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DETECTION OF APPLE CHLOROTIC LEAF SPOT VIRUS AND APPLE STEM GROOVING VIRUS BY RT-PCR AND IC-RT-PCR.

J. Kummert, G. Rufflard, D. Colinet & P. Lepoivre
Centre National de Phytovirologie IRSIA
UER de Phytopathologie, FSAGx, Gembloux, Belgique.

SUMMARY

Apple chlorotic leafspot virus (ACLSV) and Apple stem grooving (ASGV) isolates, and complexes containing these viruses have been transferred and multiplied on herbaceous host plants in the greenhouse for the development of RT-PCR and IC-RT-PCR detection methods.

Comparative analysis of nucleotide and deduced amino acid sequences published for 2 isolates of ACLSV and one of ASGV showed the presence of several short stretches of homologous amino acids although both viruses belong to different phytovirus genera. Only amino acid sequence homologies located in the 3' terminal part of the 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*. The primers pairs retained have been also tested for immunocapture RT-PCR amplification of genomic RNA from virus particles contained in crude sap of the same plant species.

As the size of amplified products are quite similar for ACLSV and ASGV, the identification of the responsible virus would require the combination of PCR and hybridisation with specific probes from cloned cDNA.

The RT-PCR and IC-RT-PCR protocols thus developed will be adapted for direct use on woody material, and specially mother tree 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 inducing, sometimes severe symptoms (pseudo-pox; bark split).

The genetic organisation, and thus the classification of these three viruses was confused and ambiguous till recently.

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).

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

These are the reason why we aimed at developing RT-PCR and immunocapture-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 should be cloned for further use, as sequencing for taxonomic studies, and/or development of non radioactive probes to be used in dot blot hybridisation tests for virus detection.

The present communication is dealing with the development of RT-PCR and IC-RT-PCR for the detection of ACLSV and ASGV in herbaceous hosts by using polyvalent degenerate primers and analysing the amplification products by electrophoresis in ethidium bromide stained agarose gels.

MATERIALS AND METHODS

Transfer of the virus isolates on herbaceous hosts plants

Young leaves taken on trees in the orchard, 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 test plants used for virus multiplication are *Chenopodium quinoa* and *Nicotiana occidentalis*, grown in the greenhouse with supplemented light (16 h).

RNA extraction and cDNA synthesis

Two g (fresh weight) of symptomatic bearing leaves of mechanically inoculated plants of *C. quinoa* or *N. occidentalis* were grounded in liquid nitrogen. After homogenisation with Ultra-Turax in guanidine isothiocyanate and β-mercaptoethanol, total RNA was extracted and purified by the method of Chirgwin (1979). For material of apple shoots or trees, total RNA was extracted from 100 mg of young leaves using the Trizol reagent (Life Technologies).

Single-stranded cDNA was synthesised from 5 µg of purified total RNA using the Amersham first strand cDNA synthesis kit, or Superscript preamplification system for first strand cDNA synthesis (Life Technologies), 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.

PCR experiments

Amplification from the cDNA were carried out with 5 µl of cDNA preparation 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 $MgCl_2$ (1.5mM), each dATP, dCTP, dGTP, dTTP (200 mM), 0.05 or 0.1 nmol of each upstream and downstream primers and 2.5 or 5 units of Taq DNA polymerase (Amersham, or Boehringer). Thermal cycling was realised in a triothermoblock cycler (Biometra, Göttingen). 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 analysed by electrophoresis in 1% agarose gel stained with ethidium bromide (Sambrook *et al.*, 1989).

IC-RT-PCR

The immunocapture-PCR protocol used was derived from that of Candresse *et al.* (1995). In this protocol, the reverse transcription of the viral RNA and the PCR reaction are performed sequentially in the same 500 µl Eppendorf tube previously coated with specific anti-virus immunoglobuline.

Hundred µl of anti-ACLSV or anti-ASGV IgG (Loewe Phytodiagnostica, D-83624 Otterfing) in carbonate coating buffer pH 9.6 were added to each tube and these were incubated for 3 hours at 37°C. After 2 washings with 150 µl PBS containing 0.5% Tween 20, 100 µl of clarified plant extracts were added, and the tubes incubated overnight at 4°C. Plant extract consisted in the supernatant obtained after grinding leaf samples (1 g/10 ml) in PBS containing 0.5 % Tween 20, 2% polyvinylpyrolidone, 20 mM sodium diethyldithiocarbamate and 0.2% $MgCl_2$, and centrifugation for 10 min at 6000 rpm.

After 2 washings with PBS-Tween, RT-PCR reactions were conducted in each tube in 100 µl with the GeneAmp RNA PCR kit (Perkin Elmer) according to the manufacturer. Primer for reverse transcription was oligo dT_{16}. For short degenerate primers, cycling conditions were the same as described for RT-PCR; a pair of specific primers for ACLSV was also used, with 30 cycles at a annealing temperature of 50°C. Amplification products were analysed by electrophoresis in 1% agarose gels, stained with ethidium bromide.

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 1 present the origin and composition of the different isolates and the symptoms induced on the test plants used.

Table 1. Isolates maintained on herbaceous host plants in the greenhouse

	Origin	Symptoms observed on		Virus isolate
		C. quinoa	*N. occidentalis*	
p863	prune(1)	LLt; (Mo)	-	ACLSV
9L300	apple	LLc-n; Mo	-	ACLSV
8572	apple	LLc; Mod	Sc,r; Sc,r	ASGV+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	-	ASGV
91.297	apple	LLc; Mod	Sc,r; (Mo)	ACLSV + ASGV
PA66	pear(2)	-	Sc,r; Mo	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.

-;-: inoculated leaves; systemic symptoms.

(1) received from Candresse T. (INRA, Bordeaux, Fr.) and (2) received from Jelkmann W. (BBA, Dossenheim, Ge.) as dried leaf pieces of infected herbaceous host; all the other isolates have been transferred from tree shoots.

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. In particular, as demonstrated by ELISA tests conducted on leaves taken on the symptomatic herbaceous test plants, or even

directly on the shoots from which they were isolated, only ASGV has been successfully isolated from shoots received as infected by ASPV. This is the reason why later we asked for, and obtained, reference ASPV and PVYV (pear vein yellow virus) isolates which are not included in the work presented here.

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 (Roberstson *et al*; 1991) or geminiviruses (Ribicky and Hughes, 1990), and maybe also viruses from related genus in a family like Potyviridae or Potexviridae.

At the beginning of the work, ACLSV was considered to be a closterovirus; the first comparative analysis of nucleotide or amino acid sequences thus concerned ACLSV (plum isolate p863; German *et al.*, 1990), beet yellows virus (Agranovski *et al.*, 1991) and *Citrus* tristeza virus (Sekiya *et al.*, 1991). No significant homologies allowing design of correct primers were found.

On the contrary, several stretches of sequence homology were found in the putative RNA polymerase gene of ACLSV and ASGV (Yoshikawa *et al*;, 1992) allowing the design of degenerate primers for PCR experiments although the 2 viruses were considered to belong to different virus genus. Later, the comparison was extended to the sequences published for an apple isolate of ACLSV (Sato *et al.*, 1993) and for ASPV from pear (Jelkmann, 1994). Quite the same homologies were found with the previous published sequences of ACLSV p863 and ASGV (figure 1) and further primer pairs were defined.

Adapter sequences containing a restriction site were added to the short first selected primers (figure 2a) to allow a higher temperature for annealing, from cycle 6 in the polymerase chain reaction. They would also facilitate the further cloning of the obtained amplification products. As the further selected primers (figure 2b) were longer, they did not contain adapter sequences.

Table 2 presents the expected sizes of amplification products obtained for the different selected primers pairs.

Table 2. Expected size of the amplified products

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

Size expressed as the number of base pairs. For sequences of the primers and their localisation 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 or a mixture of both viruses. As the targeted viruses

possess a polyadenylated 3' end, reverse transcription was primed with oligo-dT, and the PCR reaction conducted with the primers pairs defined in Table 2.

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aclsv-a REFREFKGNN GWSNQFREEA GPNWKHPYRV NQAMSYEAIY PRHRMDDDLT
aclsv-p REYREFKGKN GWSNQFREEA GPNWKFPYKV NQAMSYEAVY PRHKMDDDLT
aspv    REDREFRIGD ITTEQFTDDH SKNRGQEL.T NAAERYEAIY PRHKGTDTAT
asgv    KEKREFQSVL GLSNQFLDME KNGCKIDI.. .LPFARQNVF PHHQASDDVT

aclsv-a FLAAIKKRLR FDNLANNYAK FKAAESRGKY LAKVFLRHVP IKCGRDQRLL
aclsv-p FLAAIKKRLR FDNVANNYAK FKAAESRGKY LTKIFLKHVP IKCGRDQRLL
aspv    FLMAVKKRLS FSSPAAEHAK LRRAKPFGKF LLDTFLKRVP LNSSHDEKMM
asgv    FWAGVQKRIR KSNWRREKSK FEEFESQGKE LLQEFISMLP FEFKVNIKEI

aclsv-a DQCRQEFEET KLSKSAATIG AHSQRSDSDW PLDKIFLFMK SQLCTKFEKR
aclsv-p DQCRQEFEET KLSKSAATIG AHSQRSDSDW PLDKIFLFMK SQLCTKFEKR
aspv    QEAVHAFEEK KLSKSMATIE NHSGRSCEDW PVDKALIFMK SQLCTKFDNR
asgv    EDGEKSFLEK RKLKSEKMWA NHSERSDIDW KLDHAFLFMK SQYCTKEGKM

aclsv-a FTEAKAGQTL ACFPHKILVE FSPWCRYTEK VLTANLPDNY YIHQRKNFSE
aclsv-p FTEAKAGQTL ACFPHKILVE FSPWCRYTEK VLTANLPDNY YIHQRKNFSE
aspv    FRSAKAGQTL ACFQHSVLCR FAPYMRYIES KVTEVLPKNL YIHSGKNIDD
asgv    FTEAKAGQTL ACFQHIVLFR FGPMLRAIES AFLRSCGDSY YIHSGKNFFC

aclsv-a LEDFAKR..F SNGSMCVESD YTAFDVSQDH TILAFEVELL KHFGWDDKVL
aclsv-p LEDFARR..F SNGSICVESD YTAFDVSQDH TILAFEVELL RHFGWDDRVL
aspv    LAAWVTT..S KFNGVCTESD YEAFDASQDH FILAFELEVM KFLGLPSDLI
asgv    LDSFVTKNAS VFDGFSIESD YTAFDSSQDH VILAFEMALL QYLGVSKEFQ

aclsv-a QSYIKMKCTL GCRLGGFAIM RFTGEFSTFL FNTLANMVFT FCRYEV.PDG
aclsv-p QSYIKMKCTL GCRLGGFAIM RFTGEFSTFL FNTLANMVFT FCRYEV.PDG
aspv    ADYTFIKTHL GSKLGSFAIM RFTGEASTFL FNTMANMLFT FLRYDL.NGR
asgv    LDYLRLKLTL GCRLGSLAIM RFTGEFCTFL FNTFANMLFT QLKSKIDPRR

aclsv-a TPICFAGDDM CALRNL..RE IDTHECILSK LSLKAKVNRT KVPMFCGWRL
aclsv-p TPICFAGDDM CALRNL..RE IDTHEFILSK LSLKAKVNRT KVPMFCGWRL
aspv    EAICFAGDDM CANSRL..KV TNRFSNFLDK IKLKAKVQFT ATPTFCGWGL
asgv    HRILFAGDDM CSLSSLKRRR GERATRLMKS FSLTAVEEVR KFPMFCGWYL

aclsv-a CFDGLIKEPC LIYERLQVAI ENGRLMDVID SYFLEFSFAY KLGERLYSHL
aclsv-p CCDGLIKEPC LIYERLQVAI ENGRLMDVID SYFLEFSFAY KLGERLYSHL
aspv    CEHGVFKKPD LVLERLQIAR ETRNLENCID NYAIEVSCAY KMGENLNLYL
asgv    SPYGIIKSPK LLWARIKMMS ERQLLKECVD NYLFEAIFAY RLGERLYTIL

aclsv-a EIEQLNYHQV LTRFFIRNKH LLRGDSRHNI SELE...... WLSDEDSDDD
aclsv-p EIEQLNYHQV LTRFFIRNKH LLRGDSRHNI SELE...... WLSDEDGDND
aspv    TPQEVDAHYN CVRFIVQHNH LLKSNIRDLF KG.E...... SLPASS....
asgv    KEEDFEYHYL VIRFFVRNSK LLTGLSKSLI FEIGEGIGSK WLSSTSTASS

aclsv-a KGSQIEDRRR GYSNCWGEKL QNLF...... .......... ..........
aclsv-p KGSQIEDRRR GYSNCWGEKL QNLF...... .......... ..........
aspv    .......... .......... .......... .......... ..........
asgv    RRSNLQTSKL MLSRPQSFTR MQPFSNQTCL IASKGLNQTS RFPLDLVTAS
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Figure 1. Sequence homologies at the amino acid level of the 3' end of the putative RNA polymerase gene of ACLSV (apple and plum isolates), ASGV and ASPV; arrows indicate selected primers used in this work.

a) ACG1a

F L F M K S

5' 3'

gtcgaaTTC CTN TTY ATG AAR TC

ACG2a

M F C G W

3' 5'

TAC AAA ACA CCN ACCtaggatc

ACGP1a

I(L) F M K S Q

5' 3'

atgctgcagMTN TTC ATG AAR TCV CAA

ACGP2a

F A G D D M C

3' 5'

AAR CGN CCH CTR CTR TAC ACGagctcatc

b) ACP1

Y E A V(I) Y P R H

5' 3'

TAT GAR GCT ATA TAY CCM AGR CA

ACGP2

F A G D D M C A

3' 5'

AAR CGN CCN CTR CTR TAC ACR CG

ACGP3

A K A G Q T L A C

5' 3'

GCY AAR GCN GGH CAR ACT YTN GCY TG

ACGP6

A I M R F T G E

3' 5'

CG NTA DTA CKC NAA RTG NCC NCT

Figure 2: 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 letter represent nucleotides not derived from the viral sequences (adapter sequences). R = A or G; 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, G, C or T.

Figure 3 presents the analysis of amplification products from transcripts of total RNA preparations from leaves of *C. quinoa* or *N. occidentalis* inoculated with isolates of ACLSV, ASGV, or a complex containing both viruses, and of two apple trees from the orchard. For established isolates multiplied in greenhouse by multiple passage in *C. quinoa*, the two selected pairs of degenerate primers allow the amplification of one specific DNA fragment with a length corresponding to that expected from the position of the primers on the sequences published for ACLSV and ASGV isolates (respectively about 630 bp for primers ACG1a-ACG2a, and about 520 bp for primers ACGP1a-ACGP2a).

For two isolates recently transferred and multiplied on *N. occidentalis*, and containing ASGV as shown by ELISA tests, an amplification product of expected size has been obtained from one of both, only with primers ACG1a-ACG2a. No amplification product has been observed for transcripts of total RNA preparations from apple leaves collected in July. However, amplification products with primers ACGP1a-ACGP2a have been observed in previous experiments for transcripts of leaves from different virus-infected apple trees taken in May and June (results not shown).

Indeed, due to the relatively low temperature (37 to 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. Furthermore, the fragment of the expected size is not always consistently found for successive assays with material from the same origin. This prompted us to develop further primers.

Figure 4 shows the results of agarose gel electrophoretic analysis of amplification products obtained from transcripts of total RNA preparations of leaves of *C. quinoa* inoculated with an ACLSV isolate (91-300), an ASGV isolate (10311) and a complex containing both viruses (91-297). 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, as already mentioned.

Figure 5 shows the results of agarose gel electrophoretic analysis of amplified products from transcripts of total RNA preparations of leaves of *C. quinoa* 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 4, the primer pair ACGP1a-ACGP2a allow the amplification of one virus specific DNA fragment from transcripts of total RNA from plants infected by ASGV.

Note also the presence of non specific amplification products for the two shorter sets of degenerate primers (ACG1a-1CG2a, ACGP1a-ACGP2a) which indicate 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 cDNA to plant nucleic acid when using primers designed for the amplification of viral nucleic acid sequences has been reported by several authors and for example, 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.

IC-RT-PCR

The whole process of immunocapture RT-PCR has been carried out in Eppendorf 0.5 ml/tubes. Virus particles in crude extracts of infected *C. quinoa* were trapped by ACLSV or ASGV specific antibodies (Loewe Phytodiagnostica), and RT-PCR conducted with different sets of primers as described for total RNA preparations.

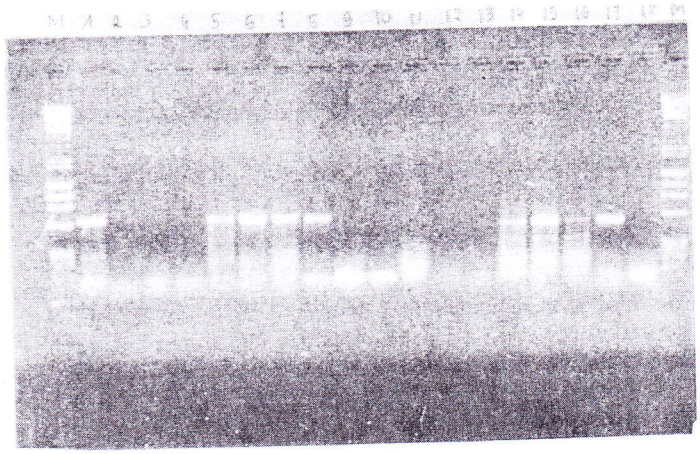

Figure 3. Agarose gel electrophoretic analysis of PCR amplification products from transcripts of total RNA preparations from leaves of *N. occidentalis* infected with isolates VDM 879 (1,10) or 8572 (2, 11), virus infected apple trees from the field (3-4, 12-13) or *C. quinoa* infected with isolates 91-300 (5, 14), 91-297 (6, 15), 10311 (7, 16) or p863 (8, 17) in the presence of primers ACG1a-ACG2a (1-9) and ACGP1a-ACGP2a (10, 18). For the composition of the different virus isolates: see table 1. Lines 9 and 18: PCR reaction medium without addition of any cDNA. Molecular weight marker (M) = DNA of phages λ and ΦX_{174} digested respectively by Hind III and Hae III.

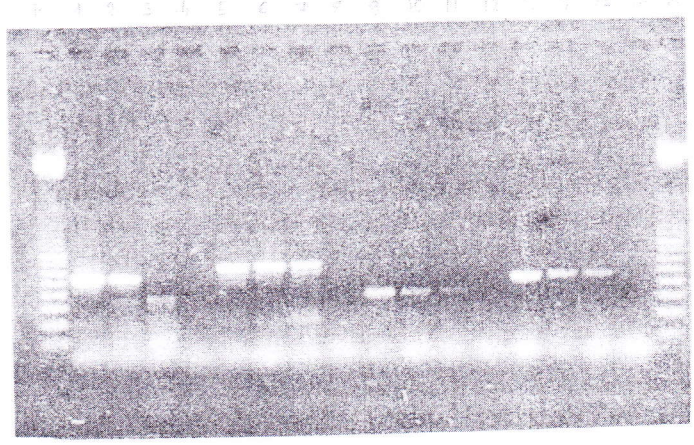

Figure 4. Agarose gel electrophoretic analysis of PCR amplification products from transcripts of total RNA preparations from leaves of *C. quinoa* infected with isolates 91-300 (ACLSV; lines 1, 5, 9, 13), 91-297 (ACLSV + ASGV; lines 2, 6, 10, 14), 10311 (ASGV; lines 3, 7, 11, 15). Lines 4, 8, 12, 16 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).

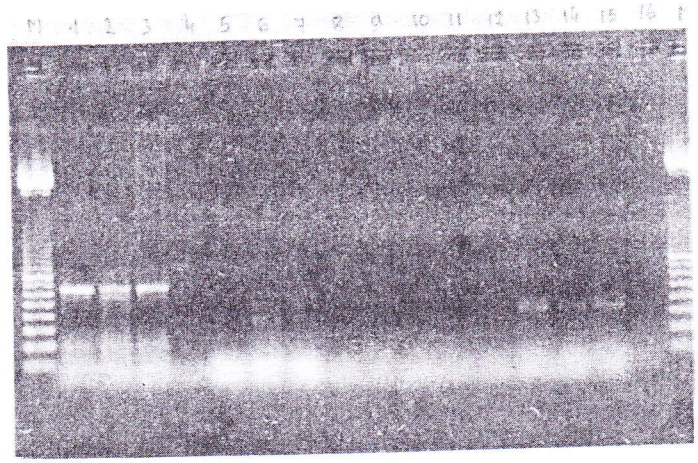

Figure 5. Agarose gel electrophoretic analysis of PCR amplification products from transcripts of total RNA preparations from leaves of *C. quinoa* inoculated with ASGV isolates 10311 (lines 1, 5, 9, 13), 10391 (lines 2, 6, 10, 14), or 10771 (lines 3, 7, 11, 15). Negative control without added cDNA (lines 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).

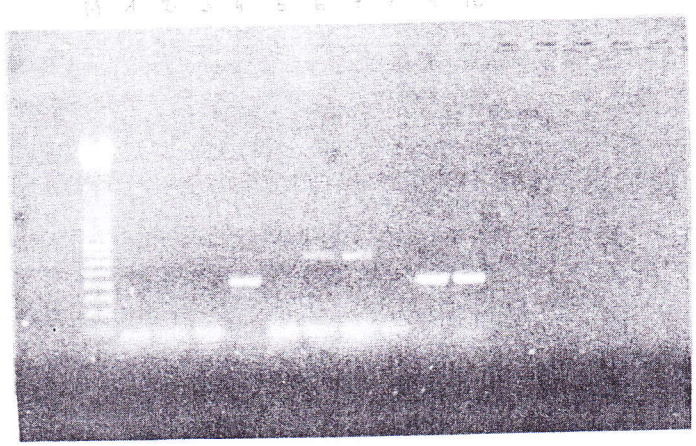

Figure 6. Agarose gel electrophoretic analysis of PCR amplification products from virus particles contained in crude sap of *C. quinoa* inoculated with ACLSV isolate 91300 (lines 2, 4, 7, 9), with ASGV isolate 10311 (lines 3, 5, 8) or with a complexe (91297) containing both viruses (lines 1, 6, 10) and trapped with polyclonal antibodies raised against ASGV (lines 1-5) or ACLSV (lines 6-10). Transcription of viral RNA was primed with oligo-dT and primers used for PCR were CLS2-CLS3 (lines 4, 5, 9, 10) or ACG1a-ACG2a (lines 1-3, 6-8). M = molecular weight marker (100 bp ladder, Life Technologies).

Figure 6 shows the results obtained for crude sap of *C. quinoa* infected with ACLSV (isolate 91-300), ASGV (isolate 10311) and the complex of both viruses (91-297) in the presence of degenerate primers ACG1a-ACG2a and 2 specific primers for ACLSV (complementary primer CLS2: TCGCGAACATAGGGATCCA and reverse primer CLS3: TTGCGAATTCAGTGTGT AAA, corresponding respectively to nucleotides 6908-6926 and 7311-7291 of ACLSV p863; German et al., 1990) which should amplify fragments of 624 nt (ACLSV) or 639 nt (ASGV) for the first set of primers and a fragment of 404 nt (ACLSV) for the second. Extracts from plants infected with the ACLSV isolate (lines 7 and 9) and the complex containing this virus (lines 6 and 10) give rise to an amplification product of correct size for tubes coated with ACLSV antiserum while no amplification products are observed for extracts of plants infected with ASGV isolate (line 8). No amplification product is observed for tubes coated with ASGV antiserum in the presence of crude sap of plants infected with ASGV, or a complex containing this virus (the product observed in line 4 of figure 6 is due to incorrect sample deposit on the gel).

Further experiments conducted with these same primers, as with primers ACGP3-ACGP2 and ACGP3-ACGP6 gave the same results i.e. specific amplification for ACLSV in ACLSV coated tubes and absence of amplification for ASGV in ASGV coated tubes, although serological ELISA tests with the same antiserum showed positive for the plant extract submitted to IC-RT-PCR.

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 gene of the RNA polymerase of either ACLSV and ASGV, from transcripts of total RNA preparations from herbaceous host plants.

When used in immunocapture RT-PCR, the same primers allowed the specific amplification from crude extracts of herbaceous hosts infected by ACLSV, for tubes coated with a polyclonal antibody raised against this virus. No amplification has been obtained so far from crude extracts of ASGV-infected plants for anti-ASGV coated tubes, although the virus can be detected in these extracts by the same antibody in ELISA test.

The RT-PCR and IC-RT-PCR protocols will be optimised for use with transcripts of total RNA preparations from woody plants from the orchards.

On the other hand, specific amplified fragments have been, or will be, cloned for sequencing in order to control the origin of the amplified product, to study the variability of the viruses concerned, and to define new primers selected either for their specificity or their polyvalence.

Probes can also be developed from cloned specific RT-PCR or IC-RT-PCR amplified products for further use in dot blot hybridisation tests for virus detection or in southern blot for more precise control and identification of amplified products from field samples after electrophoresis analysis.

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