and TEM could not detect the presence of a virus, while sap inoculation on few cereal species showed that the sample was infected with unidentified sap transmissible virus(es). Symptomatic tissues were used for RNA purification and subsequent sequencing by Roche 454 GS-FLX+ generated reads which on database search recognized the resultant sequence as *Maize chlorotic mottle virus* and *Sugarcane mosaic virus* that are collectively reported to cause maize lethal necrosis disease (Adams et al. 2013). Such NGS data even help to characterize viral strains and develop specific diagnostic assays for qPCR. NGS data has enabled the identification of viral strains in infected materials from various fields/regions in Kenya (Adams et al. 2013). Thus, NGS is an advantageous tool that allows quick detection and identification/characterization of known and novel viruses/viral strains, respectively.

7.8 Conclusion

Plant virus diagnostics is gaining importance with the increased spread of viruses and threats of epidemics by known and/or novel/unidentified viruses. Proper diagnostic assays therefore help in the timely identification or detection of viruses to help enforcement of quarantine. The diagnostic methods discussed above broadly include various serological and molecular approaches. Among molecular approaches, PCR has gained a lot of popularity. Nowadays, qPCR is preferred because of its qualitative and quantitative detection free from post-PCR contamination. Developments of isothermal amplification-based techniques including HDA, RPA, NASBA, etc., are gaining acceptance for their suitability for poorly resourced laboratories and being cost-effective, quick methodologies supporting on-site testing of plant samples. Arrays are also a good choice of those aiming for accurate and reliable detection of broad range of viruses that migrated to new areas or infected new hosts. NGS is a revolutionary technique generating huge sequence data that helps to detect and identify new viruses/viral strains.

However, the challenge remains in the development of technologies that are cost-effective and easy to perform supporting infield detection/screening of suspected plants material and are affordable to underdeveloped and developing countries.

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