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Diagnosis of plant viruses by nucleic acid hybridization

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Abstract. Nucleic acid hybridization is a powerful technique for the diagnosis of many plant viruses not easily detected by serological techniques. It is particularly effective in the detection of viruses occurring in low amount in plant tissue, viruses that are poor immunogens or contain satellites.

Molecular probes with desired specificities can be prepared by recombinant DNA techniques for large scale use. cDNA probes of potato virus X (PVX) RNA were made by molecular cloning, and the clones were ^{32}P labelled by nick translation. Hybridization of cDNA to PVX RNA revealed 1 ng of purified virus in 2 μl spots dried onto nitrocellulose filter. Infected samples of crude leaf extracts were easily detected by hybridization, while probes did not react with healthy leaf samples.

Nucleic acid hybridization research aims at replacing radiometric probes with non-radioactive methods involving enzymes which are directly or indirectly coupled to the probe and whose presence is observed with the aid of a colour changing substrate. Hybridization assay formats that can easily be automatized are under development. Sandwich hybridization is a simple test format developed for analyzing unpurified biological material, and it appears to be a powerful tool for microbial diagnostics. Sensitivity can be improved by using detection systems in which the specific activity of the probe is increased. Procedures such as 'polymerase chain reaction', in which the amount of detectable nucleic acid sequences can be increased, are promising alternatives for increasing sensitivity. It is concluded that even if probe-based assays are in their infancy, they will no doubt develop towards such easy use as have immunological test kits.

Index words: virus detection, cDNA probes, potato virus X (PVX), spot hybridization, sandwich hybridization

Introduction

Crop losses caused by plant viruses are currently controlled mainly by using virus-free plant material and disease resistant cultivars (Walkey 1985). The success of virus disease control is thus crucially dependent on the availability of accurate, sensitive but simple diagnostic techniques which enable the early detection of viral infections in plant material. In many areas of plant production there is a great need for improved procedures for the rapid and sensitive detection of important plant viruses, particularly in laboratories responsible for producing healthy horticultural and field crop plants, in plant quarantee laboratories (Symons 1984), and plant breeding stations. Different virus diagnostic tools are needed for different purposes. For instance, extremely sensitive and accurate diagnostic techniques are required to detect some viruses in berry plants and other horticultural crops when producing virus-free material because certain viruses occur in low concentration in plant tissue. When producing virus-free potato cultivars or screening breeding material for virus resistance, diagnostic procedures should be rapid but reliable to detect various strains of the target virus because large numbers of samples are analyzed in a short time.

Traditionally, plant virus diagnosis has mainly relied on electron microscopy, immunological analyses, and symptom expression on indicator plants. However, many of these methods are time-consuming and unreliable and thus unsuitable for analyzing large numbers of samples (Symons 1984). Currently, the most widely used serological technique is the ELISA because it is simple and sensitive and appropriate even for small laboratories (review by Clark and Bar-Joseph 1984). However, the ELISA or related serological techniques are not always reliable enough. For instance, some viruses which occur in low concentration in plant tissue (e.g. barley yellow dwarf virus, potato leafroll virus), are poor immunogens or difficult to purify, are not easily detected by serological techniques (Symons 1984, Hull 1986). For some viruses which have a wide host range (e.g. cucumber mosaic virus) and are unstable or contain satellite RNA encapsidated by the coat protein of the associated virus, serological diagnosis is also impossible (Harrison et al. 1983, Palukaitis et al. 1985). Viroids, the smallest known pathogenic agents of plants, have no protein coat, and are thus not detectable by immunological methods (Owens and Diener 1984).

Nucleic acid hybridization is a new, powerful diagnostic alternative for the detection of viral infections in plants (Maule et al. 1983). Hybridization techniques are based on the ability of complementary single-stranded nucleic acid sequences to hybridize under appropriate conditions. Because of the unique base pairing tendency, hybridization is an attractive method since it is highly specific, accurate and able to detect very low concentrations of viral nucleic acids in plant extract. Nucleic acid hybridization has become a realistic diagnostic technique in the past few years, when recombinant DNA techniques made it possible to produce complementary nucleic acid probes (cDNA) with desired specificity for large scale use.

Nucleic acid hybridization has been used in the detection of various human viruses and other microbes of clinical importance for several years (review by Viscidi and Yolken 1987), as well as in the detection of some food contaminating microbes (Fitts et al. 1983). It was first shown by Owens and Diener (1981) that plant viroid infections can be effectively detected by nucleic acid hybridization using cloned cDNA probes. Since then nucleic acid hybridization has been widely tested as a means of diagnosing various plant virus diseases (Hull 1986). However, there are still many problems limiting its wide diagnostic application both in medical and agricultural fields. One serious limitation is the use of radioactively labelled recombinant DNA probes, which are still more reliable than the non-radioactive ones available. However, non-

radioactive labelling techniques are rapidly being developed (Syvänen et al. 1986, Li et al. 1987).

Progress has also been made in developing hybridization test formats. Sandwich hybridization technique has been developed for the detection of nucleic acids in crude clinical samples (Ranki et al. 1983). In this hybridization method, the specimen is kept in solution, and thus sample pretreatments are easy and background problems can be reduced. Sandwich hybridization has been used to diagnose various viral and bacterial infections during the past five years (Ranki et al. 1987), and its value in the detection of plant viral infections is being evaluated.

This paper is a review of recent developments in nucleic acid hybridization and its applications for plant virus diagnostics. The use of hybridization techniques is illustrated using potato virus X (PVX) as a test virus.

Methodological aspects

In principle, the use of hybridization techniques for the detection of plant viruses is rather simple. The first step is to prepare labelled complementary DNA (cDNA) to the target viral nucleic acid. In the case of dot-blot (sap spot) hybridization, a small amount of plant extract is immobilized on a solid support, usually nitrocellulose or nylon filter, and the labelled cDNA probe is added and incubated with it. As both samples are rendered single-stranded, the probe will hybridize with homologous sequences in the plant extract on the support. The extent of hybrid formation is a measure of the concentration of viral sequences in the plant extract, which can be visualized on the filter by autoradiography.

Although most plant viruses contain RNA genomes, RNA probes are relatively little used in nucleic acid hybridization diagnostics. Viral RNA to be used as end-labelled RNA probes can be prepared from ssRNA isolated from purified virions or from dsRNA replicative forms (Garger and Turpen 1986). RNA probes can also be conveniently made *in vitro* using RNA polymerase and cloned cDNA in plasmid vectors with RNA polymerase promoter sites (Melton et al. 1984).

The use of synthetic oligonucleotides is a rapid hybridization method. Several synthetic probes have been used to diagnose various viral and bacterial infections (Hill et al. 1985, Lin et al. 1987). However, synthetic oligonucleotide probes have not been much used in plant virus diagnostics because of the lack of published sequence information and partly because of their poor sensitivity (Bar-Joseph et al. 1986).

Complementary DNA (cDNA) to viral genomic RNA is the most widely used type of probe in nucleic acid hybridization. There are several techniques for its preparation. In general, these techniques involve four steps (Palukaitis 1986): 1. the cDNA synthesis reaction, 2. the separation of the cDNA from the template and the enzyme, 3. the separation of the cDNA from the unincorporated radioisotope and other components of the reaction mixture, and 4. the concentration of the cDNA probe. The appropriate method depends on the properties of the virus, its molecular weight, structure, and 3' polyadenylate sequences. The majority of plant viruses contain single-stranded RNA genomes, and only a small percentage contains DNA (Hull and Davies 1983).

In this paper, potato virus X (PVX) was used as a test virus to prepare a cDNA probe and to use it for detecting plant viral infections.

Cloning strategies and preparation of cDNA probes of PVX RNA

Potato virus X (PVX) is world-wide distributed in potato growing countries, and it is estimated that tuber yields of infected plants can be reduced by 5—15 % (Torrance et al. 1986). The virus causes mild mosaic on potato leaves, but foliage symptoms are not reliable indications of infection (Torrance et al. 1986). Several strains of PVX can be distinguished, but in some cases they are not

easily detected by serological assays based on polyclonal antibodies (MOREIRA et al. 1980). In order to reveal their serological relationships in more detail, monoclonal antibodies have been produced from two strains of PVX (KOENIG and TORRANCE 1986, TORRANCE et al. 1986).

PVX is a potexvirus containing single-stranded RNA. Its molecular weight is 2.1×10^6, and its coding capacity is sufficient for three polypeptides (MOROZOV et al. 1983). Recently, MOROZOV et al. (1983) showed that the RNA of PVX has a poly-A tail of about 50—200 bases at its 3' end, and there is a cap m^7 GpppG at the 5' end. Part of PVX RNA has been sequenced, and its amino acid sequence has been deduced from nucleotide sequences (MOROZOV et al. 1983).

Virus purification and RNA extraction

The PVX isolate used in this work was originally isolated by Dr. A. Kurppa. The virus was purified from infected *Nicotiana glutinosa* leaves, mainly according to SHEPHARD (1972), but further purification was made by centrifugation into CsCl gradient. The viral RNA was treated with 0.5 % SDS and phenol extracted, then precipitated with ethanol. Purity of the RNA was analyzed spectrophotometrically and by agarose gel electrophoresis.

cDNA synthesis and molecular cloning

Complementary DNA (cDNA) to the genomic RNA of PVX was synthesized by the method of GUBLER and HOFFMAN (1983). In general, first-strand cDNA was synthesized by AMV reverse transcriptase (Promega Biotech) using oligo-dT as a primer and the polyadenylated RNA of PVX as a template. The second strand of cDNA was synthesized with DNA polymerase I.

Double-stranded cDNA was digested with *Sau*3 and cloned into the plasmid pBR322 at the *Bam*HI site. Recombinant clones were identified on the basis of their sensitivity or resistance to tetracycline and ampicillin. The clones were screened for the size of cDNA insert by agarose gel electrophoresis, and those containing inserts larger than 500 bases were selected for hybridization studies. The cDNA probes were labelled by nick-translation to a specific activity of approximately 10^8 cpm/μg.

Sample preparation and hybridization

For hybridization, 2 μl of crude leaf sap extract or purified virus was spotted onto nitrocellulose filters, which were first soaked in water and then in 20 × SSC. The filters were baked at 80°C for 2 h in a vacuum oven.

The filters were prehybridized in a water-bath at 45°C for 4—5 h using sealed plastic bags and then hybridized at 50°C for about 16 h. The hybridization buffer contained denatured ^{32}P labelled cDNA at a concentration of approximately 30 ng/ml. After hybridization, the filters were washed four times at room temperature for 5 min and twice at 50°C for 15 min in 0.1 SSC + 0.2 % SDS. Then filters were autoradiographed at −80°C for 24 h.

The cDNA clone 19 of PVX RNA used as the reference probe was a kind gift of Dr. D. Baulcombe, Plant Breeding Institute, Cambridge.

Preparation of reagents for sandwich hybridization

The sandwich hybridization method is based on two separate nucleic acid reagents, which are derived from two non-overlapping but adjacent regions of the target microbial genome (RANKI et al. 1983). One of the fragments is immobilized on a nitrocellulose filter in single-stranded form (filter-DNA), and the other fragment is radioactively labelled (probe-DNA). In the reaction any nucleic acid sequence homologous to the DNA reagents will hybridize both to the filter-DNA and to the probe-DNA, thus binding the probe to the filter (RANKI et al. 1983). In this system, the reagents have no common sequences, there-

fore no hybrids are formed with incorrect sample nucleic acids.

In general, the preparation of nucleic acid fragments for sandwich hybridization involves various molecular biological techniques (Fig. 1). Various restriction enzymes are usually first used to map the target DNA fragment, and then two adjacent restriction fragments are subcloned into two different vectors. DNA fragments for filter-DNA are subcloned into the plasmid vector pBR322 or its derivative pAT153 (RANKI et al. 1983). Single-stranded probe-DNA is cloned in the bacteriophage M13. In the case of subcloning of reagent pairs of starter cDNA of PVX for sandwich hybridization, the size of filter-DNA was 150 bp, and for the probe-DNA 700 bp was used.

As in the spot hybridization described previously, double-stranded recombinant plasmid DNA is denatured in 0.2 M NaOH at 100°C for 5 min, cooled at 0°C, and applied to the nitrocellulose filter in ice-cold 6 × SSC under slight pressure, then fixed onto the filter by baking under vacuum at 80°C for 2 h (RANKI et al. 1983). In sandwich hybridization reaction, each hybridization contains one filter with microbe-specific DNA and one or two control filters with calf thymus or no DNA, respectively (RANKI et al. 1983). Incubation is usually allowed to proceed overnight (16—20 h) at 65 °C, after which the filters are carefully washed. Hybrid formation is quantitatively measured by a radioactivity counter (RANKI et al. 1983).

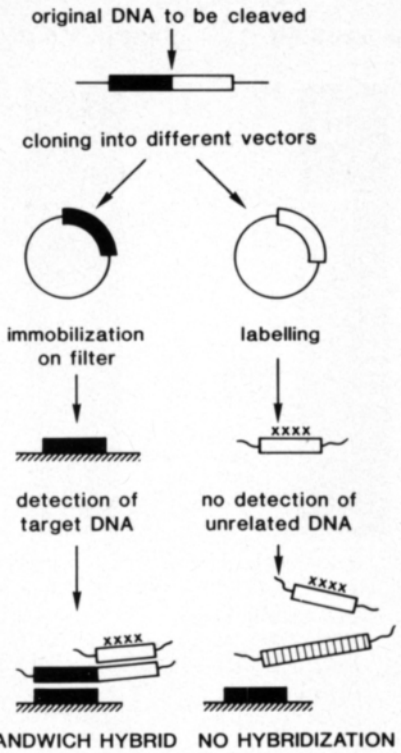

Fig. 1. Principle of sandwich hybridization assay.

Applications for virus detection

cDNA cloning of PVX RNA revealed three distinct types of inserts after *Sau*3 digestion (Fig. 2). Of these inserts, the one of 850 bp was selected as the test clone, called clone PVX 59, to demonstrate the use of cloned cDNA probes for detecting plant viral infections. After mass production of the plasmid in *E. coli* cells, it was labelled with ^{32}P by nick-translation.

In the first test, the ^{32}P-labelled cDNA probe was used to detect purified PVX virus. It was found (Fig. 3) that this probe of 850 bp complementary to PVX RNA easily revealed as little as 1 ng of purified virus containing 50 pg of RNA by hybridization to 2 µl spots dried onto nitrocellulose filters. In the second test, dilution series of crude extract from infected tobacco leaves indicated that dilutions with water of up to 500 times were readily detectable by hybridization autoradiographed for 24 h (Fig. 4). In addition, an experiment was carried out where 36 sap samples (2 µl), including random samples, infected and healthy potato leaves, were spotted onto filter. The results showed (Fig. 5) that infected samples were easily detected by cDNA probe.

The results presented here showed that cDNA hybridization is a reliable way of detecting PVX infections in crude plant sap. The sensitivity of our cDNA probes appears to be

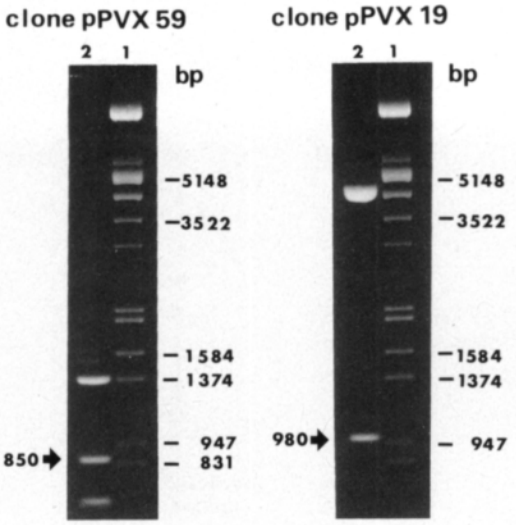

Fig. 2. Agarose gel electrophoresis of fragments of the pPVX 59 and pPVX 19 clones. Clone 59 (lane 2) was cut with *Sau*3A, clone 19 (lane 2) with *Pst*I, and the DNA markers (lane 1) with *Hind*III + *Eco*RI. The arrow indicates the position of PVX cDNA inserts.

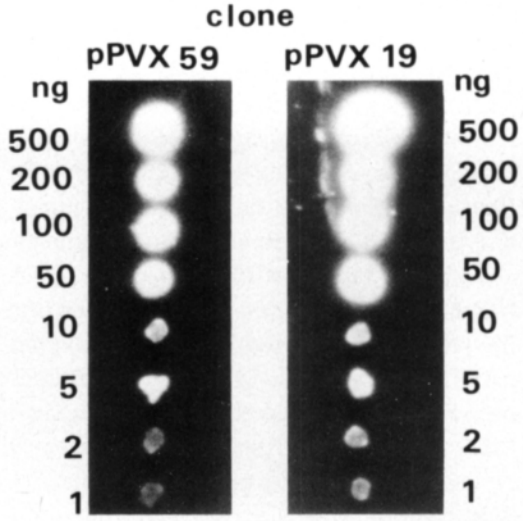

Fig. 3. Dot blot hybridization of purified PVX with ^{32}P-labelled, nick-translated probes of pPVX 19 and pPVX 59. 24 h exposure.

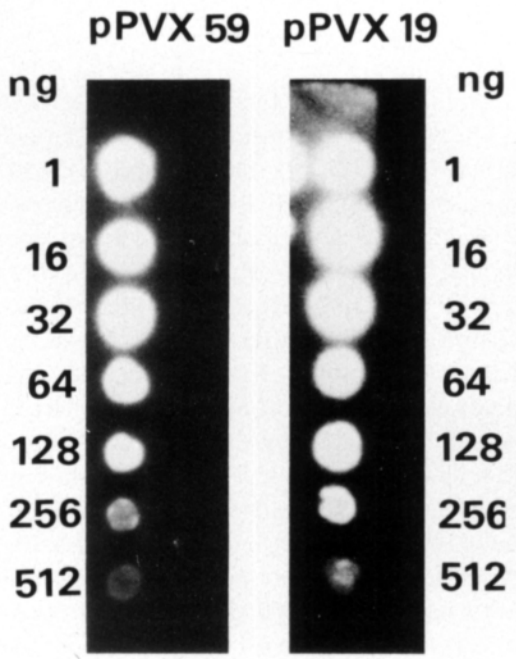

Fig. 4. Dot blot from crude sap dilutions of *N. glutinosa* leaves infected with PVX. The clones were ^{32}P-labelled by nick-translation. 24 h exposure.

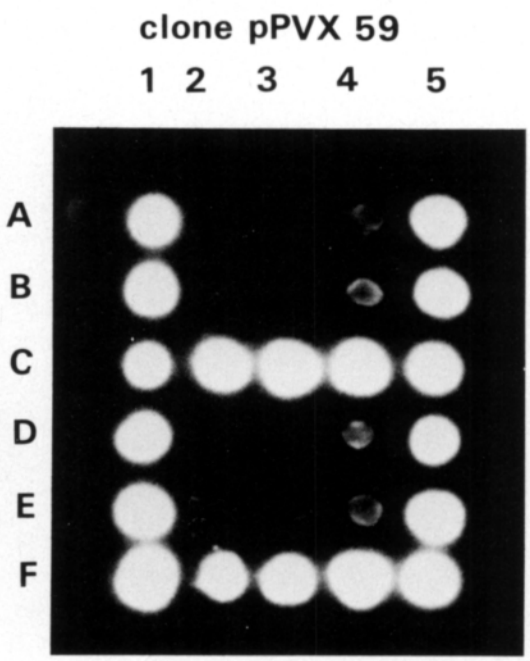

Fig. 5. Detection of PVX in sap extracts. 2 µl samples of sap from infected, healthy, and randomly taken leaves were spotted onto nitrocellulose filter. After 24 h exposure, samples containing viral RNA (lanes 1, 4, 5, spots 2C, 3C, 2F and 3F) were easily detected.

of the same order as detected by BAULCOMBE et al. (1984). In general, cDNA hybridization seems to be as sensitive as the ELISA or even more sensitive to detect PVX infections in plants (BOULTON et al. 1984). Hybridization has been found to be an efficient way of screening large numbers of potato clones for resistance to PVX. BOULTON et al. (1984, 1986) have pointed out that cDNA probes require less sap and fewer manipulations than the ELISA, and they are more rapid for screening large numbers of clones for PVX infections in a few days than the ELISA. However, PVX is highly immunogenic and occurs abundantly in plant tissue, and it is in most cases easily detectable by standard ELISA techniques (GOODWIN and BANTTARI 1984). It has been suggested that antisera against certain strains of PVX cannot always detect all strains (MOREIRA et al. 1980). In these cases, alternative strategies using either monoclonal antibodies (TORRANCE et al. 1986) or cDNA probes carefully prepared from certain regions of the PVX genome (BAULCOMBE et al. 1984) can be useful diagnostic tools and also reveal the strain diversity of PVX.

Within the past three years nucleic acid hybridization has been increasingly used for the detection of plant virus infections. For example, cDNA probes have been produced from various virus groups (Table 1), including large potyviruses, potexviruses, closteroviruses, luteoviruses, and the Fiji disease virus belonging to the small group of reoviruses. cDNA probes are not only sensitive for detecting some viruses which occur in high amounts in plant tissue, e.g. TMV and PVX, but also a powerful way of diagnosing viruses such as tobacco rattle (HARRISON et al. 1983, HUUB et al. 1986), which infect large numbers of different agricultural and horticultural crops and which are not reliably detected by serological techniques. In addition, it has been recently shown that cDNA techniques are effective in diagnosing viruses which occur in low concentration in plant tissue, such as the economically very important barley yellow dwarf (HABILI et al. 1987) and the Fiji disease

Table 1. Some of the cDNA probes prepared from different plant viruses as reported by various authors.

Virus group	Virus	Viral nucleic acid	Reference
Luteoviruses	Barley yellow dwarf virus (BYDV)	ssRNA	WATERHOUSE et al. 1986
	Subterranean clover red leaf virus (CRLV)	ssRNA	JAYASENA et al. 1984
	Potato leafroll virus (PLRV)	ssRNA	BAULCOMBE et al. 1984
Potyviruses	Potato virus Y (PVY)	ssRNA	»
	Bean yellow mosaic virus (BYMV)	ssRNA	HAMMOND and HAMMOND 1985 ROSNER et al. 1986 DEBOKX and CUPERUS 1987
Potexviruses	Potato virus X (PVX)	ssRNA	BAULCOMBE et al. 1984
Tobamoviruses	Tobacco mosaic virus (TMV)	ssRNA	SELA et al. 1984 BAR-JOSEPH et al. 1986
Tobraviruses	Tobacco rattle virus (TRV)	ssRNA (2 div.)	HARRISON et al. 1983 HUUB et al. 1986
Tombusviruses	Tomato bushy stunt virus (TBSV)	ssRNA	GALLITELLI and HULL 1985
Closteroviruses	Citrus tristeza virus (CTV)	ssRNA	ROSNER et al. 1983
Reoviruses	Fiji disease virus (FDV)	dsRNA	SKUTNICKI et al. 1986
Comoviruses	Cowpea mosaic virus (CpMV)	dsRNA	MAULE et al. 1983
Geminiviruses	African cassava mosaic virus (ACMV)	ssDNA	ROBINSON et al. 1984
Caulimoviruses	Cauliflower mosaic virus (CaMV)	dsDNA	MAULE et al. 1983
	Figwort mosaic virus (FMV)	dsDNA	»
	Carnation etched ring virus (CERV)	dsDNA	»

of sugarcane (Skutnicki et al. 1986). The present status of the sensitivity of cDNA probes compared with other diagnostic methods is not well demonstrated. Only a few direct comparisons are available between the ELISA and cDNA probes, and in general they suggest that nucleic acid hybridization is at least as sensitive as the ELISA (Maule et al. 1983) or even more sensitive for the detection of, for example, tobacco mosaic virus (TMV) and potato virus Y (PVY) (Sela et al. 1984, DeBokx and Cuperus 1987). The actual limit of sensitivity for the detection of plant viruses using cDNA probes is poorly known. Maule et al. (1983) showed using ^{32}P-labelled probes that the limit of sensitivity for several plant viruses was about 5—20 pg of purified RNA. Baulcombe et al. (1984) were able to detect 1 ng of PVX (50 pg RNA) in a 1 μl spot. Sensitivity limits for the detection of viroid infection in plant tissue are somewhat better known than those of plant viruses. For example, 80 pg of PSTV (30 ng/g tuber) has been detected (Palukaitis et al. 1985). About 300 pg of avocado sunblotch viroid (ASBV) was detected without any purification (Rosner et al. 1983), and with partial purification and concentration about 5 pg in 3—5 μl spots, which means about 20 pg ASBV/g fresh weight leaf (Barker et al. 1985).

Sandwich hybridization has been used to diagnose various animal viruses as well as bacterial pathogens (Ranki et al. 1987). Advantages of sandwich hybridization over spot hybridization are that sample pretreatments can be kept simple and crude samples can be tested without causing unspecific hybridization background. Sandwich hybridization has been shown to be as sensitive in adenovirus detection as radioimmunoassay (review by Ranki et al. 1987). So far, however, this technique is just beginning to be applied for the diagnosis of plant viruses.

Prospects for improving nucleic acid hybridization as a diagnostic tool

Any diagnostic procedures which are likely to be used on a large scale in routine plant virus testing should fulfill a number of criteria. The main requirements are a) specificity, b) sensitivity, c) simplicity to perform, and d) they should not contain decaying reagents.

In nucleic acid hybridization the specificity is an intrinsic advantage. A gene region specific for the organism or group of organisms to be detected can always be found by using recombinant DNA techniques. The cloning of specific viral nucleic acid sequences and the preparation of probes for desired specificity provide powerful tools for the detection of various isolates of the target virus and for the characterization of strain variation.

The sensitivity of nucleic acid based tests appears, in chemical terms, very good. Today the best sensitivity is obtained using ^{32}P-labelled probes, in which case down to 2×10^{-20} moles of target nucleic acid can be found. This corresponds to about 10 000 molecules of DNA or RNA (Syvänen 1986). With non-radiometric methods the detection limit is usually reduced several hundred fold. There is, however, usually only one genome per micro-organism and in many applications a test in which e.g. 100 000 bacteria per 100 μl is the detection limit, is simply not satisfactory.

A lot of work is done on improving the sensitivity of probe-based tests. One simple way is to assay for a nucleic acid present in many copies per micro-organism. Such multicopy sequences which can be assayed for include ribosomal RNA (Göbel and Standbridge 1986), multicopy plasmids (Totten et al. 1983), and repetitive DNA sequences in the genome (Gonzales et al. 1984).

Another direct way of improving the tests is to use detection systems in which the specific activity of the probe (i.e. signals generated per mass unit of DNA) is increased over those used presently. This is at least theoretically achievable using time-resolved fluorescence and Europium label (Soini and Kujala 1983, Syvänen et al. 1986).

Bioluminescent systems can in principle give extremely high sensitivity (Tanaka and Ishi-

KAWA 1986), as can coupled multienzyme reactions in which the final detectable product is amplified over the primary one (SELF 1985). The major breakthrough in improving the sensitivity of probe-based tests is, however, found in one of the unique properties of nucleic acids. The very basis of heredity is that DNA is duplicated in dividing cells and can be multiplicated in propagating organisms. Specific regions of DNA can by the same principle be enzymatically amplified *in vitro*. In a reaction called 'polymerase chain reaction' (SAIKI et al. 1985) a given DNA-sequence can be duplicated many times giving an exponential increase in the copies of the target DNA which is then easy to detect by hybridization. Even a few copies of DNA can be detected in this way. The polymerase chain reaction has the potential to solve the sensitivity problem of hybridization-based tests.

Extremely high sensitivity is only seldom required in plant virus diagnostics, perhaps most often in the detection of viruses of berry or woody plants when producing virus-free plant material. When comparing the published data on sensitivity values in detecting plant viruses and those of the potential detection sensitivity provided by nucleic acid hybridization (SYVÄNEN 1986), it is clear that the present probe-based tests are not nearly as sensitive as they could be. This is probably due to the fact that sample treatments are not optimal for obtaining high sensitivity. Using simple pretreatments for plant material and effective extraction buffers, detection sensitivity can be improved (PALUKAITIS et al. 1985). In addition, slight modifications of procedures, changes in hybridization buffers, for instance, can in some cases considerably improve the sensitivity compared to the original buffer (PALUKAITIS 1986). However, extra steps complicate procedures, and multistep pretreatments are justified only in cases where virus amounts in plants are low and high sensitivity is necessarily required.

Reaction times in minutes rather than hours or days are often important in diagnostics. Due to low concentrations, hybridization reactions are relatively slow. However, the situation has improved recently. Very high probe concentrations (LEARY et al. 1983), the use of oligonucleotides rather than large probes (JABLONSKI et al. 1986), and volume excluders like dextran sulfate and polyethylene glycol (AMASINO 1986) have all been important steps in increasing the reaction rate significantly. As hybridization time can be shortened to a few hours without any significant loss of sensitivity by using oligonucleotide probes (LIN et al. 1987), their use in plant viral diagnostics might be useful in cases where speed is more important than high sensitivity. Synthetic probes may be an attractive alternative for the diagnosis of dangerous viroid diseases because they can be prepared without the need of propagating the target organism (BAR-JOSEPH et al. 1985). Many plant viroids have been sequenced and oligonucleotides could be easily constructed (RIESNER and GROSS 1985).

The first generation of probe-based tests is now becoming available for the diagnostics of some micro-organisms. These tests have their roots in the methodology used in research laboratories and their use is still dependent on laboratory surroundings. The development of more convenient assay formats and simple tools and kits is, however, in progress.

One important aspect is the development of non-radioactive probes which do not self-decay. Several different approaches have been tried to replace ^{32}P or ^{125}I as detectors. One useful method involves enzymes, directly or indirectly coupled to the probe (LEARY et al. 1983, RENZ and KURZ 1984, TCHEN et al. 1984, JABLONSKY et al. 1986, LI et al. 1987), the presence of which is observed with the aid of a colour changing substrate. This approach will lead to tests with certain ELISA-like features. Another possibility is to use probes labelled with fluorecent or luminescent markers (MATTHEWS et al. 1985, SYVÄNEN et al. 1986). Recently, HABILI et al. (1987) had encouraging results as they showed that BYDV, which occurs in low amounts in cereal foliage, can be

effectively detected by using photobiotin-labelled cDNA probes. We conclude that even if probe-based assays are in their infancy, there is no doubt that they will develop towards such easy use as have immunochemical test kits.

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SELOSTUS

Kasvivirusten tunnistaminen nukleiinihappohybridisaatiolla

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Geeniteknologia tarjoaa uuden keinon tunnistaa kasviviruksia niiden perintöaineksen perusteella. Tätä pikadiagnostista menetelmää kutsutaan nukleiinihappohybridisaatioksi, koska se perustuu nukleiinihappomolekyylien puolikkaiden pariutumiseen. DNA-molekyyli koostuu kahdesta toisiaan tarkasti vastaavasta osasta, juosteesta, jotka toisistaan erotettuina pyrkivät pariutumaan uudelleen. Nukleiinihappohybridisaatiossa tunnistin eli koetin on tunnistettavan patogeenin nukleiinihappomolekyylin keinotekoinen puolikas, toinen juoste. Kasvinäytteessä olevien virusten nukleiinihapot 'halkaistaan' yksijuosteisiksi kuumentamalla ja kiinnitetään erikoissuodattimelle, nitroselluloosafiltterille. Tähän lisätään koetinjuosteet, jotka pariutuvat vastaavan puolikkaansa kanssa, mikäli niitä on näytteessä, eli mikäli kasvi on viruksen infektoima. Pariutuminen havaitaan esimerkiksi autoradiografisesti, tuikelaskimella tai entsymaattisten värireaktioiden perusteella. Tässä kirjoituksessa tarkastellaan nukleiinihappohybridisaation kehitysnäkymiä kasvivirusten tunnistamisessa sekä kuvataan sen työvaiheet käyttäen perunan X-virusta (PVX) testiviruksena.

Nukleiinihappohybridisaatiossa tarvittavien koetinmolekyylien valmistamiseksi puhdistettiin ensin perunan X-virus ja eristettiin sen RNA. Tämän jälkeen syntetoitiin toista juostetta vastaava eli komplementaarinen DNA-juoste (cDNA) spesifisten entsyymien avulla. Kaksisäikeiset komplementaariset DNA-molekyylit pilkottiin tämän jälkeen *Sau*3-restriktioentsyymillä ja kloonattiin pBR322-plasmidiin *Bam*HI-alueelle. Yhdistelmä-DNA -molekyylejä eli PVX:n nukleiinihappoa sisältävät bakteeripesäkkeet tunnistettiin antibioottimarkkerien avulla, ja valitut koetinmolekyylit leimattiin radioaktiivisella ^{32}P:llä nick-translaation avulla. Hybridisaatiota varten 2 µl puhdistamatonta perunan tai tupakan mehua tai puhdistettua virusta pipetoitiin nitroselluloosafiltterille, joka oli ensin käsitelty 20 × SSC -puskurissa. Tämän jälkeen nukleiinihapot kiinnitettiin filtterille kuumentamalla sitä 80°C:ssa kaksi tuntia. Tämän jälkeen filtterit esihybridisoitiin muovipusseissa 4—5 tuntia, minkä jälkeen koetin lisättiin varsinaiseen hybridisaatioliuokseen ja hybridisaation annettiin jatkua 50°C:ssa 16 tuntia. Hybridisoinnin jälkeen suodattimet pestiin useaan kertaan puskurissa, jolloin hybridisoitumaton leima huuhtoutui pois.

Tulokset osoittivat, että PVX:n RNA:sta kloonatuilla koettimilla pystyttiin tunnistamaan 1 ng puhdasta virusta 2 µl pisarassa. Koettimien avulla voitiin myös no-

peasti ja luotettavasti tunnistaa PVX:n infektoimat mehunäytteet suodattimelta, sillä koettimet reagoivat vain virusta sisältävien näytteiden kanssa. Nämä hybridisaatiotulokset tukevat viimeaikaisia DNA-diagnostiikkatutkimuksia, joiden mukaan tämän tekniikan avulla voidaan tarkasti ja luotettavasti tunnistaa monia viruksia. Monien virusten tunnistuksessa DNA-tekniikka on ELISAa herkempi ja nopeampi, joskin ELISA on toistaiseksi paljon yksinkertaisempi ja helpompi. Nopeuden ja tarkkuuden vuoksi nukleiinihappohybridisaatiota kuitenkin käytetään jo laajalti perunan jalostuksessa seulomalla sillä nopeasti virusta kestävät kloonit jalostusaineistoista. Nukleiinihappohybridisaatio soveltuu parhaiten sellaisten virusten tunnistamiseen, joihin ELISA ja muut serologiset menetelmät eivät sovellu, kuten viruksiin, jotka ovat kasvissa pieninä pitoisuuksina, joilla on huonot antigeeniset ominaisuudet, jotka sisältävät satelliitteja ja joiden genomi on moniosainen. Tällaisia viruksia on hyvin paljon, ja ne ovat hyvin haitallisia sekä maa- että puutarhataloudessa.

Nukleiinihappohybridisaation käyttöä rajoittaa toistaiseksi eniten se, että tunnistuksessa tarvittavat koettimet on leimattava radioaktiivisesti, mikä on kallista ja edellyttää erikoistiloja. Nukleiinihappohybridisaatio on kuitenkin hyvin uusi menetelmä, ja ei-radioaktiivisista koettimista on jo saatu lupaavia tuloksia. On myös kehitetty uusia hybridisaatiomenetelmiä, jotka soveltuvat entistä paremmin automatisoitaviksi. Kerroshybridisaatio on Suomessa kehitetty menetelmä (Orion-yhtymä Oy), jolla mikrobeja voidaan tunnistaa helposti puhdistamatta näytettä. Nukleiinihappohybridisaatiomenetelmät ovat nopeasti kehittymässä siihen suuntaan, että niillä voidaan tehostaa monien tällä hetkellä vaikeasti tunnistettavien kasvivirusten, viroidien ja kasvipatogeenisten bakteerien diagnostiikkaa. Tämä uusi menetelmä tulee lähivuosina helpottamaan esimerkiksi kasvintarkastustoimintaa, terveen kasvimateriaalin tuotantoa ja taudinkestävyysjalostusta.