Molecular Diagnosis of Plant Viruses

Khaled Makkouk[1] and Safaa Kumari[2]
(1) International Center for Agricultural Research in the Dry Areas (ICARDA), P.O. Box 2416, Cairo, Egypt, Email: k.makkouk@cgiar.org; (2) ICARDA, P.O. Box 5466, Aleppo, Syria.

Abstract

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Proper virus identification is always the key in developing appropriate practical solutions to manage plant virus diseases. Recent advances in biotechnology and molecular biology have played a significant role in the development of rapid, specific and sensitive diagnostic tests. The use of ELISA, by employing either polyclonal or monoclonal antibodies, was a significant step in adding sensitivity and precision to virus detection. The development of the tissue-blot immunoassay (TBIA), as a variant of ELISA, greatly simplified testing, reduced the cost and permitted virus testing at locations where facilities are limited or even absent. Immuno Chromatographic Assay (ICA), is another ELISA variant which added speed to virus identification, where results can be obtained within 10-15 minutes, as compared to 2-3 hours for TBIA. However, ICA is more expensive than TBIA. The development of nucleic acid-based tools was another new dimension of virus detection. The most common among these techniques are cDNA hybridization and polymerase chain reaction (PCR). In addition, PCR can be used as a confirmatory test for TBIA, where processed blots can be cut individually and tested by PCR. This proved to work well with both DNA and RNA plant viruses. Furthermore, unprocessed plant tissue blots on nitrocellulose membrane represent a good sample for PCR amplification. PCR products can also be used for cloning and subsequent sequencing which is extremely useful for identification of new viruses or virus strains.

Keywords: Identification, detection, ELISA, Real time PCR, TBIA, ImmunoStrips.

Introduction

Accurate diagnosis of virus diseases and diseases in general, is a first important step for any crop management system. With virus diseases, plant treatment after infection often do not lead to an effective control. Accordingly, virus diseases are managed most effectively if control measures are applied before infection occurs. The use of healthy (virus-free) plant propagation material is among the most effective approaches to adopt by farmers. One of the elements essential for successful certification programs to produce such propagation material is the availability of sensitive diagnostic methods.

Few decades ago, virus detection was based mainly on biological techniques which are too slow and not amendable to large-scale application. Advances in molecular biology and biotechnology over the last three decades were applied to develop rapid, specific and sensitive techniques for the detection of plant viruses. This review will summarize the development and use of the main immunological and nucleic-acid-based methods for virus detection.

Immunological-protein based methods

The use of serology for the detection of plant viruses was in use for more than half a century (13). However, the use of advanced immunodiagnostic methods for the identification and detection of viruses made the detection, easier, more sensitive and with reasonable cost (5). In this paper we will summarize the important features of the most commonly used techniques in recent years.

ELISA - During the last three decades, enzyme linked immunosorbent assay (ELISA) was widely used method for the detection of viruses that is highly sensitive, simple, fast and most importantly has the ability to quantify virus content in plant tissue. The binding of the virus and specific antibody is made visible through an antibody tagged with an enzyme which can react with a substrate to produce a colored, water soluble product. The first reported method was the double antibody sandwich ELISA (DAS-ELISA) where the antibody is bound to the solid phase (e.g. polystyrene microtiter plate), then the test samples, enzyme labeled antibody and the substrate are added sequentially, with unbound material removed by washing between steps (4). In a positive test, the substrate solution turns colored, whereas a negative test remains colorless. The color intensity, which is proportional to virus contents, can be measured spectrophotometrically. Since the report of Clark and Adams in 1977 (4), many ELISA variants were reported, by using different enzymes or universal conjugates. In this later case the test is known as triple antibody sandwich ELISA (TAS-ELISA). In other variants, the first step of coating the solid phase with antibodies is deleted, and consequently virus particles are adsorbed directly on the solid phase, and the test is known as direct antigen coating ELISA (DAC-ELISA). In addition, immunoassay sensitivity can be enhanced by the use of different amplification systems, with avidin-biotin being the most common.

In addition to the polystyrene plates, a number of solid phase supports were found adequate. Assays in which antibodies or virus particles are bound to nitrocellulose membrane filters were used and known as immunoblots or dot-blots. Dot blot ELISA tends to be rapid, easy to perform and conservative of reagents and often more sensitive than ELISA carried out in a microtiter plate (2). Immunoblot assays use the same reagents used in microtiter plate ELISAs, except that the substrate produces an insoluble product which precipitates onto the membrane. Positive reactions can be determined visually.

An interesting development was the printing of plant parts cut surfaces on nitrocellulose membranes and then the test continues in a way similar to dot-blot assays. The procedure is known as the tissue-blot immunoassay (TBIA) (6, 8). The major advantage of this test was the elimination of sap extraction, which is the most time consuming step in all previous techniques. In addition, once the plant tissue is blotted on the NC membrane, the test can be completed either few days or few months later. This is a big advantage in remote places, where facilities for processing NC membranes do not exist. In such locations, samples can be printed on NC membranes and then sent/mailed to distant locations for processing.

As a result of the progress made in the last two decades in the medical diagnostic industry, a number of procedures and devices have been developed that increase speed, sensitivity and ease of use of immunoassays in the field. One of these approaches for the detection of plant viruses is the "dipstick", developed earlier for the physicians' office and home use, is now being used for the detection of various pathogens, including viruses, in the field (1, 11). Small plant

tissue is placed in an extraction bag which contains an extraction buffer, then the bag is rubbed with a pen or blunt object to crush the samples, the tip of a strip (e.g. ImmunoStrips form Agdia) is inserted in a vertical position into the extract and the result will appear as a colored line within 3-5 minutes.

Molecular-nucleic acid based methods

Nucleic acid-based virus detection systems make use of cloned DNA probes in a dot-blot assay or specifically designed primers in a polymerase chain reaction (PCR) test. Both approaches have the potential to detect single nucleotide differences. These two approaches will be summarized in the following paragraphs.

Dot-blot assay - This development in nucleic acid hybridization technology offers a good potential for virus detection (9). The target viral nucleic acid from a plant sample is spotted onto a solid matrix, commonly nylon or nitrocellulose membranes, and bound by baking. Free binding sites on the membrane are blocked with a non-homologous DNA and a protein source. Thereafter, hybridization with a labeled probe is carried out. The label is then detected by autoradiography (for radioactive probes), or by a colorimetric reaction if an enzyme label is used. The sensitivity of dot-blot hybridization is about the same as ELISA (12). A modification of the dot-blot assay, squash blotting, has been used to detect some viruses (3).

PCR (polymerase chain reaction) - The polymerase chain reaction (PCR) (10) has been used as the new standard for detecting a wide variety of templates across a range of scientific disciplines, including virology. The method employs a pair of synthetic oligonucleotides or primers, each hybridizing to one strand of a double stranded DNA target, with the pair spanning a region that will exponentially reproduced. The hybridized primer acts as a substrate for a DNA polymerase, which creates a complementary strand via sequential addition of deoxyncleotides. The process can be summarized in three steps: (i) dsDNA separation at temperatures above 90ºC, (ii) primers annealing at 50-75C, and (iii) optimal extension at 72-78ºC. The rate of temperature change, the length of the incubation at each temperature and the number of times each cycle is repeated are controlled by a programmable thermal cycle. The amplified DNA fragments will then be separated by agarose gel electrophoresis and the bands are visualized by staining the resulting bands with ethedium bromide and irradiation with ultravidet light. The specificity of PCR testing is dependent on the primer sets used. There are virus species specific primers and genus specific primers. Figure 1 illustrates the use of primers that can detect all species of the genus *Nanovirus* and other primer sets that can detect an individual virus species within that genus.

The above procedure work well for DNA viruses (e.g. viruses of the genera Geminivirus, Nanovirus and Caulimovirus). For RNA viruses, an initial step to transcribe the RNA viral genome to its complementary DNA (cDNA) (Reverse Transcription) by using the enzyme reverse transcriptase (RT) is needed. Accordingly, the PCR procedure followed for the detection of RNA viruses is known as RT-PCR.

Real time PCR - The ability to visualize the progress of amplification in a quantitative manner was welcomed by research workers (7). This approach has provided insight into the kinetics of the PCR reaction and it is the foundation of "real time" PCR.

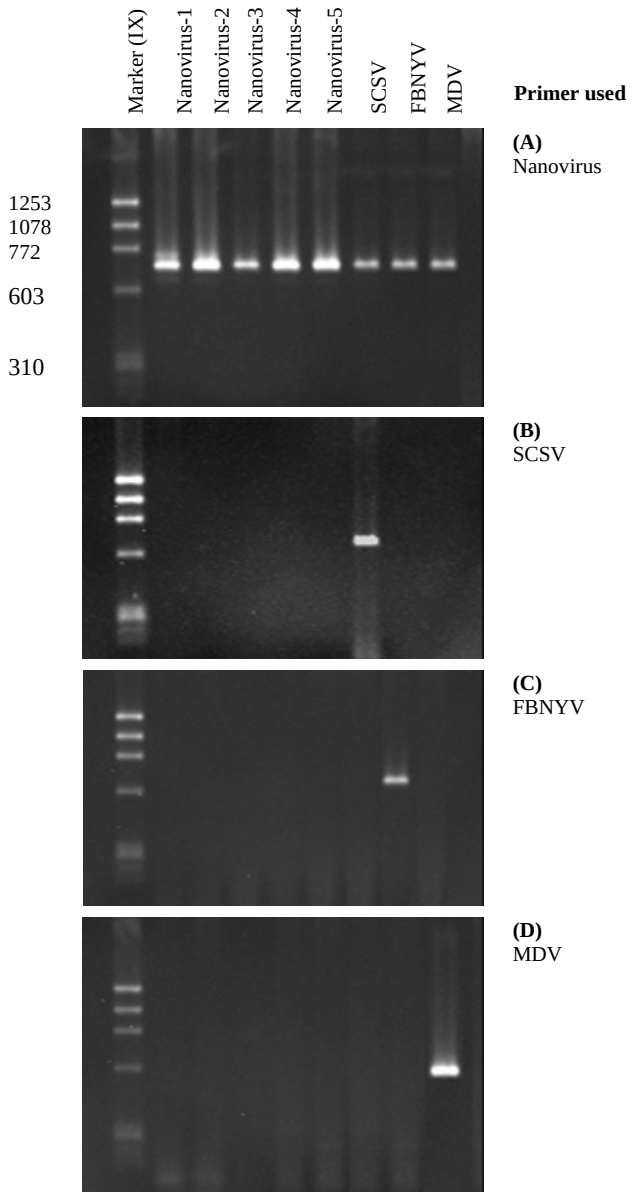

Figure 1. Broad and specific detection of Nanoviruses by PCR. (A) detection of different Nanoviruses by a broad primers set, (B) detection of *Subterranean clover stunt virus* (SCSV) by a virus species specific primers set, (C) detection of *Faba bean necrotic yellows virus* (FBNYV) by a virus species specific primers set, and (D) detection of *Milk vetch dwarf virus* (MDV) by a virus species specific primers set.

The monitoring of accumulating amplicon in real time PCR has been possible by the labeling of primers, probes or amplicon with fluorogenic molecules. The increased speed of real time PCR is largely due to reduced cycle times, removal of post-PCR detection procedures and the use of fluoregenic labels and sensitive methods of detecting their emissions (14). The reduction in amplicon size generally recommended by the inventors of commercial real-time assays may also play a role in this speed, but decreased product size does not necessarily improve PCR efficiency.

Quantitative real-time PCR is based on detection of a fluorescent signal produced proportionally during the

amplification of a PCR product. A probe (e.g. TaqMan) is designed to anneal to the target sequence between the traditional forward and reverse primers. The probe is labeled at the 5' end with a reporter fluorochrome and a quencher fluorochrome added at the 3' end. The probe is designed to have a higher T_m than the primers, and during the extension phase, the probe must be 100% hybridized for success of the assay. As long as fluorochromes are on the probe, the quencher molecule stops all fluorescence by the reporter. However, as Taq polymerase extends the primer, the intrinsic 5' to 3' nuclease activity of Taq degrades the probe, releasing the reporter fluorochrome. The amount of fluorescence released during the amplification cycle is proportional to the amount of product generated in each cycle. Similar to the conventional PCR, in case of RNA viruses, amplification can be measured after extraction of total RNA and preparation of a cDNA by a reverse transcription (RT) step. Real time PCR has proven increasingly valuable diagnostic tool for plant viruses. However, it requires an initial high capital investment to acquire the needed equipment, as compared to other techniques.

Confirmation of TBIA results by PCR - In virus identification, especially when a virus is detected for the first time in a region, it is essential to confirm the results obtained by one test (e.g. TBIA) by applying another technique on the same sample. Recent research results showed, that when TBIA positive samples where cut from the NC membrane and tested by PCR, virus-specific amplicons were produced. In addition, when the PCR test was conducted on few months old processed NC membranes, amplification of the target sequence was clearly obtained. This is an indication that the TBIA procedure had no effect on the viral genome, and it remained stable for a long period. To cut down on cost, it is possible that in large scale operations (surveys, certification program), it is possible to test the samples in the first round by TBIA, and only those samples positive for a specific virus can be re-tested by PCR.

Conclusions

Recent developments in molecular detection technology led to the development of more convenient, effective, and specific assays and permitted the use of these tests for detecting plant pathogens, including viruses. Such assays will help growers, crop agronomists, and plant-health professionals not to rely exclusively on symptomatology and/or time-consuming diagnostic procedures, and permit early detection of viral infection. These new techniques are effective management tools to be used in parallel with knowledge of the crop, understanding the biology of the pathogen and the ecology of the disease. Thus, these tools can be excellent tool to determine the point in time at which control measures should be implemented. In addition, such diagnostic assays are essential tools for programs devised to produce virus-free plant propagation materials.

Viral genome sequence data available made it very easy to design primers for different uses, for broad or specific detection of viral pathogens. Similarly, the production of monoclonal antibodies gave immunological tests increased capacity in terms of specificity, not provided earlier by polyclonal antibodies.

Among the variety of immunological tests now available, TBIA proved to be extremely helpful for large scale testing at a very low cost without much compromise on sensitivity or specificity. It is an essential test to be used in developing countries where sophisticated equipment is not available and the cost/sample is a limiting factor for any assay to be adopted for large scale testing.

الملخص

مكوك، خالد وصفاء قمري. 2006. التشخيص الجزيئي للفيروسات النباتية. مجلة وقاية النبات العربية. 24: 135-138.

يعتبر التشخيص الدقيق للفيروسات الأساس لايجاد الحلول العملية المناسبة لإدارة الأمراض الفيروسية النباتية. أسهمت التطورات الحديثة في مجال علوم التقنيات الحيوية والبيولوجيا الجزيئية بدور فاعل في تطوير اختبارات تشخيصية سريعة وذات حساسية عالية. يعد اكتشاف اختبار اليزا (ELISA)، الذي يعتمد على استخدام الأجسام المضادة وحيدة أو عديدة الكلون، خطوة فاعلة في زيادة الدقة والحساسية عند تشخيص الفيروسات. كما أن تطوير اختبار بصمة النسيج النباتي (TBIA)، وهو أحد تحويرات اختبار اليزا، أدى إلى تسهيل عملية التشخيص وتقليل تكلفته، وسمح استخدامه بالكشف عن الفيروسات في الأماكن التي تكون فيها الإمكانات قليلة أو معدومة. وسرّع اختبار الكروماتوغرافيا المناعي (ICA) – وهو تحوير آخر لإختبار اليزا – عملية تشخيص الفيروسات، حيث يمكن الحصول على النتيجة خلال 10-15 دقيقة مقارنة بـــ 2-3 ساعات لإختبار بصمة النسيج النباتي؛ إلا أن تكلفة اختبار الكروماتوغرافيا المناعي أعلى بكثير من تكلفة إختبار بصمة النسيج النباتي. وقد شكل تطوير الإختبارات التي تعتمد على الحمض النووي بعداً آخر في مجال تشخيص الفيروسات، ومن أكثرها شيوعاً إختبارات تهجين الحمض النووي المكمل (cDNA hybridization) والتفاعل المتسلسل للبوليمراز (PCR) اللذين أديا إلى الكشف عن تركيزات منخفضة جداً من الفيروسات. بالإضافة لذلك، وجد بأنه يمكن استخدام التفاعل المتسلسل للبوليمراز لتأكيد نتائج اختبار بصمة النسيج النباتي، وذلك عن طريق إعادة قص مقاطع النباتات المفحوصة باختبار بصمة النسيج النباتي ومن ثم فحصها مرة أخرى بالتفاعل المتسلسل للبوليمراز. هذا، وقد نجحت هذه الطريقة في تشخيص الفيروسات ذات الحمض النووي من النوع RNA أو من النوع DNA. علاوة على ذلك، فقد تم عزل الأحماض النووية للفيروسات (RNA و DNA) من مقاطع النباتات المطبوعة على أغشية النيتروسيلليلوز، وكانت تمثل عينة جيدة لتضاعف الحمض النووي للفيروس عن طريق التفاعل المتسلسل للبوليمراز متبوعاً بعمليات الكلونة ومن ثم دراسة تسلسل القواعد النيتروجينية للحمض النووي فيما بعد. وتعتبر هذه الطريقة مفيدة جداً لتحديد هوية فيروسات أوسلالات فيروسية جديدة.

كلمات مفتاحية: تعريف، تشخيص، اختباراليزا، Real time PCR، اختبار بصمة النسيج النباتي، ImmunoStrips.

عنوان المراسلة: خالد مكوك، المركز الدولي للبحوث الزراعية في المناطق الجافة (إيكاردا)، ص.ب. 2416، القاهرة، مصر، البريد الإلكتروني: k.makkouk@cgiar.org

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