Advances in the detection of plant pathogens by polymerase chain reaction

edited by

Charles Manceau
Laboratory of plant pathology, INRA, Beaucouzé, France

and

Josef Spak
Institute of plant molecular biology, Ceske Budejovice, Czech Republic

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USE OF DEGENERATE PRIMERS FOR RT-PCR DETECTION OF APPLE AND PEAR TREE VIRUSES

J. KUMMERT*, G. RUFFLARD, V. DE ALMEIDA MARINHO

Centre National de Phytovirologie IRSIA-Unité de Phytopathologie, Faculté Universitaire des Sciences agronomiques, B-5030 Gembloux, BELGIUM

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ABSTRACT

Apple chlorotic leafspot virus (ACLSV), Apple stem grooving virus (ASGV), Apple stem pitting virus (ASPV) isolates and complexes containing these viruses have been transferred and multiplied on herbaceous host plants in the greenhouse for the development of RT-PCR detection methods using degenerate primers. Comparative analysis of nucleotide and deduced amino acid sequences published for 2 isolates of ACLSV, one isolate of ASGV and one isolate of ASPV showed the presence of several short stretches of homologous amino acids, although the three viruses belong to different phytovirus genera. Only amino acid sequence homologies corresponding to the translation product of the 3'-terminal part of the putative RNA polymerase gene are sufficiently conserved and have a sufficient length to allow the design of different sets of degenerate primers. These primers have been used for amplification of viral sequences from reverse transcribed total RNA preparations of virus-infected leaves of *Chenopodium quinoa* or *Nicotiana occidentalis*. As the sizes of the amplified products are quite similar for the 3 viruses, the precise identification of the responsible virus would require the combination of PCR and hybridization with specific probes from cloned cDNA. Cloned PCR products have been sequenced to identify the causal agents and to design more efficient or more specific primer pairs in order to allow the detection of the various isolates of viruses (specially ASGV and ASPV). The RT-PCR protocols thus obtained will be adapted for use on woody material and specially mother trees with the aim to define certification scheme for virus free propagating material.

INTRODUCTION

Apple chlorotic leafspot virus (ACLSV), Apple stem grooving virus (ASGV) and Apple stem pitting virus (ASPV) are three latent viruses with elongated particles infecting pome fruit trees (apple and pear), ACLSV isolates also infects stone fruit sometimes inducing, severe symptoms (pseudo-pox ; bark split).

The detection of these fruit tree viruses relies on biological indexing by grafting on woody indicators in the field, or eventually in the greenhouse. These techniques which constitute the "baseline" tests for the certification of fruit tree planting material are working quite well, but are cumbersome, lengthy and expensive to perform (time, space and manpower required), they are also not always very specific and may not differentiate mixed infections with several related viruses.

There seems now to exist a good evidence that some fruit tree viruses could be assayed, with equal reliability to biological methods by laboratory tests that are becoming increasingly sensitive and refined with the accumulation of experience in serology and molecular biology.

Serological detection by ELISA tests is possible for ACLSV and ASGV with commercially available antisera, but accurate only during a short time period in the spring; no available antiserum exists for ASPV, to day.

On the other hand, the transfer of isolates of these viruses on herbaceous host plants, in the greenhouse, is possible, rendering them available for multiplication, characterization and use all year long, although purification of virus particles, even from these herbaceous hosts remains difficult.

As the genetic organization and thus the classification of these viruses was confused and quite ambiguous till recently, and the precise identification of the reference isolates collected not sure, we aimed at developing RT-PCR techniques using degenerate primers to obtain amplification products which would be specific for virus-infected plants.

The protocols developed can be used for direct detection of virus infection by analysis of amplified products in ethidium bromide stained agarose gels.

Specific amplification products have been cloned for further use, as sequencing for taxonomic identification of the isolates and design of more specific primers or primers more adapted to cope with the variability of the concerned viral agents. The cloned products also allow the development of non radioactive probes to be used in dot blot hybridization tests for virus detection.

The present communication is dealing with the development of RT-PCR amplification tests for detection of ACLSV, ASGV and ASPV in herbaceous hosts by using polyvalent degenerate primers and analysing the amplification products by electrophoresis in ethidium bromide stained agarose gels, and of it further appraisal for use with woody infected samples from apple trees.

MATERIAL AND METHODS

Transfer of the virus isolates on herbaceous hosts plants

Young leaves taken on trees in orchards, or developed on shoots taken during the vegetative rest, stored at 4°C and put into growth by dipping in water or nutritive solutions at 20-24°C under light, are grinded in 4 parts (w/vol) potassium-sodium phosphate buffer 0.04 M pH 7.2 containing 0.01 M sodium diethyl carbamate and 2 % nicotine sulphate. The solution and the inoculum are maintained on melting ice. Inoculation is made with gloves on Carborundum dusted leaves, and plants are immediately rinsed with distilled water. The tests plants used for virus multiplication are *Chenopodium quinoa* and *Nicotiana occidentalis*, grown in the greenhouse with supplemented light (16 h).

Infected reference trees

Next to the collection of virus isolates transferred on herbaceous hosts, we dispose of 10 apple-trees prepared by the research station of Gorsem planted in soil in Gembloux and infected by 1 or more generally by a complex from 2 to 3 of the viruses studied (Table 1). An extensive collection of infected trees is also available at the research station on fruit trees of Gorsem.

Table 1 - Apple trees planted in soil in Gembloux

Identification	Viruses determined by biological indexing
91-325	ASPV
91-327	ACLSV + SED*
91-328	ACLSV
73058	ASPV
10291	ACLSV + ASGV + SED
10392	ACLSV + ASGV
11043	ACLSV + ASGV + SED
J.C.D. 1390	ASPV
A.TH.1385	ASPV
LP680	ASPV

** "Spy epinasty and decline" disease

RNA extraction and cDNA synthesis

Total RNA was extracted from 100 mg samples of leaf material of herbaceous host plants or apple trees using the Tripure reagent from Boehringer, according to the manufacturer's protocol.

Single stranded cDNA was synthesized from 1 to 5 µg of purified total RNA using the Superscript preamplification system for first strand cDNA synthesis (Life Technologies) or the Expand™ reverse transcriptase (Boehringer), with oligo-dT primers. The resulting cDNAs were then diluted 5-fold with sterile water. Five microliters from these preparations were then used for the PCR amplifications. In some cases, specially for total RNA preparations from apple material, or for sequencing of PCR amplified products, more defined degenerate or specific primers were used.

PCR experiments

Amplification from the cDNA were carried out with 5 µl of cDNA preparations in a total volume of 50 or 100 µl of PCR buffer (10 mM Tris-HCl pH 9.0, 50 mM Kcl, 0.1 % Triton X-100), containing $MgCl_2$ (1.5 mM), each dATP, dCTP, dGTP, dTTP (200 mM), 0.05 or 0.1 nmol of each upstream and downstream primers and 1 unit of Taq DNA polymerase (Boehringer). Thermal cycling was realized in a triothermoblock cycler (Biometra, Göttingen) or a PTC 200 cycler from M.J. Research. Taq polymerase was added during a 5 min stage at 72°C after a denaturation step of 5 min at 94°C.

For short degenerate primers with added adapter sequences, cycling was the following : template denaturation at 94°C for 30 sec, primer annealing at 40 or 42°C (cycles 1-5) or 50°C (cycles 6-35) for 1 min and DNA synthesis at 72°C for 2 min. For degenerate primers (23 to 27 nucleotides) without adapter sequences, primer annealing temperature of 50°C was used for 30 cycles. A final 15 min elongation step was performed at the end of the 30 or 35 cycles and 10 µl of the reaction mixtures analyzed by electrophoresis in 1 % agarose gel stained with ethidium bromide (Sambrook *et al.*, 1989). For some more specific primers, particular cycling conditions may be used which are specified in the text.

Cloning and sequencing of PCR amplified fragments

After electrophoresis, the amplified DNA fragments of expected sizes were excised and eluted with the QIAEX gel extraction kit (QIAGEN). The amplified fragments obtained with the first degenerate primers were cloned in pBluescript vector (Stratagene) using the EcoRI and BamHI restriction sites located in the primers (Sambrook *et al.*, 1989). The amplified fragments obtained with further selected degenerate or specific primers were cloned in the plasmid pCRII using the TA cloning kit (Invitrogen) according to manufacturer's instructions.

Double-stranded DNA sequencing by the dideoxy chain termination method was performed with T7 DNA polymerase (Pharmacia) according to manufacturer's instructions. Long template amplification products are sequenced with the help of the double-stranded nested deletion kit from Pharmacia, using exonuclease III and S_1 nuclease.

RESULTS AND DISCUSSION

Multiplication of virus isolates on herbaceous hosts

All the isolates used in this study have been transferred and multiplied in *C. quinoa* and/or *N. occidentalis* in the greenhouse. Table 2 presents the origin and composition of the different isolates and the symptoms induced on the tests plants used.

The constitution of this collection of virus isolates has shown that the identification based on the results of serological tests and partial sequencing of PCR amplification products conducted on these herbaceous hosts may be quite different from that given for the trees or the shoots when received, and most generally based on reactions observed on woody indicators.

Table 2 - Isolates maintained on herbaceous host plants in the greenhouse

	Origin	Symptoms observed on		Virus
		C. quinoa	*N. occidentalis*	isolate
P863	prune(1)	LLt ; (Mo)	-	ACLSV
91-300	apple	LLc-n ; Mo	-	ACLSV
91-297	apple	LLC ; Mod	Sc, r ; (Mo)	ACLSV + ASGV
Gorsem 77	apple	LLC ; Mod	Sc, r ; Sc, r	ASGV
10311	apple	(LLc) ; Mod, e	-	ASGV
10604	apple	(LLC) ; Mo	Sc; Sc	ASGV
10771	apple	LLC ; Mod, e	-	ASGV
VDM879	apple	LLC ; Mod, e	N.T.	ASGV
PV0199	apple(4)	LLC ; Mod, e	(4)	ASGV
PA66	pear(2)	-	Sc, r ; Mo	ASPV
ASPV-J	pear(2)	-	Sc, r ; Mo	ASPV
3536	pear(3)	-	Sc, r ; Mod	ASPV
PSA-H	pear(2)	-	Mod ; Mod	PVYV

- : no symptoms ; LL : local lesions ; Mo : mosaic; S : spots or blots ; c : chlorotic ; n : necrotic ; d : deforming ; r : reddening ; t : translucid ; e: epinasty ; () : labile or occasional symptoms. N.T.: not tested. -;-: inoculated leaves; systemic symptoms.

Reference isolates received in herbaceous hosts from : (1) : Candresse T. (INRA, Bordeaux, Fr.) ; (2) : Jelkmann W. (BBA, Dosenheim, Ge) ; (3) : Leone G. IPO/DLO, Wageningen, NL) ; (4) DSM, (Braunschweig, Ge)

Design of degenerate primers for PCR amplification

Our approach of RT-PCR is based on the use of degenerate primers consisting in the mixture of most or all the potential nucleotide sequences coding for conserved amino acids in the products of corresponding genes from related viruses.

This approach has been shown of practical use in our laboratory as it allowed the detection and further identification of 4 different potyviruses infecting sweet potato (Colinet and Kummert, 1993 ; Colinet *et al.*, 1994). It can be used to detect most or all the isolates of one particular virus, different related viruses within a genus, as shown for potyviruses (Langeveld *et al.*, 1991) luteoviruses (Robertson *et al.*, 1991) or geminiviruses (Rybicky and Hughes, 1990), and maybe also viruses from related genus in a family like Potyviridae or Potexviridae.

Although the 3 viruses belong to different virus genus, several stretches of sequence homologies were found in the amino acid sequences of the translation products of the putative RNA polymerase gene of ACLSV (plum isolate : German *et al.*, 1990 ; apple isolate : Sato *et al.*, 1993), ASGV (Yoshikawa *et al.*, 1992) and ASPV (Jelkmann, 1994), allowing the design of degenerate primers for PCR experiments (fig. 1).

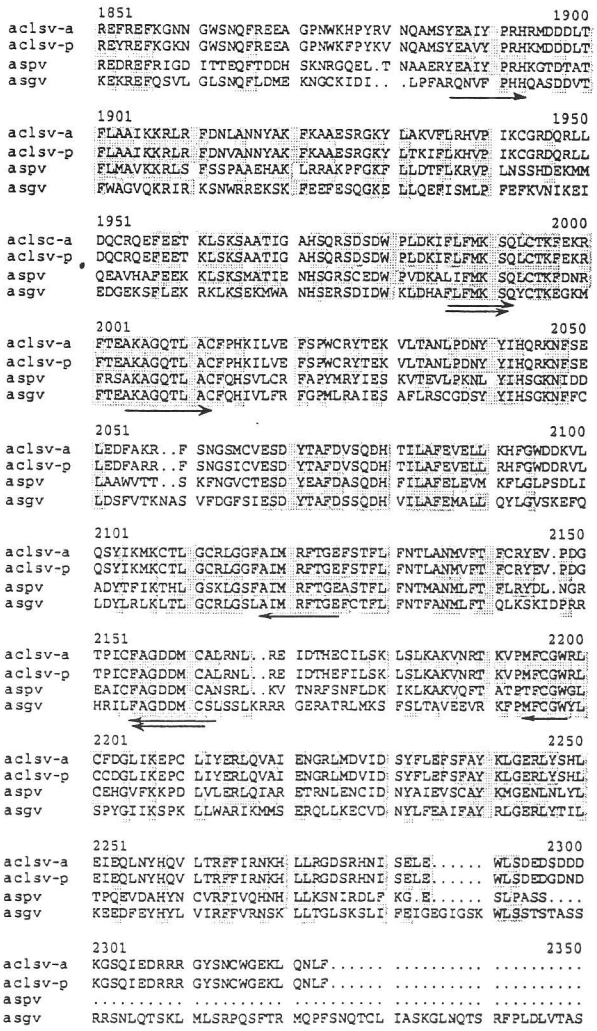

Figure 1 - Alignment of amino acid sequences of translation products from the 3' end of the putative RNA polymerase gene of ACLSVp (German *et al.*, 1990), ACLSVa (Sato *et al.*, 1993), ASGV (Yoshikawa *et al*, 1992) and ASPV (Jelkmann, 1994). Arrows indicate the selected degenerate primers used for this work.

Adapter sequences containing a restriction site were added to the short first selected primers (fig. 2a), for subsequent cloning of the amplification products thus obtained. They also allow a higher temperature for annealing from cycle 6 in the polymerase chain reaction. The availability of the TA cloning kit (Invitrogen) for PCR amplified products allowed the further selection of longer primers, without adapter sequences (fig. 2b).

Sequencing of amplification products from reverse transcribed total RNA preparations from plants infected by different isolates from the same virus allow the design of more specific and mainly more efficient primer pairs based on the comparison of nucleotide sequences. Fig. 2c shows the sequence a such a primer pair defined from the sequences of five ASGV isolates. The same approach will be used for ASPV.

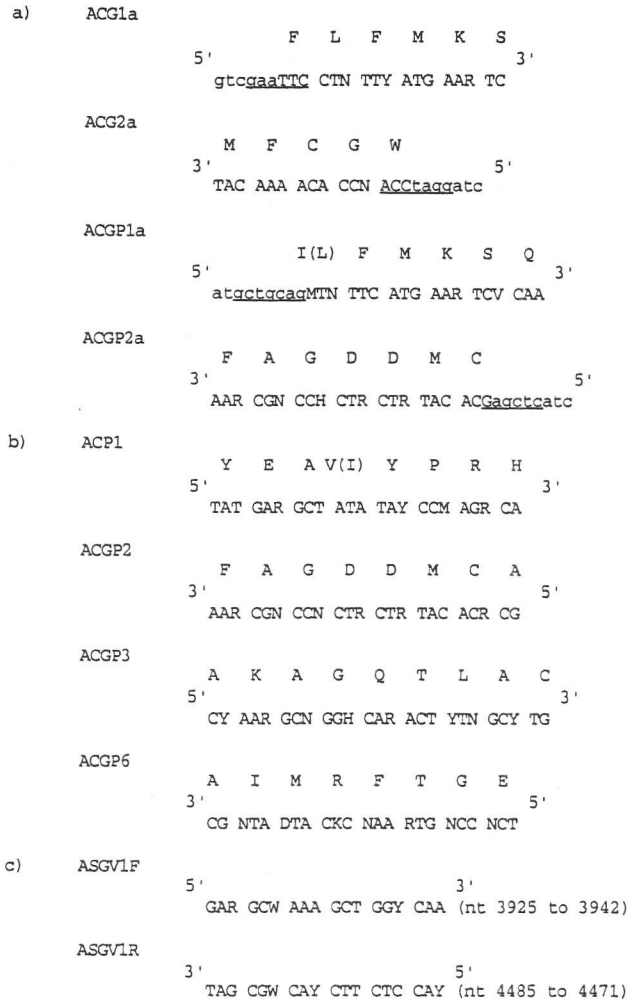

Figure 2 - Primers designed for RT-PCR reactions from cDNA transcripts from total RNA preparations from infected plants.
(a-b) : Selected conserved amino acid motifs found in the C-terminal part of the putative RNA-polymerase of ACLSV, ASGV and/or ASPV, and nucleotide sequences of deduced degenerate primers corresponding to either the coding or the complementary strand. Lower case letters represent nucleotides not derived from the viral sequences (adapter sequences)
(c) : degenerate primers corresponding to the nucleotide sequences of cloned amplification products obtained with primers ACG1a, ACG2a for plants infected by 5 ASGV isolates.
R = A or G; W = A or T; Y = C or T; M = A or C; K = G or T; H = A, T or C; D = G, A or T; V = G, A or C; N = A, C, G or T.

Table 3 presents the expected sizes of amplification products obtained for the different selected primer pairs.

Table 3 - Expected sizes of the amplified products (bp)

Primers	Virus		
	ACLSV	ASPV	ASGV
ACG1a-ACG2a	624	-	639
ACGP1a-ACGP2a	515	515	524
ACP1-ACGP2	818	817	-
ACP1-ACGP6	715	714	-
ACGP3-ACGP2	464	464	473
ACGP3-ACGP6	359	360	365
ASGV1F- ASGV1R	-	-	551

Size expressed as the number of base pairs. For sequences of the primers and their localization in the 3' terminal part of the gene coding for the viral RNA polymerase, see respectively figures 2 and 1.

RT-PCR experiments

Combined assays of reverse transcription and polymerase chain reaction (RT-PCR) have been performed on total RNA preparations from symptomatic leaves of *C. quinoa* and *N. occidentalis* infected with isolates of ACLSV, ASGV, a mixture of both viruses, or ASPV. As the genomic RNA of the targeted viruses possess a polyadenylated 3' end, reverse transcription has been primed with oligo-dT, and the PCR reaction conducted with the different primer pairs defined in figure 2.

Figure 3 shows the results of agarose gel electrophoretic analysis of amplification products obtained from transcripts of total RNA preparations of leaves of *C. quinoa* plants inoculated with an ACLSV isolate (91-300), an ASGV isolate (10311) and a complex containing both viruses (91-297) with four different pairs of degenerate primers. The respective sizes of amplification products obtained (~ 520, 630, 360 or 470 bp) correspond to those expected from the position of the different sets of degenerate primers on the sequences published for ACLSV and ASGV. The absence in this assay of a specific amplification product for the ASGV infected material with primers ACGP1a-ACGP2a illustrates the less consistent amplification often observed for this pair of short degenerate primers.

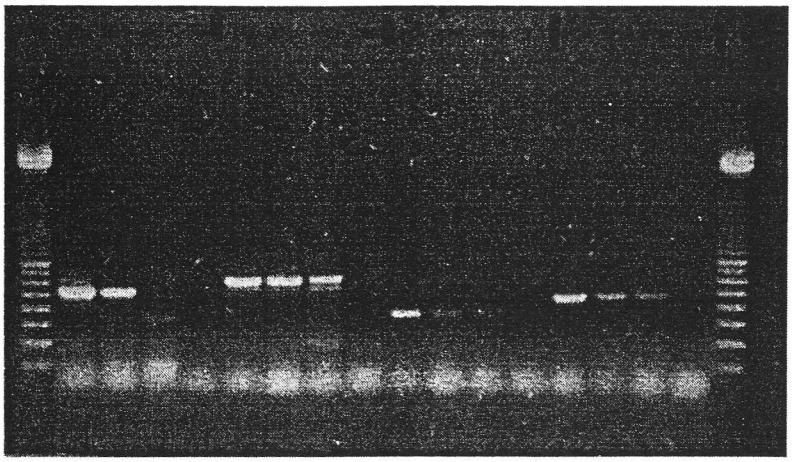

Figure 3 - Agarose gel electrophoretic analysis of PCR amplification products from transcripts of total RNA preparations from leaves of *C. quinoa* infected with isolates 91-300 (ACLSV; lines 1, 5, 9, 13), 91-297 (ACLSV + ASGV; lines 2, 6, 10, 14), 10311 (ASGV; lines 3, 7, 11, 15). Lines 4, 8, 12, 16 correspond to the negative control (no cDNA added). Primer pairs were ACGP1a-ACGP2a (lines 1-4), ACG1a-ACG2a (lines 5-8), ACGP3-ACGP6 (lines 9-12) and ACGP3-ACGP2 (lines 13-16). M = molecular weight marker (100 bp DNA ladder, Life Technologies).

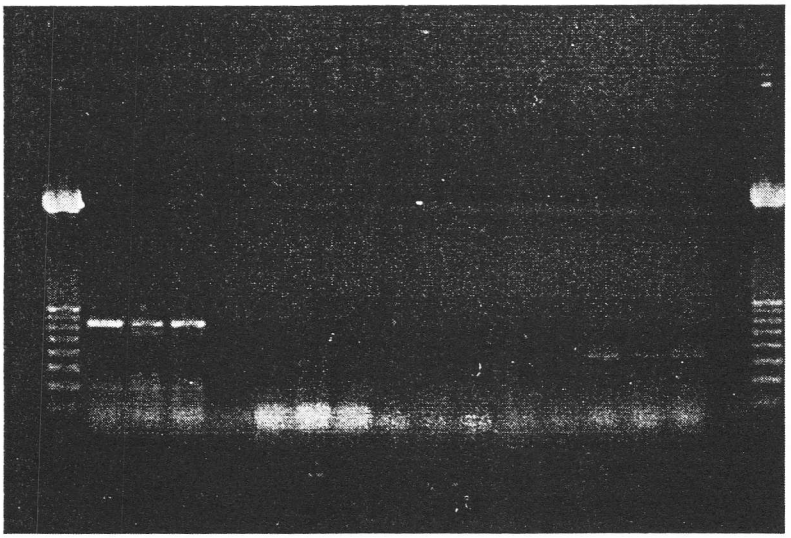

Figure 4 - Agarose gel electrophoretic analysis of PCR amplification products from transcripts of total RNA preparations from leaves of *C. quinoa* inoculated with ASGV isolates 10311 (lines 1, 5, 9, 13), 10391 (lines 2, 6, 10, 14), or 10771 (lines 3, 7, 11, 15). Negative control without added cDNA (lines 4, 8, 12, 16). Primer pairs were ACG1a-ACG2a (lines 1-4), ACGP1a-ACGP2a (lines 5-8), ACGP3-ACGP2 (lines 9-12) and ACGP3-ACGP6 (lines 13-16). M = molecular weight marker (100 bp DNA ladder, Life Technologies).

Figure 4 shows the results of agarose gel electrophoretic analysis of amplified products from transcripts of total RNA preparations of leaves of *C. quinoa* plants inoculated with 3 different ASGV isolates. Here also, the sizes of the amplified products obtained with primers ACG1a-ACG2a, ACGP3-ACGP2 and ACGP3-ACGP6, correspond to those expected from the position of the respective primers on the sequences published for an ASGV isolate. As for the experiment reported in figure 3, the primer pair ACGP1a-ACGP2a did not allow the amplification of one virus specific DNA fragment from transcripts of total RNA from plants infected by ASGV.

Figure 5 shows the results of agarose gel electrophoretic analysis of amplification products obtained from transcripts of total RNA preparations of leaves of *N. occidentalis* plants infected by ASPV isolates 3536 and ASPV-J using primers ACGP1a-ACGP2a and ACGP3-ACGP6. The sizes of the amplification products, respectively ~ 520 and 360 bp, correspond to those expected from the position of the degenerate primers on the sequence published for ASPV. The absence of visible amplification products with primers ACGP3-ACGP6 for transcripts of total RNA preparation from the leaves stored at 4°C (initial inoculum for ASPV-J) may be due to degradation of the virus particles.

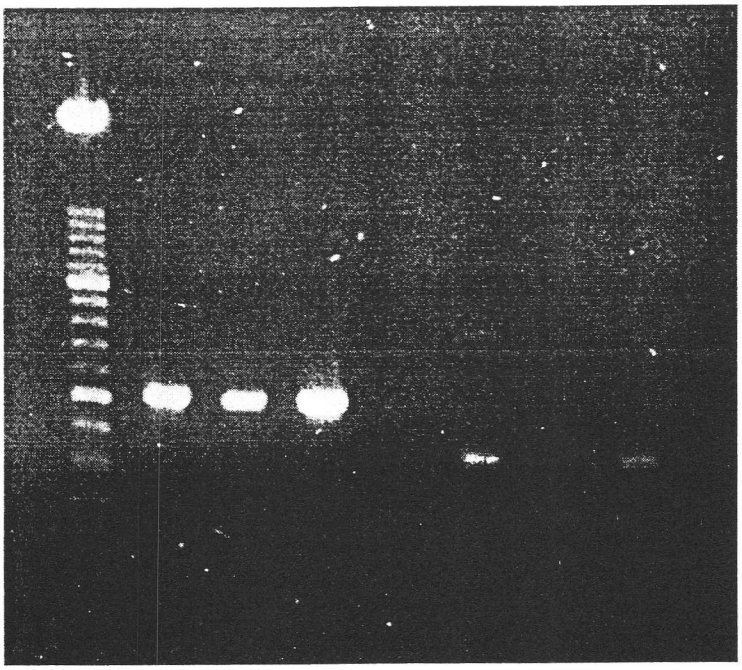

Figure 5 - Agarose gel electrophoretic analysis of PCR amplification products from transcripts of total RNA preparations from leaves of *N. occidentalis* infected by isolate 3536 (lines 1, 5), ASPV-J: initial inoculum received as fresh leaves material and conserved at 4°C (lines 2, 6), ASPV-J after several successive passages on tobacco in the greenhouse (lines 3, 7). lines 4, 8 = negative control without added cDNA. M = molecular weight marker XIV from Boehringer (100 bp DNA ladder). Primers pair ACGP1a-ACGP2a (lines 1-4), ACGP3-ACGP6 (lines 5-8).

Figures 4 and 5 show that the first selected shorter sets of degenerate primers ACG1a-ACG2a and ACGP1a-ACGP2a may lead to inconsistent amplification of specific viral sequences. Furthermore, due to the relatively low temperature (42°C) used for annealing of these short degenerate primers during the first cycles of amplification, non specific small sized amplification products are often observed which emphasizes the need to include molecular weight markers in the gel to control the correct size of amplification products in order to avoid misinterpretation of RT-PCR tests due to incomplete or unspecific amplification when using degenerate primers. As a matter of fact, the production of DNA complementary to plant nucleic acid sequences when using primers designed for the amplification of viral nucleic acid sequences has been reported by several authors and, for example, when using degenerate primers for the detection of potyviruses. Pappu *et al.*, (1993) and Pearson *et al.* (1994) have shown that with some plant species the amplification of discrete fragments of cDNA may not be indicative of virus infection.

This observation let us consider the research of more specific primers, although able to cope with the variability of the different virus isolates of the same virus.

For ASGV, amplification products obtained with the primers pair ACG1a-ACG2a for 5 different isolates have been cloned and sequenced. Comparative analysis of the sequences thus obtained allowed the design of a primers pair ASGV1F-ASGV1R (figure 2c) based on the presence of strechtes of nucleotide sequences with a minimum of variations.

Figure 6 shows that the use of these primers allows the amplification of one specific product with the expected size, from transcripts of total RNA preparations from *C. quinoa* leaves infected with the different ASGV isolates tested.

Figure 7 shows that the same primers also give amplification products for transcripts of total RNA preparations from ASGV infected apple trees. This test was performed during a dry hot period (samples collected, and total RNAs extracted the 31.05 and 10.6.96). The band of expected size was faintly visible in the ethidium bromide stained agarose gel, but clearly present by Southern blotting and detection with a digoxigenine labelled probe synthesized by PCR from cloned amplification product of isolate 10311 with primers ACG1a-ACG2a. The collected leaf material gave positive results in ELISA tests with ASGV antiserum.

Concerning cDNA trancripts from total RNA preparations from apple leaves, virus-specific amplifications have been occasionally but inconsistently observed for material taken on buds developped on dormant shoots grown in nutritive solutions and on material taken on apple trees in may and june 1995 but not in july and august of the same year; the primers pairs used were ACGP1a-ACGP2a and A52-A53 (developped by T. CANDRESSE, INRA-Bordeaux, for ACLSV) (results not shown).

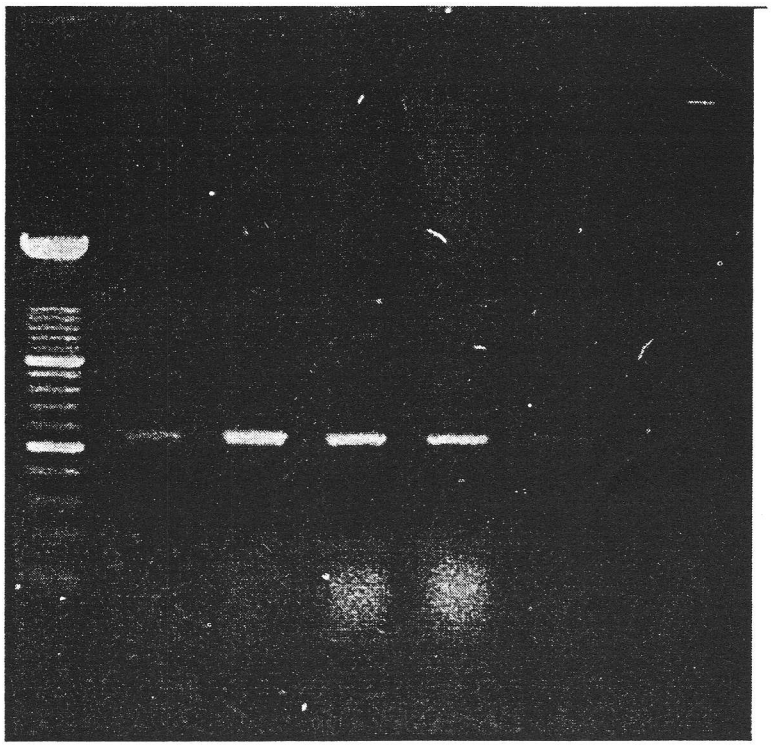

Figure 6 - Agarose gel electrophoretic analysis of PCR amplification products obtained with primers ASGV1F and ASGV1R from transcripts of total RNA preparations from leaves of *C. quinoa* infected with ASGV isolates 10391 (lines 1, 4), 10311 (line 2), 10371 (line 3), or from RNA extracted from purified virus preparation (isolate 10311; line 5). Negative control without added cDNA (line 6). M = molecular weight marker XIV from Boehringer (100 bp DNA ladder).

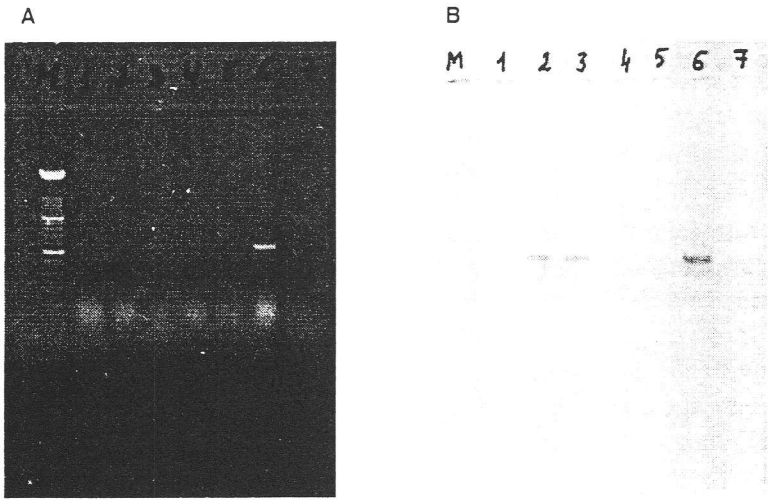

Figure 7 - Agarose gel electrophoretic (A) and Southern blot (B) analysis of PCR amplification products obtained with primers ASGV1F and ASGV1R from transcripts of total RNA preparations from leaves of *C. quinoa* infected with ASGV isolate "Gorsem 77" (line 3) or from leaves of apple trees 10291 (lines 1, 4) or 10392 (lines 2, 5); the 2 samples corresponded to two different total RNA preparations made at 15 days interval. Line 7 = negative control without added cDNA. M = molecular weight marker XIV from Boehringer (100 bp DNA ladder). DIG labelled probe used for southern blot was obtained from cloned PCR amplification product obtained for ASGV isolate 10311 with primers ACG1a-ACG2a; labelling and colorimetric detection were performed according to manufacturer's intructions (Boehringer).

As the use of degenerate primers targeting the RNA polymerase gene did not allow a reproducible and sensitive detection of latent viruses (ACLSV, ASGV, ASPV) in fruit tree material, experiments were conducted to amplify a large fragment corresponding to the 3' end of the RNA genome (from 3' end of the polymerase gene to the poly A tail), including the coat protein gene of different ASGV and ASPV isolates, for sequencing. Comparative analysis of these sequences will allow us to define specific primers to be used for the sensitive and specific RT-PCR detection of each of these viruses from woody plants (apple and pear). For that purpose, cDNA was synthesized from total RNA preparations from ASGV-infected *C. quinoa* and ASPV-infected *N. occidentalis* using oligo-dT + adapter and the Expand™ reverse transcriptase (Boehringer).

For ASPV, PCR was conducted with the cDNA thus obtained, with ACGP1 or ACGP3 degenerate primers and a primer which was complementary to the adapter sequence of the oligo-dT primer used for the cDNA synthesis, using Expand™ long template PCR system (Boehringer). Figure 8 shows the analysis of the products of Expand long template RT-PCR amplification from a total RNA preparation from leaves of *N. occidentalis* infected with ASPV-J. The discrete DNA band of approximatively 3600 nt has been purified from the gel and cloned in pCRII plasmid for sequencing. Similar products have been obtained with ASPV isolate 3536.

For ASGV, the use of degenerate primers ACGP1a or ACGP3, together with the reverse primer complementary to oligo-dT + adapter, did not allow the identification of a discrete band corresponding to the expected long template amplification product (~ 2600 nt). Although, the use of 2 specific forward primers designed from analysis of ACGP3-ACGP6 amplified products for different ASGV isolates allowed the obtention of long template PCR amplification products for 5 different ASGV isolates multiplied in *C. quinoa*, and also from the ASGV-infected tree 10291.

Sequencing of all these long template amplification products, cloned in pCRII is actually in progress.

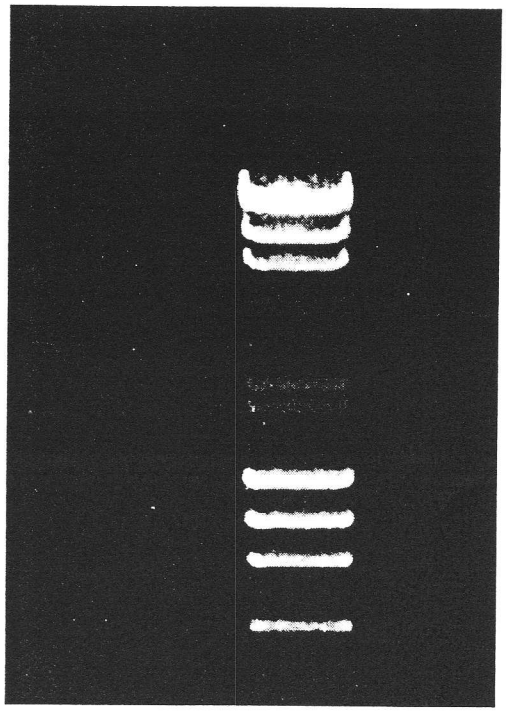

Figure 8 - Electrophoretic analysis of Expand long template PCR amplification product from a total RNA preparation from leaves of *N. occidentalis* infected with ASPV-J using primer ACGP1a and M2 (consisting in oligo-dT and an adapter sequence for cloning and sequencing of the PCR product). M = molecular weight marker (mixture of HindIII fragments of λDNA and HaeIII fragments of ΦX174, Life technologies).

CONCLUSIONS AND PERSPECTIVES

Different sets of degenerate primers have been designed allowing the specific RT-PCR amplification of fragments of the 3'-terminal part of the RNA polymerase gene of ACLSV, ASGV and/or ASPV from transcripts of total RNA preparations from herbaceous host plants.

Although the same primers did not allow the consistent and sensitive amplification of specific viral sequences from infected apple and pear trees, this approach seems of valuable use for the prime detection and identification of poorly characterized viruses. The further cloning and sequencing of the amplification products thus obtained allow the study of the variability of these viruses and the design of more specific primers for the development of sensitive RT-PCR detection protocols applicable to naturally infected (woody) host plants.

This study has also demonstrated that degenerate primers may be used for long template RT-PCR amplification allowing the sequencing of long fragments of the genome of different related isolates or viruses.

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