DEVELOPMENT OF COLORIMETRIC RT-PCR TESTS FOR APPLE STEM GROOVING VIRUS DETECTION IN APPLE TREES

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Abstract

RT-PCR technology is becoming available for diagnosis of pome fruit virus detection in their natural hosts, the amplified products being usually visualized after electrophoresis in ethidium bromide stained gels. Although highly sensitive, this technology is not amenable to routine testing of large number of samples, i.e. for certification purposes. The aim of our work was to develop a colorimetric RT-PCR (or RT-PCR ELISA) test that combines the PCR sensitivity and the convenience of ELISA. The RT-PCR amplified products were either directly labeled with digoxigenin (DIG) or, for better specificity, unlabeled amplification products were hybridized to an internal DIG-labeled detection probe. In both systems, a biotin-labeled probe was used for trapping the amplification products on streptavidin-coated microplate strips. Revelation was performed with an alkaline phosphatase labeled anti-DIG conjugate (DIG Detection Kit, Boehringer Mannheim). The results showed high sensitivity for the detection of ASGV in apple tissues. The importance of specific primers allowing high cycling temperatures, in relation with the origin and purification status of the plant extracts used for RT-PCR, and the roles of capture and detection probes on the specificity of the colorimetric RT-PCR are discussed.

1. Introduction

Apple stem grooving capillovirus (ASGV) occurs in apple trees probably worldwide, usually without causing obvious symptoms (Lister, 1970). The primary control strategy for ASGV relies on the use of virus-tested planting material and thus on the ability to detect and eliminate infected plants from basic stock material.

The most currently used and recognized technique for detection of ASGV is a bioassay based on grafting on woody indicators. This procedure is expensive and lengthy, although Howell *et al.* (1996) improved it recently by obtaining reliable response 2 months after grafting on new *Malus* indicators. Serological diagnosis (ELISA) is much more rapid, but appears reliable only during a short period in the year because it lacks sensitivity for detecting low virus concentrations in woody material. RT-PCR, which seems potentially more accurate and sensitive than serological detection, has been recently developed for this virus (Kinard *et al.*, 1996; Kummert *et al.*, 1997; McKenzie *et al.*, 1997; Magome *et al.*, 1997).

The standard RT-PCR protocols used purified total RNA or dsRNA preparations as template, which require complex and rather time consuming extractions, not easily amenable for processing of large numbers of samples. Recent improvements in sample processing for PCR, including immunocapture (Wetzel *et al*, 1992), direct binding (Rohwani *et al.*, 1995), print capture (Olmos *et al.*, 1996) and direct application of clarified plant extracts in reaction tubes (Wyatt and Brown, 1996) have been recently reported for sensitive detection of plant viruses. Specific primers developed in our laboratory allow the use of crude sap from apple trees for ASGV detection in RT-PCR tests (Kummert *et al.*, this symposium).

Another drawback hindering the development of RT-PCR for routine testing of virus infection in plants results from the use of ethidium bromide stained agarose gel electrophoresis for the detection of amplification products. Although useful for checking the size of PCR products, this technique is not very sensitive and also not amenable for

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automation; labeling PCR products either directly or by using hybridization with specific labeled probes is substantially more sensitive (Fenby *et al.*, 1995).

Novel techniques combining PCR accuracy and potential sensitivity, with ELISA convenience, have been described in medicine (Chang *et al.*, 1994; Lassner, 1995) and later in plant pathology (Rowhani *et al.*, 1996).

This paper describes the feasibility of colorimetric RT-PCR tests (RT-PCR ELISA) for ASGV diagnosis in field collected apple tree samples.

2. Materials and methods

2.1. Virus-infected material

Reference isolates for ASGV were maintained in the greenhouse on *Chenopodium quinoa* and *Nicotiana glutinosa*. Four isolates (10311, 10391, 10771 and G77) were originally obtained from the Research Station on Fruit Trees of Gorsem, Belgium. Isolate PV199 has been obtained from DSM, Braunschweig, Germany.

For the control of the specificity of the colorimetric detection of RT-PCR amplification products, apple chlorotic leaf spot (ACLSV) and apple stem pitting virus (ASPV) isolates, infecting apples trees or herbaceous hosts have been used.

2.2. Preparations used as template for colorimetric RT-PCR

Total RNA preparations were obtained from 0.2 g of plant tissue ground in liquid nitrogen and then homogenized with Ultra-Turrax in the presence of 2 ml of Tripure isolation reagent (Boehringer), and the extraction was conducted according to the manufacturer's instructions. The final pellets were resuspended in 100 µl of DEPC-treated water; the amount and purity of nucleic acids obtained was estimated by UV absorbance at 260 and 280 nm, and the samples stored at -20°C until use. One to 2 µg of total RNA were used for RT-PCR.

ds-RNA preparations from infected plant material were obtained by using the method of Valverde *et al.* (1990) applied to approximately 20 g of plant tissue, or adapted for use with 0.5 to 1 g of plant tissue. The final pellets were resuspended in 40 µl (20 g extracts) or 10 µl (1 g extracts or less) of DEPC-treated water and 5 µl used for RT-PCR.

Preparation of crude plant extracts was adapted from the method described by Wyatt and Brown (1996). Fifty mg of plant tissues (leaves or bark scrapings from current year apple tree shoots) were ground in 1.5 ml microtubes containing 0.5 ml of TE buffer (50 mM Tris, pH 8.0, 10 mM EDTA). After grinding, 0.5 ml of the same buffer was added and the content homogenized with a vortex. After centrifugation for 10 min at 10,000 g, one µl of th solution, or dilutions of it in TE buffer, was directly added to RT-PCR mix. Alternatively, 50 µl of the same extracts were incubated for 30 min in melting ice in PCR tubes, and after rinsing 3 times with TE buffer (200 µl), the RT-PCR mix was added.

2.3. Primers and probes

Two primer pairs designed to cope with the variability of partial sequences determined for 4 ASGV isolates maintained in *C. quinoa* or *N. glutinosa* in the greenhouse (10311, 10392 and 10771) and one from an apple tree in the field (10392), and the sequence published by Yoshikawa *et al.* (1992) were used:

ASGV1F = 5'-GARGCWAAAGCTGGYCAA-3' (nt 3925-3942)
ASGV1R = 5'-YACCTCTTCYACWGCAGT-3' (nt 4485-4468)
ASGV4F = 5'-GTTCACTGAGGCAAAAGCTGGTC-3' (nt 3918-3940)
ASGV4R = 5'-CTTCCGTACCTCTTCCACAGCAG-3' (nt 4491-4469)

Primer pairs ASGV1F-ASGV1R and ASGV4F-ASGV4R amplify respectively fragments of 560 and 574 bp located at the 3' end of the putative RNA polymerase gene, just before the variable region described by Magome *et al.* (1997).

The 5' biotin- and 3' digoxigenin-labeled capture and detection probes correspond to internal fragment parts of the amplified products, and will be part of a detection kit in development.

2.4. RT-PCR amplification

Reverse transcription was made either by using Expand™ reverse transcriptase system (60 min at 42°C, Boehringer) with oligo-dT priming, or AMV RT-enzyme (30 min at 50°C) contained in the Titan™ one step RT-PCR system (Boehringer) with the reverse specific primer, according to manufacturer's instructions.

Amplification from cDNA was performed in 50 µl in a Triothermoblock cycler (Biometra) as follows: template denaturation for 5 min at 94°C; 30 cycles at 94°C for 30 sec (denaturation), 52°C (primers ASGV1F-1R) or 62°C (primers ASGV4F-4R) for 1 min (annealing), and 72°C for 2 min (elongation); and final elongation at 72°C for 15 min.

For digoxigenin-labeled amplicons, the dNTP mix contained a 19:1 dTTP: DIG-dUTP ratio (PCR DIG labeling mix, Boehringer)

2.5. Detection of PCR products

2.5.1. Electrophoretic analysis

PCR amplification products (10 µl) were analyzed by electrophoresis (1 h in TAE buffer at 120V) in 1% agarose gel, stained with 0.01% ethidium bromide and photographed under UV light (306 nm) with Polaroid films.

2.5.2. Colorimetric detection

PCR amplification products were also analyzed by using the PCR ELISA DIG detection kit (Boehringer) with minor modifications. Ten µl of PCR products, and adequate controls, were mixed with 40 µl of denaturation solution and incubated for 10 min at room temperature.

If PCR amplification products are labeled with digoxigenin, 450 µl of hybridization solution containing 100 ng of biotin-labeled capture probe were added, and the mix was then put in duplicate (200 µl/well) in microtiter plate strips coated with streptavidin, before the incubation for 3 h at 52°C.

For unlabeled PCR amplification products: 450 µl of hybridization solution containing 100 ng/ml of the digoxigenin-labeled probe were added, and tubes incubated for 3 hours at 52°C, under shaking. While hybridization of the amplification products with the DIG-labeled probe was running, the biotin-labeled probe at 100 ng/ml in PBS/Tween was added (200 µl/well) in microtiter plate strips precoated with streptavidin and incubated for 1 h at 37°C. The microtiter plate strips were then washed 4 X with 250 µl of PBS-Tween and 200 µl of the hybridization product obtained between PCR amplicons and DIG-labeled probe added, in duplicate, before further incubation for 1.5 h at 37°C, under shaking.

In both cases, microtiter plate strips were finally washed again with PBS-Tween, and 200 µl of conjugate buffer (PBS-Tween plus 0.5% blocking reagent) containing a 1/3000 dilution of anti-digoxigenin alkaline phosphatase conjugated antibody (Fab fragment) were added to each well before incubation at 37°C for 30 min, under shaking. After washing, 200 µl of substrate buffer (10 mM diethanolalanine-HCl, pH 9.5) containing 0.7 mg/ml p-nitrophenyl-phosphate were added. Absorbance at 405 nm were measured in a Multiscan plus colorimeter, after 1 h at room temperature, and after 16 h at 4°C. All reagents and the precoated microtiter plates strips were purchased from Boehringer.

3. Results and discussion

3.1. Colorimetric RT-PCR with DIG-labeled amplification products

The assay is based on amplification of specific sequences by PCR and detection of the amplicons by ELISA, after capture by a specific biotin-labeled probe on a streptavidin-coated microplate.

Preliminary experiments had shown that DIG-labeled amplicons obtained with 5' end biotin-labeled primer ASGV1F and primer ASGV1R, from transcripts of total RNA preparations (herbaceous hosts) or dsRNA preparations (apple trees) from ASGV-infected plants, can be easily detected on streptavidin-coated microplate strips by using PO-conjugated anti-digoxigenin antibody. However, in this case, unspecific amplicons obtained from transcripts of total RNA preparations from ACLSV-infected *C. quinoa* (but not from ASPV-infected *N. occidentalis*, or from healthy corresponding herbaceous plants) also gave a signal, indicating the need of more specific detection procedures, based on the use of a specific biotin-labeled capture probe. When the RT-PCR experiments were conducted with unlabeled primers ASGV1F-ASGV1R, the only amplicons observed on the gels corresponded to ASGV infected material (herbaceous hosts or apple trees). Colorimetric detection of these RT-PCR products, after hybridization with a biotin-labeled probe captured on streptavidin coated plate strips was also specific for ASGV-infected material.

In the example showed in Figure1, the only fluorescence signal visible after agarose electrophoresis (Fig. 1a) corresponded to amplification products generated by total RNA preparation of ASGV infected *C. quinoa*. By colorimetric detection after capture of amplification products by a biotin-labeled oligonucleotide, homologous to primer ASGV 1F, positive signals are also observed from transcripts of ds-RNA preparations from ASGV-infected *C. quinoa* and apple tree, but not from comparable extracts from ACLSV- or ASPV-infected material (Fig. 1b), indicating the higher sensitivity of this detection method.

3.2. Colorimetric RT-PCR with capture and detection probes

The above method, although accurate and sensitive, presented limitations based on the need of complex template preparations (purified nucleic acids) and the use of costly PCR nucleotide labeling mix; all the amplicons being labeled, it also presented a potential risk of detection of false positive results. To improve the efficiency and reduce the costs, longer primers (ASGV4F-ASGV4R; 23 nt) and biotin- and digoxigenin-labeled probes (respectively 30 and 29 nt) were selected to cope the variability of partial sequences established from amplification products of the genomic RNA corresponding to a fragment of the 3' end of the gene coding for the viral replicase obtained for local isolates (Kummert *al.*, this symposium) and the sequence published for ASGV by Yoshikawa *et al.* (1992). For these probes particularly, attention has been given to avoid possible formation of secondary structures.

Different protocols have been used to perform the colorimetric detection of these RT-PCR amplification products obtained with specific primers ASGV4F-ASGV4R. The more sensitive and the only protocol allowing detection from plant material with low virus concentrations included 5 successive steps as follows: (i) denaturation of RT-PCR amplified products in microtubes, (ii) hybridization of the DIG-labeled probe to its complementary single-stranded sequence, (iii) immobilization of the biotin-labeled probe to streptavidin coated microtiter plate strips, (iv) hybridization of the preformed hybrid RT-PCR amplicon/ DIG-labeled probe with the immobilized biotin-labeled probe, and (v) colorimetric detection with anti-digoxigenin antibody conjugate and enzymatic substrate.

Although the detection probe only contains one molecule of digoxigenin, OD signals above 1 to 2 absorbance units were obtained. No false positive results were observed for healthy samples or from plants infected by ACLSV or ASPV.

As demonstrated elsewhere (Kummert *et al.*, this symposium), the primers ASGV4F-4R allowed the detection of specific viral sequences, by agarose gel electrophoretic analysis of

amplification products obtained with the Titan™ one tube RT-PCR system, directly from diluted plant sap of either ASGV-infected herbaceous hosts or apple trees. When the products of such tests were submitted to the 5 steps colorimetric detection protocols, positive signals have been obtained from ASGV-infected materials, sometimes even in the absence of visible bands on electrophoretic analysis of these same PCR products.

4. Conclusion

Although useful for detection and typing of human papillomavirus (Adams *et al.*, 1996), the simplest protocol for RT-PCR ELISA, using DIG labeling of the amplification products, and the forward primer labeled with biotin, could not be used for detection of ASGV infection, due to its lack of specificity (positive signals corresponding to unspecific amplicons obtained for ACLSV-infected material).

By using unlabeled primers for PCR amplification with digoxigenin-labeled nucleotide mix, and a biotin-labeled probe for capture of amplicons, the test became accurate and sensitive.

To reach the optimal specificity, needed to avoid false positive results in testing certified multiplication material, an improved colorimetric detection method using two specific labeled probes (respectively with biotin and digoxigenin) has been developed. Coupled with primers ASGV4F and ASGV4R, it allows the detection of ASGV infection from crude diluted sap from either leaf or bark tissue from apple trees.

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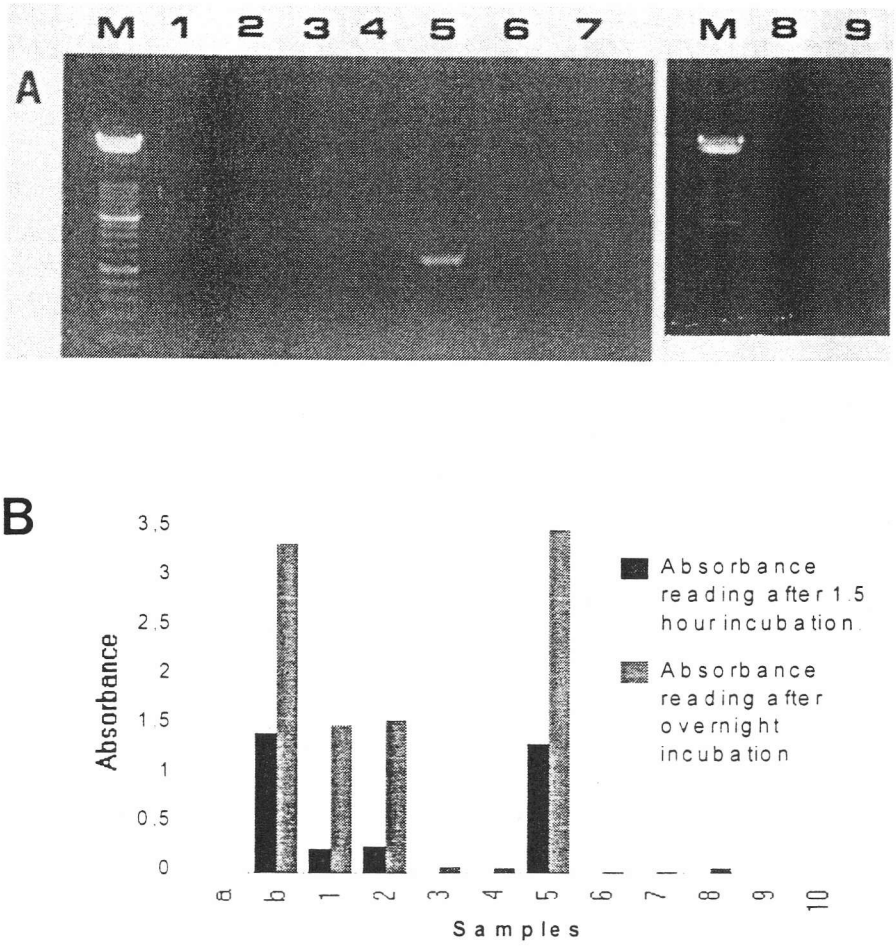

Figure 1 - Analysis of digoxignin-labeled two-steps RT-PCR products obtained with primers ASGV1F-ASGV1R from: ds-RNA preparations of ASGV-infected *C. quinoa* isolate 10311; (lane 1) or from apple trees 10392 (lane 2), LP680 (lane 3) or LP679 (lane 4) containing respectively ASGV, ASPV or ACLSV; total RNA preparations of ASGV-infected *C. quinoa* isolate G 77; (lane 5), ASPV-infected *N. occidentalis* (isolate ASPV-J; (lane 6), ACLSV-infected *C. quinoa* (isolate 91300, Lane 7), or healthy *C. quinoa* (lane 8). Lane 9 = negative control (no cDNA added). A. Analysis by electrophoresis in ethidium bromide agarose gel; Lane M = molecular weight marker (100 bp, Boehringer; lower heavy band = 500 bp). B. Absorbance values after capture of the digoxigenin-labeled RT-PCR products by biotin-labeled oligonucleotide homologous to primer ASGV1F, and revelation wit the PCR-ELISA detection kit from Boehringer (samples a,b = negative and positive controls from the kit; sample 10 = no PCR product added). Mean values for 2 repetitions.