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Further studies on the use of molecular probes to grapevine closterovirus A

by

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Weitere Untersuchungen über die Verwendung von Molekülsonden beim Grapevine-Closterovirus A

Zusammenfassung: Zwei klonierte cDNA-Sonden für genomische RNA des Grapevine-Closterovirus A (GVA) wurden mit Erfolg zum Nachweis von Virussequenzen in infizierten krautigen Wirtspflanzen (*Nicotiana benthamiana*) und Reben benützt. Die eine Sonde (pGA112) war komplementär zum zentralen Teil des Virusgenoms und lieferte schwache falsch-positive Nachweisreaktionen mit Extrakten aus gesunden Reben; die andere Sonde (pGA240), die vermutlich kolinear mit dem 3'-Terminus ist, war dagegen virusspezifisch und hybridisierte nur mit Extrakten aus infizierten Proben. In elektrophoretisch aufgetrennten *N. benthamiana*-Extrakten „erkannten" die beiden Sonden RNAs von weniger als Genomgröße; sie hybridisierten differenziert mit den Banden, so daß diese subgenomische RNAs darstellen könnten. Die Sonde pGA240 kann für den GVA-Nachweis verwendet werden; für Routinetests muß die Vorbereitung der Proben für die Hybridisierung noch verbessert werden.

Key words: Vitis, closterovirus, virosis, subgenomic RNAs, cDNA, molecular probes, diagnosis.

Introduction

Grapevine closterovirus A (GVA) is the best known of the closteroviruses infecting grapevine. Its biological, physico-chemical and epidemiological properties have extensively been investigated (for review see GUGERLI 1991) and antisera produced in several laboratories. Some of these antisera are also commercially available as kits for ELISA testing.

In spite of this wealth of information and diagnostic tools, the detection of GVA in infected vines is not easy and often yields doubtful results, at least in our experience. To overcome this difficulty, a cDNA probe was synthesized years ago and was successfully used for identifying GVA RNA sequences in grapevine tissue extracts (GALLITELLI *et al.* 1985). Unfortunately, this probe was not cloned. More recently, other probes were produced which gave hybridization signals with GVA-infected *Nicotiana benthamiana* but not with grapevine samples (MINAFRA *et al.* 1991).

In the present paper the selection of a new probe and improvements in the extraction method are reported, which enabled the recognition of GVA in infected grapevines and the presence of subgenomic RNAs in *N. benthamiana*.

Materials and methods

Molecular probes

Two cDNA probes were used, denoted pGA112 (about 2900 base pairs) and pGA240 (about 1600 base pairs), respectively.

Probe pGA112 was obtained by random priming genomic RNA extracts with hexanucleotide primers and was the same utilized in previous investigations (MINAFRA *et al.* 1991). Probe pGA240 was the largest clone among those of a cDNA library obtained by artificial polyadenilation of genomic RNA templates and priming with oligo(dT)10-12. cDNA in double stranded form was synthesized using 'cDNA System Plus' (Amersham), starting from 1 ng viral RNA. Size selection of double stranded cDNAs was made by electrophoresis in low melting point agarose. cDNA fragments were ligated in the Sma I site of a pUC 18 plasmid and cloned in cells of *Escherichia coli* strain JM 103, which were plated in an ampycillin-containing medium. To identify polyadenilated cDNA clones, oligo(dT) labelled at the 5' end with ^{32}P was used as a probe in colony lifting assays (BARRY *et al.* 1989).

The pGA240 insert proved to belong to the same family of clones that were 3' co-linear with one another and, presumably, also with viral RNA since the sequence upstream the poly(A) tail was the same as that of the other clones (A. MINAFRA, unpublished data). Thus, pGA240 was from a genomic region differing from that of clone pGA112, which derives from sequences located in a somewhat central position of the GVA genome, towards its 5' terminus (MINAFRA *et al.* 1991).

Extraction and hybridization of tissues samples

Total nucleic acid extracts from healthy and GVA-infected *N. benthamiana* and grapevines were obtained by the method of WHITE and KAPER (1989) encompassing phenol extraction and ethanol precipitation. These preparations were spotted on nylon membranes (Hybond N, Amersham) after dilution 1 : 4 in formaldehyde-SSC (NaCl 0.15 M, sodium citrate 0.015 M, pH 7.0) buffer (12 × SSC and 15 % formaldehyde) and denaturation at 60 °C for 10 min.

Aliquots (100 µl) of partially clarified sap from *N. benthamiana* and grapevine leaves which had been chloroform extracted according to PALUKAITIS *et al.* (1984) were also spotted onto membranes without previous denaturation. Grapevine samples were from glasshouse-grown plants or *in vitro*-cultured explants. All membranes were exposed to pGA112 and pGA240 labelled with ^{32}P by nick translation or random oligo-labelling synthesis. The membranes were hybridized overnight at 42 °C using a probe activity of 5×10^5 cpm/ml, then washed at 65 °C for 2 h in 2 × SSC containing 0.1 % SDS.

For Northern blots, nucleic acids were electrophoresed in formaldehyde-containing denaturing agarose gels, then blotted onto nylon membranes by capillary elution (SAMBROOK *et al.* 1989). Usually, autoradiographic exposure for dot blots and Northern blots did not exceed 18 h.

Results

Regardless of the method used for ^{32}P labelling (nick translation or random oligo-labelling synthesis) both molecular probes (pGA112 and pGA240) hybridized with puri-

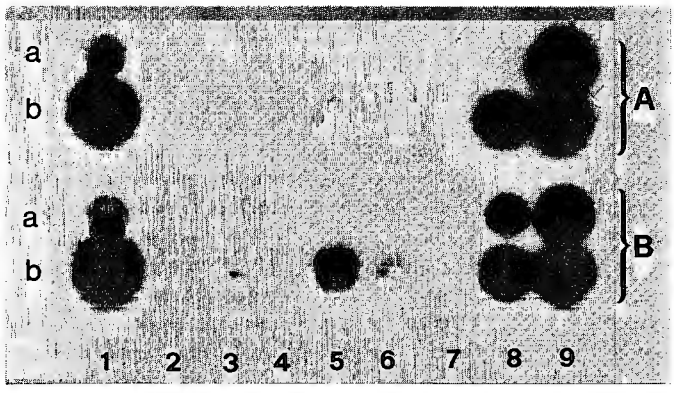

Fig. 1: Spot hybridization of healthy and GVA-infected *N. benthamiana* and grapevine extracts. Probes were ^{32}P-labelled by nick-translation. — A) Spots hybridized by pGA112. B) Spots hybridized by pGA240. Chloroform extracts on upper rows (a); total nucleic acid extracts on lower rows (b). — Lane 1: GVA-infected *N. benthamiana*; 2: healthy *N. benthamiana*; 3: grapevine cv. Perricone (glasshouse); 4: grapevine isolate 421 (glasshouse); 5: cv. Perricone, isolate Pa-2 (*in vitro*); 6: cv. Perricone, isolate Pa-3 (*in vitro*); 7: healthy cv. Mission seedling (glasshouse); 8: GVA-infected *N. benthamiana*, Colmar isolate; 9: viral RNA (2 ng in lower and 0.2 ng in upper spots).

Fleckhybridisierung von Extrakten aus gesunden und GVA-infizierten *N. benthamiana* und Reben. Die Sonden wurden durch nick-translation mit ^{32}P markiert. — A) Flecken mit pGA112 hybridisiert. — B) Flecken mit pGA240 hybridisiert. Obere Reihen a) Chloroformextrakte, b) Totalextrakte der Nukleinsäuren. — Spalte 1: GVA-infizierte *N. benthamiana*; 2: gesunde *N. benthamiana*; 3: Rebsorte Perricone (Gewächshaus); 4: Rebe, Isolat 421 (Gewächshaus); 5: Perricone, Isolat Pa-2 (*in vitro*); 6: Perricone, Isolat Pa-3 (*in vitro*); 7: gesunder Sämling der Sorte Mission (Gewächshaus); 8: GVA-infizierte *N. benthamiana*, Isolat Colmar; 9: Virus-RNA (unterer Fleck 2 ng, oberer 0,2 ng).

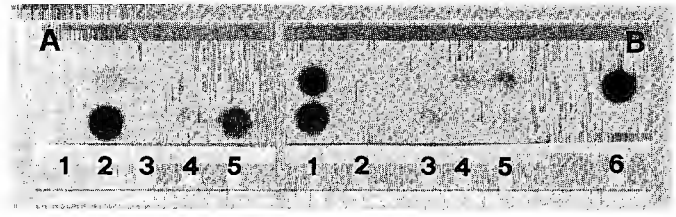

Fig. 2: Spot hybridization of healthy and GVA-infected *N. benthamiana* and grapevine extracts. Probes were ^{32}P-labelled by random oligolabelling synthesis. — A) Spots of chloroform extracts hybridized by pGA240. Lane 1: healthy *N. benthamiana*; 2: GVA-infected *N. benthamiana*; 3: healthy cv. Mission seedling (glasshouse); 4: GVA-infected cv. Perricone (glasshouse); 5: GVA-infected cv. Perricone (*in vitro*). — B) Spots of chloroform (upper row) and total nucleic acid extracts (lower row) hybridized by pGA112. Lane 1: GVA-infected *N. benthamiana*; 2: healthy *N. benthamiana*; 3: GVA-infected cv. Perricone (glasshouse); 4: healthy cv. Mission seedling (glasshouse); 5: GVA-infected cv. Perricone (*in vitro*). 6: viral RNA (1 ng) spotted as control. — Chloroform and total nucleic acid extract spots are equivalent to 25 and 10 mg of leaf tissues, respectively.

Fleckhybridisierung von Extrakten aus gesunden und GVA-infizierten *N. benthamiana* und Reben. Sonden durch random oligo labelling mit ^{32}P markiert. — A) Flecken von Chloroformextrakten mit pGA240 hybridisiert. Spalte 1: Gesunde *N. benthamiana*; 2: GVA-infizierte *N. benthamiana*; 3: gesunder Sämling der Sorte Mission (Gewächshaus); 4: GVA-infizierte Sorte Perricone (Gewächshaus): 5: GVA-infizierte Sorte Perricone (*in vitro*). — B) Flecken von Chloroformextrakten (oben) und Nukleinsäure-Totalextrakten (unten) mit pGA112 hybridisiert. Spalte 1: GVA-infizierte *N. benthamiana*; 2: gesunde *N. benthamiana*; 3: GVA-infizierte Sorte Perricone (Gewächshaus); 4: gesunder Sämling von Mission (Gewächshaus); 5: GVA-infizierte Sorte Perricone (*in vitro*); 6: Virus-RNA (1 ng) als Kontrolle. — Chloroform- und Totalextrakte entsprechen 25 bzw. 10 mg Blattgewebe.

fied viral RNA giving very strong signals (Fig. 1, lane 9; Fig. 2 B, lane 6). Comparable heavy hybridizations were obtained when purified intact virus preparations were spotted onto membranes (not shown). The limits of detection of viral RNA and purified virus were 10 pg and 120 pg, respectively.

The two probes recognized also viral RNA sequences in extracts from leaves of *N. benthamiana* infected with different GVA isolates either homologous or heterologous to the probe. As shown in Fig. 1, a French isolate of the virus (Colmar isolate, lane 8) hybridized with pGA112 and pGA240 equally well as the Italian isolate (lane 1) used to produce the probes. However, the RNA extraction method had a bearing in determining the extent of hybridization, as judged by the intensity of the signal. Total RNA extracts gave a clearer and stronger signal (Fig. 1, lanes 1 and 8 of rows b) than chloroform extracts (Fig. 1, lanes 1 and 8 of rows a). No hybridization was ever observed with either type of extracts from healthy *N. benthamiana* tissues (Fig. 1 A and B, lane 2; Fig. 2 A, lane 1; Fig. 2 B, lane 2).

As to grapevine samples, probe pGA240 hybridized specifically with infected tissues only, but with an intensity of reaction that varied with the RNA extraction method

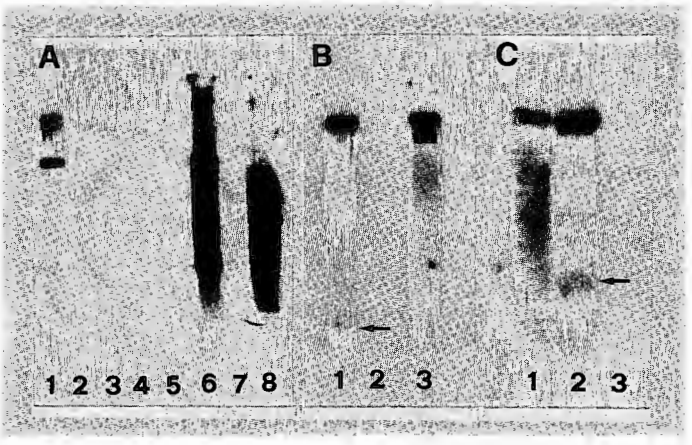

Fig. 3: A) Autoradiography of a Northern blot hybridization of healthy and GVA-infected grapevines and *N. benthamiana*. The probe was nick-translated pGA240. Lane 1: dsRNA (10 ng) from GVA-infected *N. benthamiana*; 2: healthy cv. Mission seedling; 3: cv. Perricone, isolate Pa-2; 4: cv. Perricone, isolate Pa-3; 5: infected undetermined grapevine cultivar; 6: GVA-infected *N. benthamiana*; 7: healthy *N. benthamiana*; 8: viral RNA (10 ng). Each grapevine lane is equivalent to 50 mg of leaf tissue. Exposure was 48 h. — B and C) Autoradiography of Northern blot hybridizations of total nucleic acid extracts from healthy and GVA-infected *N. benthamiana*. The blots, derived from formaldehyde denaturing gels, were hybridized by nick-translated pGA112 (B) and pGA240 (C). (B) lane 3 and (C) lane 1: viral RNA (10 ng); (B) lane 1 and (C) lane 2: GVA-infected *N. benthamiana*; (B) lane 2 and (C) lane 3: healthy *N. benthamiana*. Each lane is equivalent to 10 mg of leaf tissue extract. Arrows indicate putative subgenomic RNA bands.

A) Autoradiogramm einer Northern-blot-Hybridisierung von gesunden und GVA-infizierten Reben und *N. benthamiana*. Die Sonde ist pGA240, markiert durch nick-translation. Spalte 1: dsRNA (10 ng) aus GVA-infizierter *N. benthamiana*; 2: gesunder Sämling von Mission; 3: Perricone, Isolat Pa-2; 4: Perricone, Isolat Pa-3; 5: unbekannte infizierte Rebsorte; 6: GVA-infizierte *N. benthamiana*; 7: gesunde *N. benthamiana*; 8: Virus-RNA (10 ng). Jede Spalte entspricht 50 mg Blattgewebe. 48 h Inkubationszeit. — B und C) Autoradiogramme von Northern-blot-Hybridisierungen der Nukleinsäure-Totalextrakte gesunder bzw. GVA-infizierter *N. benthamiana*. Die Blots wurden von Formaldehyd-denaturierten Gelen angefertigt und mit nick-translatierter pGA112 (B) bzw. pGA240 (C) hybridisiert. (B) Spalte 3 und (C) Spalte 1: Virus-RNA (10 ng); (B) Spalte 1 und (C) Spalte 2: GVA-infizierte *N. benthamiana*; (B) Spalte 2 und (C) Spalte 3: gesunde *N. benthamiana*. Jede Spalte entspricht 10 mg Blattgewebeextrakt. Die Pfeile markieren mögliche subgenomische RNA-Banden.

and the conditions under which the source material was grown. Samples from glasshouse-grown vines gave visible hybridization reactions (Fig. 2 A, lane 4) but these were inconsistent and much lighter than the signals obtained with extract from *in vitro*-grown explants (Fig. 1 B, lanes 5 and 6; Fig. 2 A, lane 5). Probe pGA112 behaved in a somewhat similar manner but, contrarily to probe pGA240, it hybridized, though slightly, also with extracts from healthy grapevine seedlings (Fig. 2 B, lane 3).

Similarly to *N. benthamiana*, total nucleic acid extracts from grapevine tissues yielded more consistent hybridization signals than chloroform extracts.

The specificity of clone pGA240 was confirmed by Northern blot analysis where, after autoradiographic exposures as long as 48 h, no signals were detected in uninfected controls (Fig. 3 A, lanes 2 and 7). Infected samples (50 mg of leaf tissues) gave a strong hybridization reaction in the case of *N. benthamiana* (Fig. 3 A, lane 6) but no signal at all in the case of grapevine (Fig. 3 A, lanes 3 and 4).

When blots of viral RNA and total RNA extracts for healthy and infected *N. benthamiana* were hybridized with both probes after electrophoresis in denaturing gels, there was no reaction with healthy extracts (Fig. 3 B, lane 2; 3 C, lane 3) but a clear-cut signal was visible with viral RNA and infected extracts. However, whereas viral RNA occurred as a single band (Fig. 3 B, lane 3; 3 C, lane 1), infected *N. benthamiana* extracts, in addition to the genomic RNA band, showed faster migrating bands that hybridized selectively with either probe (Fig. 3 B, lane 1; 3 C, lane 2).

Discussion

Both molecular probes used in the present investigation did recognize RNA sequences of GVA isolates from Italy and France in leaf extracts of infected *N. benthamiana* and, limited to the Italian isolates, grapevine. Of the two probes, however, pGA240 gave specific reactions hybridizing strictly with infected samples even after prolonged autoradiographic exposures. Although probe pGA112 did not seem to produce false positives with healthy *N. benthamiana* occasionally it gave weak hybridization signals with healthy grapevines, which may make it unsuitable for diagnostic use.

With probe pGA240 there was a distinct improvement over the results previously reported, which indicated lack of reactivity of molecular probes with infected grapevine extracts (MINAFRA *et al.* 1991). It is now ascertained that failures to hybridize were not due to the quality of the probes but, rather, to the exceedingly low concentration and, perhaps, erratic distribution of GVA particles and/or viral RNA in infected grapevines. The results of several of the experiments made seem to support this likelihood: (a) the influence of the extraction method used on the intensity of the hybridization signal; (b) the absence of hybridizing bands in agarose-fractionated nucleic acid extracts; (c) the differential reactivity of extracts from tissues collected from vines growing under different conditions. With reference to this latter point, it was interesting to note that, with grapevines, the strongest hybridization signals were obtained with tissue culture samples, which is in line with the finding that *in vitro* plant cultures are better sources of GVA for purification (MONETTE and JAMES 1991).

An interesting feature of the hybridization pattern of electrophoresed extracts from infected *N. benthamiana* is the presence of bands smaller than genomic RNA, which were recognized differentially by the two probes. Similar bands were not seen in purified viral RNA extracts, which may tentatively be explained by admitting that these smaller fractions represent non-encapsidated subgenomic RNAs. This possibility

is supported by the notion that with other closteroviruses (e.g. citrus tristeza closterovirus) more than one species of subgenomic dsRNA is commonly found *in vivo* (DODDS and BAR-JOSEPH 1983; GUERRI *et al.* 1991) and that the coat protein gene may be translated *in vitro* using subgenomic RNA (SEKYIA *et al.* 1991). Incidentally, the absence of a poly(A) tail in viral RNA seems to confirm the closterovirus status of GVA and distinguishes it from presumed closteroviruses, like apple chlorotic leaf spot virus, which has a 3' terminal poly(A) tract (GERMAN *et al.* 1990).

Notwithstanding the improvements reported in this paper, the use of molecular probes for GVA detection in infected vines has not yet been optimized for routine testing. Room for further amelioration may reside in the choice of tissues and timing of sample collection, and/or in the use of methods (e.g. polymerase chain reaction) for increasing the quantity of RNA sequences in test samples. Both these areas are now being investigated.

Summary

Two cloned cDNA probes to genomic RNA of grapevine closterovirus A (GVA) were utilized successfully for the detection of viral sequences in infected herbaceous hosts (*Nicotiana benthamiana*) and grapevines. One of the probes (pGA112) was complementary to the central part of the viral genome and gave light false positive signals with healthy grapevine extracts, whereas the other (pGA240), which is presumably colinear with the 3' terminus, was virus-specific and hybridized only with infected sample extracts. The two probes recognized smaller than genome RNAs in electrophoresed *N. benthamiana* extracts and hybridized differentially with the bands, thus suggesting that these represent subgenomic RNAs. Probe pGA240 may be used for GVA detection, but the preparation of samples for hybridization needs further improvement for routine testing.

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