Diagnosis of plant viral pathogens

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In the last ten years there has been an explosion in plant virus outbreaks, because of either direct or indirect activities of humans. The ease of transporting plant materials and virus vectors, and collecting relatives of crop plants from the wild, has spread viruses widely throughout the world. The most important step in managing a virus disease is correct identification. Tests for diagnosis should be fast, accurate and inexpensive. Current diagnosis techniques are broadly divided into serological procedures, nucleic acid procedures and combinations of both. This article presents a review of the current and upcoming methods of virus diagnosis in plants.

CROP production losses attributed to plant viruses can be enormous, especially when high-value cash crops, turf grass or ornamentals are at stake. For example, *Tomato spotted wilt virus* causes estimated losses of one billion US dollars per annum[1]. Even where overall losses do not appear to be great, local areas may be severely affected when conditions are favourable to virus spread. Control of viruses is limited largely to agronomic practices; using virus-free propagules, rouging of infected individuals, elimination of alternative hosts and vectors, and by quarantine practices. Although some viral diseases can be diagnosed quickly by visual examination of symptoms, others require molecular tests for diagnosis because they are symptomless or a number of different viruses cause similar symptoms in a plant. Several techniques are available for plant virus diagnosis, each with its own particular advantages and disadvantages. A brief introduction into each method is given, together with a pertinent example of each.

Serological procedures

Enzyme-linked immunosorbent assay (ELISA)

ELISA is not a new technique, although it is widely used throughout the world because of its accuracy, simplicity and low cost. The technique utilizes the ability of antibodies raised in animals to recognize proteins, usually the coat protein, of the virus of interest. Antibodies are fixed to the surface of a well within a microtitre plate, and a sap extract from the plant is added to the well. If the virus of interest is present in the plant, it will bind to the antibodies fixed on the surface. Any unbound extract is washed-off before a secondary antibody that recognizes the first antibody is added. The secondary antibody allows for indirect detection of the virus because it has a reporter molecule attached to it, usually an enzyme that acts on a substrate that changes colour, which is detected visually by a calibrated microtitre plate spectrophotometer[2]. With careful calibration, ELISA can be quantitative as well as qualitative.

This method can be used for testing multiple plants for a single virus using one well per plant sample, or alternatively a single plant can be simultaneously tested for many viruses on a single plate with different antibodies coated to each well in duplicate or triplicate for reproducibility.

The major constraint of the method is the requirement for polyclonal or monoclonal antibody sera specific for each virus of interest that does not cross-react with plant proteins, but cross absorption with plant sap avoids this problem substantially.

A modification of ELISA named voltametric enzyme immunoassay, detects the change in electrical conductivity of the substrate, rather than a colour change, when acted upon by an enzyme attached to a secondary antibody. This method is claimed to be an order of magnitude more sensitive than ELISA. It was used to detect *Cucumber mosaic virus*[3].

Tissue blot immunoassay (TIBA)

Tissue blotting, like ELISA, utilizes antibodies raised against viruses. Sap from the plant tissue is expressed onto blotting paper; nitrocellulose or nylon membranes and the virus is detected by labelled probes, often chemiluminescent. The procedure is less labour-intensive than ELISA, rapid, sensitive, simple (no virus extraction is required), inexpensive (minimal equipment is needed), suitable for surveys of 1000 to 2000 samples per day, and the samples can be taken in the field and processed some time later. Kits are available for a number of viruses, notably from the International Centre for Agriculture Research in the Dry Areas, which offers kits for 19 viruses of legumes.

Quartz crystal microbalance (QCM) immunosensors

In this novel technique for plant virus detection, a quartz crystal disk is coated with virus-specific antibodies. Volt-

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age is applied across the disk, making the disk warp slightly via a piezoelectric effect. Adsorption of virus particles to the crystal surface changes its resonance oscillation frequency in a concentration-dependent manner. It is therefore qualitative and quantitative. The developers of the technique claim that it is as sensitive but more rapid than ELISA, and economical. In the first described use of QCM for plant viruses, as little as 1 ng of particles of *Cymbidium mosaic virus* and *Odontoglossum ringspot virus* were detected in crude sap extracts[4].

Nucleic acids procedures

Reverse transcription–polymerase chain reaction (RT–PCR) and PCR

RT–PCR and PCR are popular techniques for detection and identification of RNA and DNA plant viruses respectively. The procedures are extremely sensitive, fairly inexpensive and require minimal skill to perform. In the case of RNA viruses, a cDNA strand complementary to the virus is made with reverse transcriptase (RT). Oligonucleotide primers, flanking part of the genome of the virus, are extended by a thermostable DNA polymerase in a series of denaturation and extension steps that exponentially increase the target DNA. For DNA viruses, the RT step is unnecessary.

PCR-based methods can be adapted to high-throughput applications[5]. In addition to detection of the virus, an additional advantage of the method is that the amplicon can be sequenced to provide further data about strain types. Possible drawbacks of the method are the need for a thermocycler, which can be expensive, and sequence information for design of primers. With databases containing ever-growing numbers of virus sequences, access to sequence information for many viruses is possible. Careful primer design is crucial, whether to detect only a single strain, or all the members of a genus.

The sensitivity of the method is its major advantage. An RT–PCR assay of *Cucumber mosaic virus* in lupin grain was able to reliably detect one infected seed in one thousand healthy seeds[6]. High sensitivity can easily lead to false positive results from contamination; so adequate controls are essential.

Knowledge of the nucleotide sequence is required in order to design oligonucleotide primers. Reverse transcriptase synthesizes a cDNA strand from which a fragment flanked by the primers is amplified by a thermostable DNA polymerase under a cyclical temperature regime. The amplicon is visualized on an agarose gel. There are a number of variations on the basic technique, designed to increase sensitivity, alter specificity or allow automation of detection. The most common are listed below.

Multiplex RT–PCR: Multiple species or strains are detected in a single reaction by combining oligonucleotide primers specific for different viruses. It is important that the amplicons are of different lengths and that there is no cross-reactivity among them. This method was used to detect six citrus viroids and one virus[7].

Fluorescence RT–PCR using Taqman™ technology: Two primers flank the sequence of interest and a third fluorescently labelled primer anneals between them. As the flanking primers extend, the labelled primer is released and fluorescence occurs. The advantages of this method are that no post-reaction processing is required to detect the reaction product and that it is quantitative. However, unless large-scale testing is envisaged, the cost of a Taqman™ ABI Prism 7700 Sequence Detection System and the labelled primers may be prohibitive. A Taqman assay to detect *Potato spindle tuber viroid*, a quarantine pathogen in Europe, was 1000 times more sensitive than a chemiluminescent assay[8]. The thrips vector of *Tomato spotted wilt virus* was successfully screened for the virus by this method[8]. One group used multiplex fluorescence PCR to simultaneously detect two orchid viruses[9].

Competitive fluorescence PCR (CF–PCR): This is a variation on the above technique. It is used to simultaneously differentiate between virus strains and multiple virus infections. Several primer sets, each labelled with a different fluorescent marker, are added to the reaction mixture. Virus strains are differentiated with primers that differ only at the 3′ end, complementary to a nucleotide position that is polymorphic between strains. Extension occurs only where the 3′ nucleotide is complementary. Only primers that generate amplicons fluoresce and the wavelength emitted identifies the primers that have been extended. Potatoes infected with multiple strains of *Potato virus Y* were identified using this method[5].

Immunocapture PCR (IC–PCR): This combines capture of virus particles by antibodies with amplification by PCR. In this method, the virus is adsorbed by the antibody bound to a surface, then removed by heating with a non-ionic surfactant such as Triton X-100. The nucleic acids are then amplified using RT–PCR. This method is especially useful in concentrating virus particles from plant species where virus titre is low, or where compounds that inhibit PCR are present; for example, plum tree sap containing *Plum pox virus*[11] and sugarcane sap containing *Sugarcane streak mosaic virus*[12]. It has also been used for detection of the episomal *Banana streak virus*, parts of whose genome are naturally present within the banana genome, and therefore there is a high chance of false positives from standard PCR tests[13].

Nested PCR: In this method, two PCRs are carried out with the first reaction increasing the amount of template for the second. The method is particularly useful where the virus has very low titre or inhibitors of DNA poly-

merase are present in the plant extract. Low-specificity oligonucleotides, usually degenerate, are used in the first rounds of amplification. Then, an aliquot of the reaction is placed into a fresh tube for a second PCR with primers that anneal within the first amplicon. This, at once, increases the target molecule and dilutes inhibitors. This method has been used successfully to detect members of *Vitivirus* and *Foveavirus* species in grapevines[14].

Restriction fragment length polymorphism (RFLP)

RFLP is used in combination with PCR to identify differences between viruses based on the presence or absence of restriction enzyme-recognition sites. After PCR amplification, the amplicon is digested with a restriction enzyme(s) and the fragment sizes analysed by gel electrophoresis.

RFLP is a method that can be used to differentiate isolates of viruses without the expenses of cloning and sequencing. Its effectiveness relies on polymorphisms within restriction enzyme-recognition sites. RFLP was used to show that only members of subgroup 2 of *Cucumber mosaic virus* were present in Western Australian lupin crops[6].

Labelled probes

Nucleic acid hybridization of DNA or RNA probes has the advantage of being able to detect the nucleic acid of the virus in both forms, single-stranded and double-stranded. cRNA probes can be labelled with isotopes or non-radioactive probes. cRNA probes are preferable to cDNA probes when used to detect RNA viruses, because RNA/RNA hybrids are more stable than DNA/RNA hybrids. An RNA extraction from infected tissue is blotted onto a membrane and the probe hybridized to it and detected. Orchid sap containing *Cymbidium mosaic virus* and *Odontoglossum ringspot virus* was slot-blotted onto nylon membrane and 50 and 250 pg respectively, could be detected[15].

Arrays

Arrays, both microarrays and macroarrays, have been used for some years as a tool for visualizing relative changes in global expression levels of mRNAs, as well as single nucleotide polymorphism typing and host–pathogen interactions[16,17]. There are as yet a limited number of publications describing arrays for plant virus detection[18,19]. A number of groups have extended its use to include diagnosis and genotyping of human pathogens, including retroviruses[20], *Human immunodeficiency virus* and *Hepatitis virus*[21], and assorted human pathogens[22].

ssDNA probes are irreversibly fixed as an array of discrete spots to a surface of glass, membrane or polymer[23]. Microarrays are high-density arrays with spot sizes smaller than 150 microns. A typical microarray slide can contain up to 30,000 spots. Macroarrays are generally membrane-based with spot sizes of greater than 300 microns.

Arrays printed with probes corresponding to a large number of virus species (or indeed, any type of pathogen) can be utilized to simultaneously detect all those viruses within the tissue of an infected host. Viral nucleic acids are extracted from the host, reverse-transcribed and amplified where appropriate, then labelled with a probe – either radioactive or fluorescently tagged nucleotides such as fluorescin, Cy3 or Cy5 during the RT reaction[24]. The labelled target molecule is denatured and allowed to hybridize with the arrayed probes. Excess target is washed from the surface and spots where labelled target molecules have bound, become fluorescent under appropriate lighting conditions. The position of a visible spot corresponds to the presence of a particular virus in the plant sample.

Probes for microarrays

Because the employment of array technology for plant virus detection is recent, commercial plant virus arrays are not available and therefore must be made individually. Of primary importance in making an array is probe design. Probes determine the sensitivity of the array and the amount of information that they provide. Access to sequence databases and powerful sequence alignment software is therefore essential. Of importance is a knowledge of the genomic strategy of each target species, whether it is single-stranded or double-stranded, RNA or DNA, positive or negative sense.

Two probe types can be used to construct arrays, cDNAs and oligonucleotides.

cDNA probes are denatured PCR amplicons derived from the virus of interest. Both strands of the amplicon are fixed to the membrane. The advantage of this strategy is that long probes (100–500 bases in length) can be synthesized more cheaply by PCR than by oligonucleotide synthesis. However, there are a number of drawbacks to this method over synthesis of oligonucleotide probes (see below). The probe must be purified from other amplicons, nucleotides and enzymes. It is important to determine the sequence of the amplicon to ensure that it is not an unintended amplification product of PCR. There is little flexibility in its use for differentiating strains. Access to the virus is essential in order to amplify the probe.

Oligonucleotide probes are synthesized single-stranded DNA fragments of 20–70 nucleotides. Unlike cDNA probes, only one strand is present; so it is important that the probe corresponds to the coding strand of RNA viruses in order to hybridize to the labelled anticoding cDNA strand of the virus. It is not generally necessary to purify the probe, particularly where HPLC has been carried out

after synthesis to remove partial-length oligonucleotides. Specificity is easier to achieve with shorter probes than longer cDNA probes.

The main limitation of arrays, especially microarrays, is the high cost of both the spotting and detection equipment and the labelled nucleotides, and the need for dust-free rooms.

Conclusion

As has been seen in the last ten years, plant viruses are becoming more widespread and there are real threats of new virus epidemics. It is therefore essential that the movement of viruses around the world be documented and quarantine restrictions put in place where necessary. Among the methods of detection outlined above, arrays capable of detecting a wide range of viruses show the most promise of accurately identifying new viruses as they move to new geographical areas and to new hosts. At present, however, the costs and technical difficulties of designing, constructing and utilizing microarrays limit its use to government quarantine and agricultural organizations, and large companies. Hopefully, costs will reduce as chips become available commercially and as economies of scale are realized. In the meantime, organizations ideally should utilize more than one diagnostic technique, and they should screen for high-risk viruses even where they are not known to exist in the region. This would have prevented the catastrophic outbreak of *Wheat streak mosaic virus* that was first detected in eastern Australia in 2003, resulting in the destruction of most of the publicly funded wheat breeding programme.

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