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Detection of grapevine closterovirus A in infected grapevine tissue by reverse transcription-polymerase chain reaction

by

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Summary: Reverse transcription-polymerase chain reaction (RT-PCR) was successfully applied to detection of GVA RNA in nucleic acid extracts of infected grapevines. In particular, an artificially synthesized DNA primer set designed to amplify a GVA cDNA fragment of 430 base pairs, specifically detected GVA RNA sequences in extracts from infected grapevine tissues such as leaves from *in vitro*-grown explants, leaves from greenhouse-grown rooted cuttings, and bark scrapings of mature canes from field-grown vines. The detection limit of GVA RNA by RT-PCR was estimated to be 200 fold higher than that obtained by molecular hybridization or ELISA.

Key words: Grapevine closterovirus A, cDNA, reverse transcription polymerase chain reaction, molecular diagnosis.

Introduction

Grapevine closterovirus A (GVA) is one of the several closteroviruses associated with economically important diseases of *Vitis*, such as rugose wood and/or leafroll, in Italy (CONTI *et al.* 1980) and other leading viticultural countries. Direct detection of GVA in infected grapevines poses considerable problems because the virus is irregularly distributed and found at low titer in infected tissues. Recently, MINAFRA *et al.* (1991, 1992) have attempted to detect GVA in nucleic acids extracts of infected grapevine tissue by molecular hybridization with a cloned GVA cDNA probe. Virus detection was difficult and unreliable from woody tissue. In contrast, it was successful in nucleic acid extracts of infected herbaceous hosts.

The nucleotide sequence of cloned GVA cDNA has recently been determined (MINAFRA, unpublished). The availability of the nucleotide sequences of viroids and viruses affecting woody hosts such as viroid of citrus, pome and stone fruit trees (HADIDI and YANG 1990; LEVY *et al.* 1991; HADIDI *et al.* 1992; YANG *et al.* 1992) as well as plum pox virus (KORSCHINECK *et al.* 1991; LEVY and HADIDI 1991; WETZEL *et al.* 1991) has made possible the development of reverse transcription-polymerase chain reaction (RT-PCR) assays for improving the detection of these pathogens from their respective hosts. Based on these findings, we have tested RT-PCR to amplify a portion of GVA RNA from grapevine extracts. This paper describes the detection and identification of GVA RNA from nucleic acids extracts of infected grapevine tissue.

Materials and methods

Plant materials and nucleic acids extraction

Grapevine (*Vitis vinifera*) cv. Perricone infected with two different GVA isolates, namely PA-2 and PA-3, was used in this study. The following tissue samples were utilized: (i) leaves of rooted explants grown *in vitro* at 25 °C; (ii) mature leaves of green-

house-grown vines; (iii) bark scrapings from cuttings of field-grown vines. Uninfected samples from *Vitis rupestris* St. George were used as controls. Uninfected and GVA-infected *Nicotiana benthamiana* plants were maintained in a greenhouse at 25 °C. Leaf tissue from infected plants were collected 15 d after inoculation.

Total nucleic acids were isolated from GVA-infected and uninfected tissue (1 g samples) by the phenol-chloroform method, and purified through RNase-free Elutip-R minicolumns (Schleicher and Schuell Inc., Keene, NH) as described by YANG *et al.* (1992). Occasionally, total nucleic acids were dissolved in STE buffer (100 mM Tris-HCl, pH 9.0, 100 mM NaCl, 10 mM EDTA) and then purified on DEAE cellulose, as described by HADIDI and YANG (1990).

Primer selection

Three sets of DNA primers (Table) were designed by computer analysis (Primer Selection Program, Henry M. Jackson Foundation, Bethesda, MD) of nucleotide sequence of the 1529 bp long GVA cDNA clone pGA 240a (MINAFRA *et al.* 1992). The DNA primer sets were designed to produce by RT-PCR amplification, cDNAs with the following properties: (i) 400—600 bp in length; (ii) 49—51 % G+C content; (iii) melting temperature between 83 and 84.5 °C. In addition, the designed primers assured absence of homology in the last bases at the 3' or 5' end of each primer set pair (LOWE *et al.* 1990). Primers were synthesized by Synthecell Inc., Rockville, MD.

Table

GVA DNA primers

Set	Primer	No. bases	Sequence	Size of amplified DNA (bp)
1	c1137	22	5'TGACTGAGCGTTGAATACTCCC 3'	524
	635	22	5'AAGAGTTGGTGTTGGCGAAAGC 3'	
2	c1197	20	5'TACCCGTGAGAAATGATGGG 3'	451
	766	20	5'GGGGAGGTAGATATAGTAGG 3'	
3	c995	22	5'AAGCCTGACCTAGTCATCTTGG 3'	430
	587	18	5'GACAAATGGCACACTACG 3'	

cDNA synthesis and amplification

Viral cDNA was synthesized and amplified from 1 µg or less of purified GVA RNA and from 1 µg or less of minicolumn-purified or non-purified nucleic acid extracts of GVA-infected tissue according to HADIDI and YANG (1990). Primer set No. 3, which amplified a GVA cDNA fragment of 430 bp was used in most of the experiments.

Sensitivity of GVA detection by RT-PCR amplification was assessed with ten fold dilutions (from 1 µg to 1 fg) of purified viral RNA, or minicolumn-purified nucleic acid extracts of infected grapevine. Polymerase chain reactions were performed for 40 cycles with a programmable DNA thermal cycler (Perkin-Elmer Cetus Corp., Norwalk, CT) as described by HADIDI and YANG (1990).

Analysis of amplified products

Aliquots of PCR amplified products were analyzed on 6 % polyacrylamide gels in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2.5 mM EDTA, pH 8.5) at 10 V/cm for 2.5 h. pGEM DNA markers (Promega, Madison, WI) were used to determine the size of RT-PCR amplified GVA cDNA. Gels were stained with silver nitrate (SCHUMACHER *et al.*

1986) or denatured with 0.5 M NaOH and 1M NaCl for 15 min, then electrotransferred to Nytran membranes (Schleicher and Schuell, Keene, NH) in TAE buffer (40 mM Tris-acetate, 1 mM EDTA) at 0.6 A for 16 h at 4 °C. PCR products for dot blot were mixed 1:1 (v/v) with denaturing buffer (3 vol. of 20 × SSC, 2 vol. of 37 % formaldehyde; 1 × SSC: 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0) heated at 65 °C for 30 min, chilled on ice for 2 min and transferred to Nytran membranes equilibrated with 20 × SSC. PCR products were crosslinked to membranes by irradiation (1,200 μJ for 45 sec) in a U.V. crosslinker (Stratagene, LaJolla, CA). Membranes were hybridized with ^{32}P-labelled SP 6 RNA polymerase-generated GVA cRNA probe (Riboprobe Gemini System, Promega Corp., Madison, WI) synthesized according to the manufacturer. Recombinant used for hybridization was Pst I fragment of pGA 240a (MINAFRA *et al.* 1992) subcloned in pGEM-4z (Promega Corp.). Nytran membranes were prehybridized and hybridized according to the manufacturer directions for riboprobe (Schleicher and Schuell) in a Hybaid mini hybridization oven (National Labnet Co., Woodbridge, NJ). Membranes were exposed to X-ray film (Hyperfilm-MP, Amersham Corp., Arlington Heights, IL).

Results

Efficiency of primers for RT-PCR amplification of purified GVA RNA

Three sets of primer pairs (Table) were compared for their efficiency to reverse transcribe and amplify purified GVA RNA. Sets No. 2 and No. 3 successfully amplified GVA cDNA of the expected size, i.e. 451 bp and 430 bp, respectively (Fig. 1, lanes b and c). No GVA cDNA product of the expected size (524 bp) was obtained for primer set No. 1 (lane a). Primer set No. 3 was then routinely used for further experiments.

RT-PCR detection of GVA RNA from grapevine infected tissue

To check the specificity and efficiency of GVA cDNA amplification in nucleic acid extracts of infected tissue using primer set No. 3, we reverse transcribed and amplified purified GVA RNA or purified cucumber mosaic virus (CMV) RNA, total nucleic acids, or minicolumn-purified total nucleic acids of GVA-infected or uninfected grapevine, and total nucleic acids of GVA-infected or uninfected *N. benthamiana*.

Fig. 2 shows gel electrophoresis analysis of RT-PCR amplified products. A major GVA cDNA product of 430 bp (expected size) was observed after RT-PCR amplification of purified GVA RNA (lane a), minicolumn-purified total nucleic acids of GVA-infected grapevine (lane b), and total nucleic acids of GVA-infected *N. benthamiana* (lane g). This product was not observed after RT-PCR amplification of purified CMV (lane f), non-purified total nucleic acids of GVA-infected grapevine (lane c), minicolumn purified total nucleic acids of uninfected grapevine (lane d), total nucleic acids of uninfected grapevine (lane e). Total nucleic acid extracts of healthy *N. benthamiana* gave a product of about the same size as that of GVA cDNA (Fig. 2, lane h). This product, however, did not hybridize with a GVA cRNA probe (not shown).

Fig. 3 demonstrates that the detection of GVA from infected grapevine tissue by RT-PCR is specific regardless of the source of plant material. A major GVA cDNA product of 430 bp (Fig. 3 A, lanes a—d) that hybridized with ^{32}P-labelled GVA cRNA probe (Fig. 3 B, lanes a—d) was observed after RT-PCR amplification of purified viral RNA (Fig. 3 A and B, lane a), or total nucleic acids of GVA-infected leaves of grapevine cv. Perricone rooted explants grown *in vitro* (Fig. 3A and B, lane b), mature leaves of

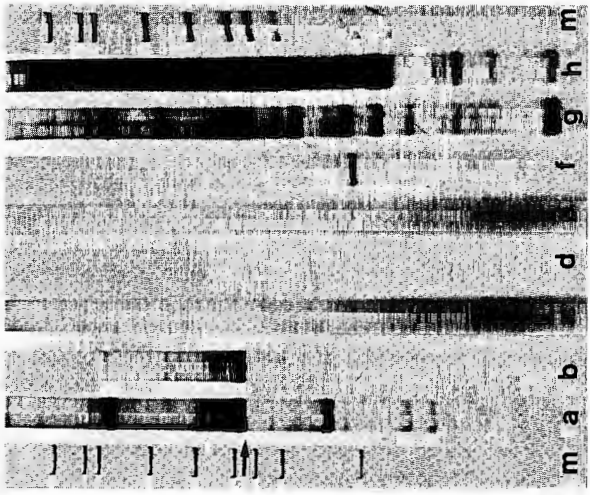

Fig. 2: PAGE of RT-PCR amplified GVA cDNA in nucleic acid extracts of GVA-infected or uninfected grapevine, or *N. benthamiana*. GVA cDNA of purified viral RNA (a); GVA cDNA of nucleic acid extracts of GVA-infected grapevine cv. Perricone: minicolumn-purified (b) and non-purified (c); cDNA of nucleic acid extracts of GVA-free *Vitis rupestris* St. George: minicolumn-purified (d) and non-purified (e); cDNA of purified cucumber mosaic virus RNA (f); nucleic acid extracts of *N. benthamiana*: GVA cDNA of infected tissue (g) and uninfected tissue (h). pGEM DNA marker (m). Arrow: 430 bp GVA cDNA synthesized with primer pair No. 3.

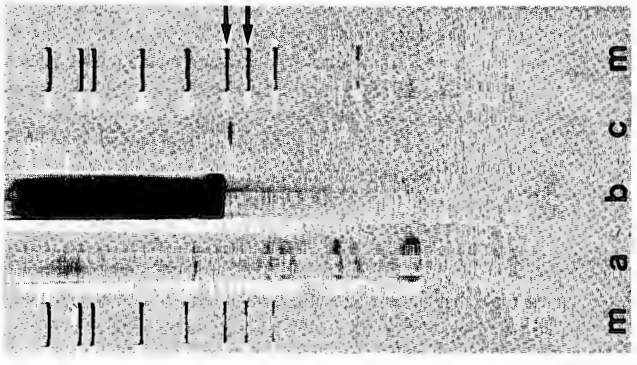

Fig. 1: Polyacrylamide gel electrophoresis analysis (PAGE) of RT-PCR amplified products of purified GVA RNA synthesized by three different sets of DNA primer pairs (see Table): primer set No. 1 (a); No. 2 (b); No. 3 (c). pGEM DNA marker (m), arrows indicate 460 bp and 396 bp. One µg of purified RNA was used in the reverse transcription reaction.

greenhouse-grown grapevine (Fig. 3 A and B, lane c), and bark tissue of field-grown grapevine (Fig. 3 A and B, lane d). The 430 bp product was not detected in purified total nucleic aicds of uninfected grapevine (Fig. 3 A, lane e), nor hybridization signals were observed (Fig. 3 B, lane e).

Sensitivity of RT-PCR assay for GVA RNA

Fig. 4 shows the sensitivity of GVA RNA detection from purified viral RNA by gel electrophoresis analysis of RT-PCR amplified products. A 430 bp GVA cDNA product was detected from 1 µg to as little as 100 fg of purified viral RNA utilized in the reverse transcription reaction. Due to the fact that one tenth volume of the reverse transcription reaction was used for PCR amplification and one tenth volume of the PCR reaction was used for polyacrylamide gel electrophoresis analysis, 1 fg of purified viral RNA is the limit of sensitivity of this technique.

The limit of detection of GVA RNA by RT-PCR amplification in purified total nucleic acids of GVA-infected grapevine was 100 pg in the reverse transcription reaction and 1 pg for polyacrylamide gel electrophoresis analysis (not shown).

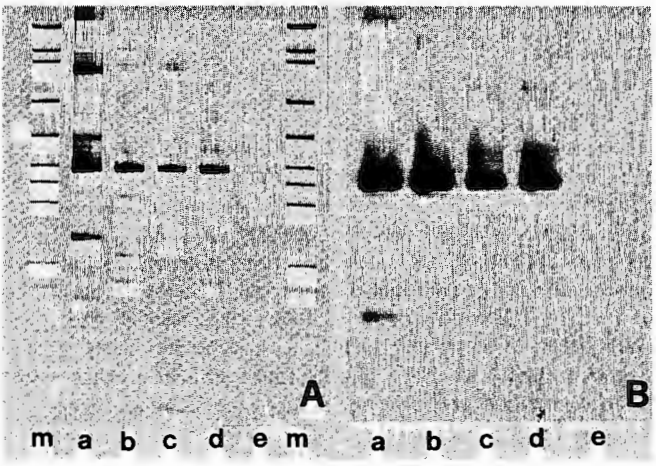

Fig. 3: PAGE (A) and an autoradiograph of Southern blot hybridization analysis (B) of RT-PCR amplified GVA cDNA in minicolumn-purified nucleic acid extracts of GVA-infected and uninfected grapevines. GVA cDNA of purified viral RNA (a); GVA cDNA of leaf nucleic acid extracts from: *in vitro*-grown GVA-infected grapevine cv. Perricone (b), greenhouse-grown grapevine cv. Perricone (c); GVA cDNA nucleic acid extracts from bark scrapings of GVA-infected grapevine growing in the field (d); cDNA of nucleic acid extracts from GVA-free *V. rupestris* (e). One µg of minicolumn-purified nucleic acid extracts was used for cDNA synthesis.

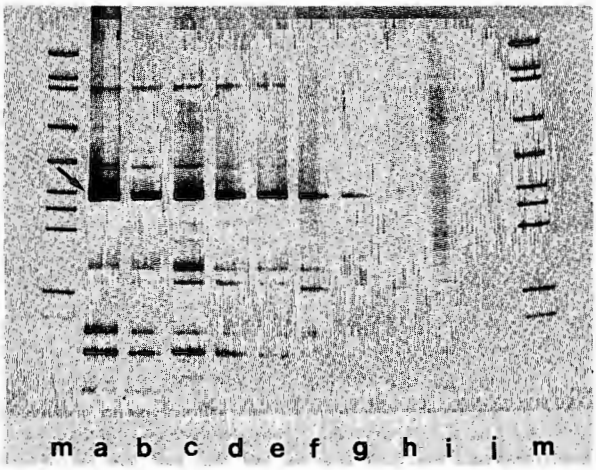

Fig. 4: PAGE of RT-PCR amplified GVA cDNA synthesized from different amounts of purified GVA RNA. Purified viral RNA (0.1 pg to 1 µg) was reverse transcribed in 50 µl of RT reaction mixture. 5 µl of each RT mixture were then transferred to 45 µl of PCR reaction mixture for cDNA amplification. 5 µl of each amplified product (obtained from 1 fg to 10 ng of purified GVA RNA) were analyzed on polyacrylamide gels. Amplified GVA cDNA products from the following amounts of purified viral RNA utilized in the RT reaction were analyzed: 1 µg (a), 100 ng (b), 10 ng (c), 1 ng (d), 100 pg (e), 10 pg (f), 1 pg (g), 100 fg (h), 10 fg (i), 1 fg (j). pGEM DNA marker (m). Arrow indicates the major amplified 430 bp GVA cDNA fragment.

Discussion

The present investigation demonstrates the successful application of RT-PCR to detection of GVA RNA in nucleic acid extracts of infected grapevine tissue. The available nucleotide sequence of a cDNA clone of GVA RNA enabled us to design three DNA primer pairs, two of which specifically primed the reverse transcription and amplification reactions of GVA RNA. The amplification of 430 bp GVA cDNA in nucleic acid extracts of GVA-infected grapevine tissue was specific, as the size of this product was that expected for the transcript synthesized in the presence of primer pair set No. 3, and the GVA cDNA hybridized with ^{32}P-labelled GVA-cRNA probe. Furthermore, the 430 bp transcript was absent in nucleic acid extracts of uninfected tissues. Successful RT-PCR amplification of GVA RNA in nucleic acid extracts of infected grapevine, however, is dependent on removal of inhibitors interfering with RT-PCR reactions. Interference of inhibitors with successful reverse transcription and amplification of nucleic acid extracts of woody hosts had already been observed with pome and stone fruit viroids (HADIDI and YANG 1990; HADIDI *et al.* 1992), citrus viroids (YANG *et al.* 1992), and plum pox viroids (KORSCHINECK *et al.* 1991). The detection limit of GVA RNA from infected grapevine tissue by RT-PCR was 200 fold higher than that obtained by molecular hybridization (MINAFRA *et al.* 1992) or ELISA (BOSCIA *et al.* 1992).

As discussed by WETZEL *et al.* (1991), the relatively low efficiency of cDNA transcription, especially in plant extracts, makes it difficult to reach the theoretical yield of PCR application. However, notwithstanding this limitation, which was experienced also in the course of this study, PCR remains a most powerful tool for detecting viral sequences present in exceedingly low titer in field samples. With grapevines, the major shortcoming of RT-PCR application is the necessity of using purified nucleic acid extracts to obtain recognizable amplification products. A wider use of RT-PCR could be envisaged (e.g. large scale field surveys for certification programmes) by simplifying sample manipulation and/or adapting the technique to diluted nucleic acids or crude sap extracts. These goals are now being pursued with further studies.

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