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Recent Development in Detection and Identification of Fruit Tree Viruses

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Trees in general could be categorized into the longest-lived organisms ever to grow and have been doing extensively well in adapting to environmental changes in natural forest. Human attempts to grow trees in non-forest conditions and breached natural barriers in modern times resulted in rapid epidemics and development of new diseases and continues to cause severe problems in their respective industries. After the liberalization of regulations for import of plants and plant materials that followed General Agreement on Tariffs and Trade ratification by participating countries, there is a potential risk of introducing several destructive pathogens and pests, especially viruses, viroids, and phytoplasmas. Implementation of adequate diagnostic methods has become imperative to prevent the introduction of new pathogens and results in saving millions of dollars.

During the last fifty years, viruses have been increasingly recognized as major threats to many crops and trees. Fruit tree viruses were often regarded as minor causal agents for tree diseases since viruses infecting fruit trees frequently cause few symptoms. Virus-infected cells are not generally killed but they replicate and produce progeny viruses that further infect tree. Unlike virus-infected crops and fodder that still can be used, fruit trees and ornamental crops can be totally lost or can cost a massive economic damage by adversely affecting fruit quality and/or productivity. At the same time there are and will be, increasingly so, considerable pressure to improve quality of human foodstuffs and there is a increasing pressure from consumers and the environment to reduce the levels of agro-chemicals that are routinely applied. The requirement of tests applied for the Certification of fruit trees is particularly important. Infection is often latent and vegetative propagation from infected apple scionwood mother trees, for example, providing material to propagate several hundred trees per year. This can result in widespread infection of viruses that are often very difficult to locate if detected several years after it has arisen. The availability of cheap, easy to use, and totally dependable diagnostic techniques for field use would provide ideal tools to improve economics of fruit crop production, reduce unnecessary use of chemicals, improve food quality, and protect total agriculture/horticulture environment.

The control of plant virus diseases can not be achieved along the lines followed for animal and human viral infections since plants do not have immune system. Vaccination or chemotherapy can not protect plants and usually they do not recover once they have become infected. When the disease situation develops to a certain degree such that the need for treatment becomes obvious, often losses are irretrievable. Preventing a viral disease is always better than curing it. Hence diagnostics must provide earliest possible warning of the presence of viruses capable of producing economic yield loss. The highest grade of 'Virus-Free' fruit trees requires testing on woody indicator plants that requires long time (some tests take 3 years to complete) and very costly. In order to achieve effective fruit tree virus disease control and the implementation of certification schemes for the improvement of fruit trees it is essential to replace these tests with quick, convenient, and reliable laboratory diagnosis and elimination of viruses from diseases propagules.

Diagnosis of plant viruses has been greatly assisted by the main characteristics of the infections including symptoms, mode of transmission, particle morphology, and serological relationships among others since viruses within group share similar properties. Bioassay, electron microscopy (EM) and serology were the methods routinely used to detect and diagnose virus diseases. Virus detection in trees was not an easy task due to the facts that woody perennials usually contain low virus concentration and show discontinuous virus distribution as well as some impact of extracts on serological assays. Increased sensitivity of recently developed methods for virus detection and identification overcome those limitations and expanded our knowledge of fruit tree virus diseases.

In Korea, a large number of economically important fruit tree viruses are yet to be fully identified and their relationships to similar viruses characterized in other countries are still to be determined. This paper is an overview of some of the recently developed approaches in diagnosing and characterizing viruses that causing damages in fruit trees. It is focused on serological and nucleic acid-based tests and discusses advantages and disadvantages of each diagnostic

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procedure.

Fruit Trees and Viruses

Fruit trees are grown in temperate, subtropical, and tropical regions of the world. Among the fruits of temperate and subtropical regions, oranges are the major tree fruits in terms of quantity produced (Table 1), with large quantities being utilized to make juice. The second is apple, probably the temperate tree fruit most often eaten fresh, although large portions are also juiced, dried, and canned (FAO, 1998). Fruits are vital sources of essential vitamins, minerals, and dietary fibers and the production of tree fruits is rapidly increasing every year.

Fruit tree viruses were often regarded as minor causal agents for cells and not generally killed but the viruses replicate and produce progeny viruses that further infect trees. The subtle nature of symptoms and dissemination through grafting and propagation in the past led to an accumulation of viruses in fruit trees and resulted in contaminating many cultivars (Cropley, 1968). This can result in widespread of viruses that are often very difficult to locate if detected several years after it has arisen. The early diagnosis and prevention of virus diseases that the certain fruits are very sensitive and can cause severe economic loss is very important. There are increasing number of viruses that can infect fruit trees. Table 2 shows most common viruses isolated from fruit trees and describes some of their biological characteristics.

Identification and Assay

The early and accurate diagnosis of plant diseases is a crucial component of all crop-management systems. Simultaneous application of several methods that depend on different properties of the virus for detection and diagnose virus diseases is useful and frequently requires two or more different experiments. Symptoms are of major importance because they are the main means by which a viral disease is determined but precise identification of a virus is not feasible on symptoms alone. Several unrelated viruses produce similar symptoms and different strains of the same virus group can also produce very different symptoms. When there is a mixed infection of unrelated viruses, it is a lot more difficult to identify viruses by symptom developments alone.

The choice of plant materials to be sampled is of great importance for successful assay and detection. Transmission of a virus from infected to healthy tissue is often used to isolate viruses from diseased plants. The distribution of virus in an infected plant tissue may be very uneven (Olmos et al., 1997; Singh and Singh, 1996; Stein et al., 1987). Therefore it is very important to harvest tissue samples from which the maximum concentration of a virus can be isolated. Note that the correct tissue samples has to be determined in view of either optimum sensitivity or reliability. Actively growing plants show an increased reliability due to the enhanced virus spread whereas plants in the stationary growth stage accumulated higher virus titer thus allowing a better discrimination of infected plants, but being less reliable due to a higher variability of samples (Knapp et al., 1997).

The EM proved to be a valuable tool for the routine diagnosis of viral diseases. The major advantages of EM method are relative simplicity in sample preparation and minimum time requirement. Most viruses could be identified by simple absorvance preparates and negative staining. In many cases, however, viruses within same group or species can be morphologically very related and thus requires additional identification tests.

Recent developments in molecular biology have brought us more convenient, effective, and sensitive assays for the detection and identification of viruses. Various recently developed techniques that can be commonly used for assay and identification of fruit tree viruses are described.

Serological tests. Antigenic properties of virions represent the single most useful criterion for reliable virus identification. Serodiagnosis of plant viruses precisely helps to relate the unknown virus isolate with known viruses and also contribute significantly to mass indexing of plants used in crop improvement and production. Serological methods are currently being used for rapid detection of virus infections. Its specificity and reliability more increased use of serological assays for virus detection (van Regenmortel, 1992; van Regenmortel et al., 1993).

Several different serological assays that utilize solid phase support exist (Bar-Joseph et al., 1979; Llacer et al., 1985; Rocha-Pena and Lee, 1991). These include dot immunoblotting assay, western blotting, radio-immunoassay, immuno electron microscopy, immuno-fluorescence microscopy, immuno-gold EM, but the most common is the enzyme-linked immunosorbent assay (ELISA)-based methods. ELISA-based assays utilize enzyme labeled antibodies, antigens or secondary reagents as a detection system. One of the most common ELISA assays is so called double antibody sandwich ELISA (DAS-ELISA) which uses antibody coated microtiter plate (Clark and Adams, 1977; Voller et al., 1976). It is possible to coat ELISA well with sample extract directly thus omitting the coating antibody stage which is called as plate-trapped antigen ELISA assay. The widespread development and adoption of ELISA-based assays has greatly facilitated routine virus diagnostics by reducing test times and permitting large-scale sample surveys. For example, Edwards and Cooper (1985) detected

Table 1. Production and yield of major tree fruits in 1998 (FAO, 1998)

Country	Apple		Cherry		Orange & Citrus	
	production (MT)	yield (HG/HA)	production (MT)	yield (HG/HA)	production (MT)	yield (HG/HA)
Argentina	1,280,000	241,509	6,000	50,000	700,000	129,967
Australia	280,147	147,446	4,783	30,272	373,000	124,603
Austria	392,000	206,316	21,329	68,803		
Brazil	787,414	294,812			23,020,900	227,035
Bulgaria	189,000	126,000	50,000	60,241		
Chile	880,000	220,000	25,000	51,760	135,000	187,240
China	17,508,250	46,062			3,380,622	83,720
Colombia					407,000	191,981
Egypt	425,000	163,462			1,572,950	174,447
France	2,500,000	320,513	66,000	47,143	1,400	171,429
Georgia	200,000	26,316	18,000	60,000	115,000	76,667
Germany	1,978,000	231,887	140,000	48,276		
Greece	373,323	237,030	50,000	51,020	1,014,110	255,250
Hungary	500,000	131,579	21,795	29,856		
India	1,250,000	56,818	4,500	26,471	2,180,000	155,160
Indonesia					623,110	89,290
Iran	2,000,000	88,889			1,872,000	157,975
Italy	1,988,776	278,423	111,967	40,321	1,884,967	164,701
Japan	900,000	193,133	18,900	54,000	396,000	184,615
Korea DP Rep	630,000	92,647				
Korea Rep	651,778	162,965			7,000	70,000
Lebanon	120,000	92,308	90,000	150,000	160,000	264,463
Mexico	639,900	102,422	331	66,200	4,025,265	130,342
Morocco	384,000	147,692	3,100	34,444	1,193,000	197,190
Netherlands	470,000	345,792	300	7,500		
Nigeria					2,200,000	36,667
Pakistan	600,000	133,333	1,800	36,000	1,410,000	102,920
Poland	1,750,000	218,750	35,626	42,412		
Portugal	265,000	108,163	3,256	9,303	175,000	83,333
Romania	610,000	75,285	74,197	60,313		
Russian Fed	1,200,000	28,571	65,000	26,210		
South Africa	500,000	238,095	500	41,667	900,000	225,000
Spain	783,700	174,156	81,900	32,760	2,507,000	202,177
Switzerland	365,000	331,818	25,000	50,000		
Syria	323,933	119,975	41,315	51,644	656,659	305,608
Turkey	2,250,000	185,950	200,000	96,618	748,790	198,093
Ukraine	1,200,000	40,000				
USA	5,060,000	255,556	175,000	79,545	12,571,000	369,030
Venezuela					527,930	155,274
Vietnam					380,000	63,333
Yugoslavia	264,000	93,286	31,929	46,680	1,792	41,674
World	56,180,310	79,293	1,629,474	53,229	70,483,440	153,822

prune dwarf virus (PDV) in 18-36% of tested *Prunus avium* seeds using protein A sandwich ELISA and DAS-ELISA. The DIBA was adapted for detection of citrus tristeza virus (CTV). Comparing to DAS-ELISA, it was as sensitive as ELISA for CTV diagnosis (Rocha-Pena et al., 1991).

Polyclonal antisera are still the most important detection tools for large-scale routine diagnosis and detection of many isolates of a virus as they can be purchased at reason-

Table 1. Continued

Country	Pears		Peaches & Nectarines		Plums	
	production (MT)	yield (HG/HA)	production (MT)	yield (HG/HA)	production (MT)	yield (HG/HA)
Argentina	588,000	326,667	200,000	60,606	56,000	29,167
Australia	156,022	209,145	93,000	72,565	31,000	72,093
Austria	69,858	120,445	9,579	68,421	76,731	127,460
Brazil	18,892	84,415	149,867	73,979		
Bulgaria	21,000	229,008	60,000	69,767	80,000	66,667
Chile	250,000	200,000	285,000	156,593	150,000	120,000
China	6,727,703	53,394	2,996,413	33,192	2,716,826	32,705
Egypt	56,000	101,818	62,000	38,750	53,000	196,296
France	256,000	182,857	470,000	176,030	210,000	95,455
Georgia	20,000	66,667	20,000	66,667	38,000	76,000
Germany	447,488	186,453	19,000	54,286	396,004	87,297
Greece	55,000	56,324	530,000	91,379	9,000	102,857
Hungary	36,779	52,541	53,819	36,862	115,000	50,000
India	135,000	60,000	87,000	47,027	57,000	40,714
Iran	184,000	87,619	126,000	78,750	160,000	84,211
Italy	969,143	192,138	1,525,252	145,408	116,333	85,039
Japan	427,800	226,349	175,500	162,500	136,200	78,276
Korea DP Rep	120,000	95,238	100,000	71,429		
Korea Rep	260,168	118,350	146,793	134,771	36,006	115,182
Lebanon	65,000	185,714	49,000	272,222	25,000	172,414
Mexico	35,000	68,627	140,000	35,897	77,766	50,432
Morocco	40,000	114,286	34,000	87,179	46,000	68,657
Netherlands	130,000	243,400			6,000	100,000
Pakistan	36,000	128,571	46,000	109,524	80,000	121,212
Poland	58,025	65,938			126,770	65,010
Portugal	165,000	132,000	85,000	77,273	17,500	76,087
Romania	69,873	105,341	17,140	32,230	394,356	39,988
Russian Fed	50,000	29,940	20,000	25,000	155,000	31,000
South Africa	150,000	166,667	220,000	100,000	30,000	65,217
Spain	585,300	158,189	887,500	126,786	148,700	74,350
Switzerland	95,000	296,875	190	31,667	13,000	34,974
Syria	18,909	67,532	23,517	46,940	22,863	90,047
Turkey	400,000	105,263	340,000	159,624	195,000	106,557
Ukraine	160,000	31,683	50,000	34,483	170,000	31,481
USA	833,000	333,200	1,300,000	152,941	815,800	158,223
Venezuela			9,000	34,615		
Yugoslavia	94,469	72,390	58,394	67,120	619,000	49,520
World	14,368,700	78,064	11,087,660	64,278	7,998,450	47,060

able prices. In many cases, virus sample extracts contain some plant contaminants that raise anti-plant antibodies following immunization. Therefore, assays using polyclonal antisera exhibit varying degree of response to plant sap proteins to give a high background signals that can cause problematic diagnosis. A desire to produce more defined reagents led to the development of methods for the production of monoclonal antibodies (MAb). The MAb-based serological assays are proved to be very useful as diagnostic tools. Use of MAbs reduces background reaction. MAbs generated against apple chlorotic leaf spot virus (ACLSV) were possible to detect less than 0.1 ng/ml of purified virus by ELISA and recognized 17 different strains of ACLSV representing most of known strains (Poul and Dunez, 1989; Poul and Dunez, 1990). Hilgert et al. (1993) generated MAbs to plum pox virus (PPV) after immunization of mice with purified

Table 2. Taxonomic groups, isolates designations of fruit tree viruses and their biological characteristics

Group	Virus[a]	Acronym	Symptoms	Transmission[b]	References
Capillovirus	Apple stem grooving virus	ASGV	stem grooves, abnormal graft union	Me/Gr/Se	Uyemoto and Gilmer, 1971 Plese et al., 1975
	Cherry A Virus	CAV	unknown	Gr	Jelkman et al., 1995
	Citrus tatter leaf virus	CTLV	tatter leaf, interveinal chlorosis blotch and malformed leaf	Me/Gr/Se	Zhang et al., 1988 Magome et al., 1997
Closterovirus	Citrus tristeza virus	CTV	quick decline, pitted stem stunt, seedling yellows	Me/Gr/In/Se	Raccah et al., 1976 Garnsey et al., 1977
Ilarvirus	Apple mosaic virus	ApMV	mosaic, mottling, necrotic ring spots	Me/Gr/Po	Gotlieb and Berbee, 1973 Wood et al., 1975
	Citrus leaf rugous virus	CiLRV	leaf flecking and malformation	Me/Gr/Se	Gonsalves and Garnsey, 1975
			small and lumpy fruit		Gonsalves and Garnsey, 1976
	Citrus varigation virus	CVV	leaf flecking and malformation	Me/Gr/Se	Desjardins et al., 1969
	Prune dwarf virus	PDV	leathery, strap-like leaves, stunting	Me/Gr/Se/Po	Torrance and Dolby, 1984
			leaf yellowing and abscission		Kelley and Cameron, 1986
	Prunus necrotic ringspot virus	PNRSV	dark, brown, or chlorotic lines and rings	Me/Gr/Se/Po	Civerolo and Mircetlich, 1972
			bright mosaic		Sweet, 1980
Nepovirus	Cherry leaf roll virus	CLRV	chlorotic mosaic, leaf rolling and death	Me/Gr/ Ne	Horvath et al., 1974
			ring pattern and die-back		Larsen et al., 1990
	Cherry rasf leaf virus	CRLV	enation, stunting, decline, flat fruit	Me/Gr/Se/Ne	Jones et al., 1985
	Peach rosette mosaic virus	PRMV	rosetted shoot, mosaic, stunting	Me/Gr/Se/Ne	Stobbs and Barker, 1985
	Tomato ringspot virus	ToRSV	mosaic or ringspots, rasp leaf, yellow	Me/Gr/Se/Po/Ne	Stouffer et al., 1977
			bud or vein, ringspots and chlorosis		Parish and Converse, 1981
Potyvirus	Passionfruit woodliness virus	PWV	necrotic and chlorotic local lesions or streaks, mottling, rugosity	Me/In	Shukla et al., 1988
	Plum pox virus	PPV	fruit pale rings and deformation	Me/Gr/In/Se	van Oosten, 1970
			leaf mottling, necrotic spots and lines		Varveri et al., 1988
			fruit drop, shoots split and die back		
Sobemovirus	Apple latent virus	ALV (type II)	latent infection	Me/Gr/Se/Po/In	Franki and Miles, 1985
	(Sowbane mosaic virus)				Hardi and Teakle, 1992
Trichovirus	Apple chlorotic leaf spot virus	ACLSV (ALV type I)	chlorotic leaf spots or rings, stem pitting	Me/Gr/Ne	Chairez and Lister, 1973
			stunning, line patterns, chlorosis		Dunez et al., 1975
	Cherry mottle leaf virus (?)	CMLV	chlorotic mottling, distortion of foliage	Me/Gr/Mite	Li et al., 1996
Ungrouped	Apple stem pitting virus	ASPV	die back, inner bark necrosis, decline	Me/Gr	Fridlund and Aichele, 1987
			epinasty, vein yellowing, latent infection		Koganezawa and Yanase, 1990
	Citrus ringspot virus	CRSV	epinasty, chlorotic flecks or pattern	Me/Gr	Desjardins et al., 1969
			mottling, ringspots		Garcia et al., 1997

[a] Viruses with (?) indicate tentative candidates within listed groups. Some fruit tree viruses were not included in this table since there was not enough information. Those include apple necrosis ilarvirus, citrus enation woody-gall luteovirus, peach enation nepovirus (?), peach leaf closterovirus, passionfruit ringspot potyvirus, passionfruit Sri Lankan mottle potyvirus (?), passionfruit yellow mosaic tymovirus, passionfruit rhabdovirus, passionfruit vein-clearing rhabdovirus (?), plum American line pattern ilarvirus, citrus leprosis rhabdovirus, and prunus S carlavirus (?).
[b] Possible transmission patterns. Me = mechanical; Gr = grafting; Se = seeds; Po = pollens; In = insects; and Ne = nematodes.

PPV-W isolate. They showed that four different PPV isolates (W, A, D, and M) can be distinguished by these MAbs and thus suggested that these MAbs can be used for routine diagnostics of plums, peaches, and apricots. Thus it is almost certain that the sales of virus specific antisera and MAbs for research purpose will increase but development and growth of commercial kits are questionable. The costs and time involved in transferring a research assay into commercial kits are significant. Therefore, it is possible that commercial serological assay kits will be used for the early detection of viral diseases of high value fruit crops. Lengthy purification and concentration protocols limit more use of these assays. In addition, the cross-reaction between antisera reduces specificity of the test and precludes detection of closely related

viruses or strains of the same virus. Another major disadvantage was that these conventional serological assays could not be used for the detection of viroids that lack coat proteins (CPs). Antibodies specific for naturally occurring dsRNAs have also been described (Garcia-Luque et al., 1986).

Using an antiserum against polyinosinic-polycytidylic acid, dsRNAs extracted from cucumber mosaic virus and CTV infected plants were readily detected by indirect ELISA and DIBA (Aramburu et al., 1991). Concentrations as low as 1 ng/ml were detected by DIBA and showed similar or higher sensitivity for detection of dsRNA than separation by polyacrylamide gel electrophoresis and silver staining. For CTV, considerably higher ELISA titers were obtained by injecting partially purified native antigens after priming with recombinant antigens (Bar-Joseph et al., 1997). Hinrichs et al. (1997) reported new way of antibody induction techniques which using DNA sequence of interest proteins. They introduced appropriate nucleotide sequence of tobacco mosaic virus CP and potato virus Y P1 proteins and showed the induction of antibodies. This new technique can be used for the induction of antibodies against many other proteins, if the nucleotide sequence of the gene encoding the protein is known and eliminate time, labor, and the technically demanding steps of antigen purification.

Recently, a number of studies shown that detection of plant viruses can be easily accomplished by 'direct tissue blotting immunological assays' (Hsu et al., 1995; Knapp et al., 1995). Knapp et al. (1995) used immuno-tissue printing (ITP) method for the localization of ACLSV, apple stem grooving virus (ASGV) and PPV in shoots of *Prunus* and *Malus* species. They suggested that ITP could be used as a rapid and accurate immunological method for diagnosis and the localization of these viruses within woody species *in vitro*. This technique proved to be highly advantageous over other serological methods, especially when the phytosanitary status of plant organs, as tiny as meristems, had to be recorded. ITP was a more reliable method than ELISA for diagnosis of ASGV. Because of its extremely localized and limited occurrence in the stem tissues, ELISA might provide false negatives. The combination of grafting techniques with ITP will open a wide range of model experiments with fruit tree cultivars.

Nucleic acid-based tests. It is generally considered that closely related viruses share a greater nucleotide sequence similarity than those that are distantly related. A highly specific nucleotide sequence present in an isolate or strain of virus but absent or different in other strain of virus or species can be used for detection of viruses. Recently, an enzymatic reaction procedure named polymerase chain reaction (PCR) was described which allows the amplification of very low amounts of target nucleic acids (Saki et al., 1985). This technique has been used successfully to detect very low amounts of viral nucleic acids and viroids. Nucleic acid-based tests have several advantages over serological assays. The antigenic determinants of viral coat proteins used for most serological assays represent only about 2 to 5% of the viral genome. Many characteristics in virus strains and isolates are governed by major portions of other viral genomes and thus cannot be differentiated by serological assays. The cloned or cDNA probes with appropriate common or specific sequences of nucleotides can be prepared and labeled in different ways. The polyvalence of the molecular hybridization assay was further improved by using RNA probes corresponding to structural and nonstructural protein genes, which has been shown to diagnose and differentiate virus strains. The sensitivity can be increased by amplification of desired sequences by using PCR.

PCR. PCR is an *in vitro* method in which DNA sequences are rapidly amplified with very high specificity and fidelity using oligonucleotide primers and thermostable DNA polymerase (Fig. 1). For many plant viral pathogens with RNA genomes, reverse transcription reaction coupled with PCR (RT-PCR) proved much more effective than ELISA assay. The availability of nucleotide sequences of many viruses and viroids has enhanced the use of PCR-based assays as diagnostic tool. The PCR is a very powerful method that has greatly facilitated detection of plant viruses that would be difficult or time consuming to detect using conventional assays (for review, see Hadidi et al., 1995). The PCR products can be used a) as a target for hybridization, b) for direct sequencing of DNA, and c) as a specific probe. The advantage of PCR-based assays includes high sensitivity, high specificity, and high sample throughput. It has been reported that using PCR-based assay one can claims the detection of around 10 femtograms (fg) of viral RNA (Romaine and Schlagnhaufer, 1995). In comparison with serological assays, PCR primers with any degree of selectivity can be synthesized at a much lower cost than that associated with the development of monoclonal or polyclonal antibodies. Because very small amounts of nucleic acid are needed for PCR amplification, the development of rapid, small-scale procedure would allow testing of many samples and increase the efficacy of PCR as a tool for routine diagnostics. Ironically, high sensitivity also increases the risk of sample carry-over contamination restricting PCR-based assays for routine usage.

The RT-PCR assays have been used for the detection of several viruses infecting woody plants. PPV was detected by PCR in infected bark of trees so that the assay can be performed throughout the year (Korschineck et al., 1991; Wetzel et al., 1991). They showed that the PCR-based assay was a lot more sensitive than ELISA or nucleic acid molec-

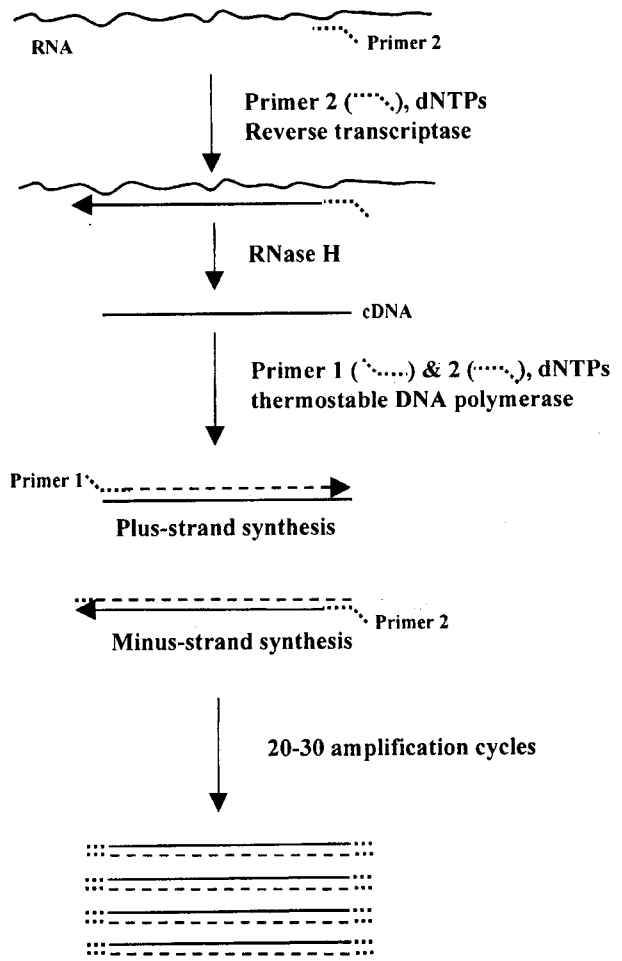

Figure 1. Reverse transcription and subsequent PCR of the synthesized cDNA using thermostable DNA polymerase.

ular hybridization assays. As few as 10 fg of viral RNA could be detected in plant extracts. Borja and Ponz (1992) also detected cherry leaf roll virus (CLRV) in infected walnut buds and twigs using virus specific probes that amplified a specific fragment of 448 bp from 3 nontranslated region of viral RNAs. The RT-PCR assays have been employed for the detection of several other fruit tree viruses (Candresse et al., 1995b; Kokko et al., 1996; Nolasco et al., 1993; Rosner et al., 1997; Spiegel et al., 1994; Sugieda et al., 1998; Vitushkina et al., 1994).

Application of RT-PCR for the detection of plant RNA viruses is considerably limited by production of secondary metabolic products in sample extracts. This is especially crucial limiting factor for use of PCR-based assay in the case of many fruit tree varieties of *Malus*, *Prunus*, and *Pyrus* origin (Korschineck et al., 1991). Woody plant extracts not only contain very low concentration of virus in many cases but also may contain many components that degrade viral RNA and/or prevent RT enzyme reaction. It has been reported that plant extracts contain components which interfere with the extraction of intact RNA (Newberry and Possingham, 1979) or inhibit synthesis of cDNA complementary to viral RNA present in the infected tissue (Rowhani et al., 1993; Vunsh et al., 1991).

The development of rapid methods for RNA extraction from infected tissue samples helped overcoming these limitations in the diagnosis and characterization of viruses using RT-PCR. Methods using spin column enables a rapid and efficient RNA extractions and eliminate the use of hazardous chemicals. This type of extraction procedure was recently shown to be very efficient for high quality RNA extraction and subsequent virus detection using RT-PCR (Levy et al., 1994).

Several other approaches have been tried to overcome extraction related limitation, among them immuno-capture (IC) of virus particles (Candresse et al., 1995a; Jacobi et al., 1998; Jansen et al., 1990) and silica capture (SC) of total nucleic acids seemed to be the most successful assays. Detection of viral pathogens becomes more sensitive when antibody binding and PCR are combined. The sensitivity of detection is 250 times that of direct PCR (Wetzel et al., 1992). For IC-RT-PCR, plant extracts were pre-incubated with specific antiserum in PCR tubes in a fashion reminiscent of ELISA assay. This step concentrates and pre-purifies the virus particles. Immuno-captured samples were then used for RT-PCR omitting the need for nucleic acid extractions. This method shows increased detection sensitivity compared to ELISA by several orders of magnitudes (Candresse et al., 1995b; Hadidi et al., 1995; Jacobi et al., 1998; Werner et al., 1997; Wetzel et al., 1992). IC-RT-PCR is reliable over a large part of the growing season for the detection of ACLSV strains taken from orchard trees of apple, pear, plum, cherry, apricot, peach, and quince (Candresse et al., 1995a). Recently, Werner et al. (1997) applied this sensitive method in the detection of CLRV. IC-RT-PCR assay was sensitive enough to detect minute amount of CLRV in several woody plant samples. For SC-RT-PCR, total nucleic acid extracts prepared by reversible binding on silica particles in the presence of guanidium thiocyanate proved to be suitable for RT-PCR detection of PPV, ACLSV, PDV, and apple stem pitting virus belonging to different virus groups (Malinowski, 1997). SC-RT-PCR seems to be useful. Immuno-PCR is another highly sensitive assay that uses straptavidin-labeled DNA fragments linked to antigen-antibody (protein A linked) complex. This complex is then bound to biotin-labeled DNA sequences followed by PCR amplification. This assay is shown to be 10^5 times more sensitive than ELISA (Sano et al., 1992) and only require antigen-specific antibody.

Recently, the PCR-ELISA assay has been introduced which enable immunoenzymatic determination of PCR products in the liquid phase without the need for electro-

phoresis, thereby simplifying the analysis of the results with an ELISA reader. These highly sensitive assays have been used for the diagnosis of PPV-D and PPV-M isolates in plum trees and tobacco (Poggi Pollini et al., 1997). When serial dilutions of infected plant extracts were assayed, PCR-ELISA was found to be 100 times more sensitive than relatively conventional IC-PCR (Olmos et al., 1997). This high specificity was also demonstrated for the detection of phytoplasma species in trees and shrubs with different capture probes (Poggi Pollini et al., 1997). The PCR-ELISA assay is simple to use, capable of processing large sample numbers, and eliminates the use of hazardous chemicals during electrophoresis procedures, especially if restriction fragment length polymorphism analysis of the amplified products is necessary. Its biggest drawback, at present, is the cost.

Limitations still exist for the use of PCR-based assays for large scale virus detection although it is likely that microtiter plate formats or other adaptations of amplified fragment analysis will soon be available (Hataya et al., 1994). RT-PCR proved to be most sensitive of all the compared method but PCR, being labor-consuming and expensive, at present it is recommended for testing only of the most valuable mother plants and breeding sources, and not for large scale screening.

Molecular hybridization. Sensitivity and reliability of the molecular hybridization methods depend on the concentration and distribution of the viruses, the virus recovery during sample preparation, and the quality of probes used to detect viral nucleic acids. Detection of viral pathogens in infected samples is based on the production of nucleic acids by specific hybridization between the single-stranded target nucleic acid sequences and complementary single-stranded probes, mostly cDNA. The cloned probes with varying specifications and in unlimited quantities can be produced to meet desired requirements for different assays.

Dot-blot hybridization (DBH) is extensively used for the detection of plant viruses and viroids. Eventhough this test generally does not distinguish types and sizes of nucleic acids, it can be very useful for qualitative detection since this method can discriminate closely related but different target sequences. Citrus exocortis viroid was detected by using both radioactive and non-radioactive probes (Flores, 1986; Fonseca et al., 1996). PPV-D was detected in infected orchards by using various lengths of radioactively labeled probes (Wetzel et al., 1990). The detection limit was of about 5 pg of purified virus per assay. The DBH using radiolabeled RNA probes were able to detect serotypes of prunus necrotic ringspot virus in peach, cherry, and herbaceous hosts (Crosslin et al., 1992). Borja and Ponz (1992) detected the CLRV in crude plant extracts. The non-radioactive DBH assay using digoxigenin-labeled RNA probes was shown to be as sensitive as DBH using radioactively labeled probes for CLRV (Mas et al., 1993). The presence of apple scar skin group viroid in infected sap extracts could be detected by DBH, which detected a minimum of 2.0-2.5 pg of purified viroid (Podleckis et al., 1993).

Imprint-hybridization (IH) assay was used for the detection of viroids that are difficult to detect using serological methods and showed that IH is fast and sensitive, and provides additional information on the sites of viroid accumulation (Romero-Durban et al., 1995). Nucleic acid hybridization including IH is now preferred detection method for viroid indexing, especially when handling a large number of samples.

Conclusions

For last twenty years, identifications and characterization of the plant viruses has revolutionary changed. Though symptoms are still the major criterion for virus identification, it should never be based on symptoms alone because symptoms vary with strains of viruses, the kind and age of hosts, and environmental stresses. The effectiveness of a detection method is highly influenced by the way the tissue samples were collected. Because of its simplicity and possibility of handling a large number of samples at one time, ELISA-based tests were one of the most frequently used diagnostic tools. However, recent developments of PCR-based tests and molecular hybridization tests will probably change the testing methodology for virus diagnostics. Each detection test is a compromise between sensitivity and specificity. It is very difficult to develop tests that are very specific and very sensitive. Especially for methods with a very high sensitivity there is a high risk of contamination due to the carry-over from one sample to another. This can give false positive results. Depending on the crops, the nature of the viruses, and the interests of grower and consumer, one has to make a decision on the test to be used. It is safe to use more than one-detection methods for important viral diseases. One of the primary selection criteria for detection techniques is their cost of the reagents, chemicals, required equipment, and labor. In addition, useful methods should be rapid, simple to use, reliable, and specific enough to detect virus strains or mixed infections.

Good detection methods should have a high signal/noise ratio. Collecting sample tissues that contain high virus titers can increase the signal in a test. PCR and nucleic acid sequence-based amplification detection methods are also increasing the amount of genetic material of the virus one is looking for. Preparing highly specific antibodies, primers and probes can reduce noise in a test. Detection and identification of viruses has to be done with a particular objective in mind: sanitation. The interaction between phytosanitory

requirements and diagnostic techniques is very important. No proper quarantine measures are possible without a solid diagnostic basis. The assays described in this paper can be used to distinguish closely related pathogens and in many cases to identify viruses in extracts made directly from infected plant material or soil.

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