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Detection of arabis mosaic virus using the polymerase chain reaction (PCR)

by

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Summary: A new method is described for detecting arabis mosaic virus (ArMV) in infected plants. Specific sequences of ArMV-RNA present in total nucleic acid extracts of infected *Vitis vinifera* or *Chenopodium quinoa* were initially reverse-transcribed into a complementary DNA (cDNA), then amplified by PCR using specific oligonucleotide-primers. Different primer combinations distinguished between an ArMV infection and an infection with grapevine fanleaf or raspberry ringspot virus. The amount of nucleic acids obtained from 5 mg grapevine leaves resp. 1 mg leaves of *Ch. quinoa* were sufficient for detecting ArMV.

Key words: Polymerase chain reaction, arabis mosaic virus, nepovirus, virus detection, grapevine fanleaf virus, Chenopodium quinoa, Vitis vinifera.

Introduction

Arabis mosaic nepovirus is an economically important pathogen of strawberry, cherry, raspberry, hop and grapevine (MURANT 1981). It is also one of the causal agents of the fanleaf disease-complex of grapevine (court-noué, Reisigkrankheit) and widespread in Europe. In German vineyards ArMV, grapevine fanleaf virus (GVF) and raspberry ringspot virus (RRV) are the most important virus-pathogens.

ArMV consists of icosahedral particles with a diameter of 28 nm and has a bipartite ssRNA genome. Currently, the detection and characterization of ArMV is based on biological and serological tests. However, molecular hybridization with recombinant cDNA has proved an excellent method for the detection and comparison of plant viruses (BURGERMEISTER *et al.* 1986; STEINKELLNER *et al.* 1989). This is a sensitive and highly specific method but is rarely used in laboratory practice because the probes are frequently used with a radioactive label (FELDNER 1990). Nevertheless, the sensitivity of this technique is reduced by the fact that a minimum amount of nucleic acids is required for hybridization.

However, DNA sequences not detectable by conventional standard methods can be amplified by polymerase chain reaction (PCR) which enables them to be visualized by gel electrophoresis (HAGEN-MANN 1990; NIENHAUS and GEHRMANN 1991; XU and LARZUL 1991). In plant virology, first successful trials have led to the detection of a potyvirus (BOL *et al.* 1991), the bean yellow mosaic virus (VUNSH *et al.* 1990) and the hop stunt viroid (PUCHTA and SÄNGER 1989).

To detect RNA viruses, a reverse transcriptase reaction (RT) must precede the PCR to transform the RNA into a complementary DNA (cDNA) before amplification can be started.

Here we describe a PCR method for the detection of the arabis mosaic virus in infected plant tissue. The method is capable of preventing cross-reaction with other nepoviruses.

Materials and methods

Virus origins

The material for virus propagation was obtained from virus-infected vineyards from the viticultural areas of Rheinpfalz (Südliche Weinstraße and Mittelhaardt). Individual stocks were tested serologically by ELISA according to the instructions of the firms Loewe (ArMV test kit) and Bioreba (GFV and RRV test kit). Only plants infected with a single virus (ArMV = arabis mosaic, GFV = grapevine fanleaf or RRV = raspberry ringspot virus) were used.

Virus propagation in test plants

Ch. quinoa served as test plants. 1 g shoot tip leaves or rootstock shavings of infected grapevines were each homogenized in 4 ml 2.5 % nicotine aqueous solution. Subsequently, the extract was rub-inoculated onto corundum-dusted plants at the 2- to 4-leaf stage.

Virus isolation

Four weeks after inoculation the plants were harvested. The frozen material was homogenized in a blender with 1.5 volume of extraction buffer (0.135 M Na-phosphate buffer pH 6.3 containing 0.1 % thioglycol acid, 0.5 % ascorbic acid and 0.01 % EDTA) and filtered through 3 layers of gauze (200 µm). The sap was clarified by stirring with 8.5 % n-butanol followed by low-speed centrifugation and filtration through glass-wool. The virus was concentrated by precipitation with 1 % NaCl and 10 % polyethylene glycol (PEG, 6,000 mol. wt.) and resuspended in 0.033 M Na-phosphate buffer pH 7.2. Further purification of the virus was achieved by two cycles of low- and high-speed centrifugation followed by centrifugation in linear sucrose density gradient (10—50 %) at 25,000 rpm for 5 h in a Beckman SW 28 rotor (modified according to RALPH and BERGQUIST 1967; MAYO *et al.* 1971 and 1982; PINCK *et al.* 1988; FUCHS 1989). The fractions containing the B components of the virus were pooled, dialysed against buffer, sedimented and resuspended in buffer.

RNA isolation from virions

The RNA was isolated according to the pronase/SDS method (MURANT *et al.* 1972). The RNA was recovered by ethanol precipitation and resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.9).

Preparation for PCR

Ch. quinoa plants were inoculated with different isolates of ArMV, GFV or RRV. The positive plants, examined after 8—10 d by ELISA, were prepared for PCR according to JELKMANN (1991). 50 mg leaves were pulverized with liquid nitrogen in 1.5 ml tubes and 500 µl Proteinase K buffer (JELKMANN 1987) was added. After centrifugation (5 min) 1 µl Proteinase K (Sigma, stock solution 20 mg/ml H_2O) per 100 µl supernatant was added and incubated for 1 h by 37 °C in a water bath. The mixture was extracted twice with an identical volume of buffer-saturated phenol/chloroform/isoamyl alcohol (25:24:1, v/v) and once with chloroform/isoamyl alcohol (24:1, v/v). The nucleic acids were ethanol-precipitated from the aqueous phase, dried and resuspended in 10 µl TE buffer.

100 mg leaves of ArMV-infected grapevines were pulverized with liquid nitrogen and the nucleic acids were isolated with 0.4 ml medium 2 according to REZAIAN and KRAKE 1987. Subsequently they were resuspended in ethanol:STE (50 mM Tris-HCl

pH 7, 100 mM NaCl, 1 mM EDTA), 35:65 (v/v), purified with CF-11-Cellulose powder (Whatman) according to REZAIAN *et al.* 1988 and resuspended in 20 µl TE-buffer.

PCR Primer

A 22 nucleotide primer (5'-GAGTTCGATGATAGGGAGAACG-3') was selected as antisense primer (A871) complementary to nucleotides 850—871 of the coat protein gene of an ArMV isolate from lilac (BERTIOLI *et al.* 1991). A sequence 21 bases long served as sense-primer (S418, 5'-AAAGAATTGGCAGCGGATTGG-3') corresponding to nucleotides 418—438 of the coat protein gene. The oligonucleotides were synthesized by Appligene, Heidelberg.

cDNA synthesis

Annealing: 2.5 µl primer A871 (10 µM), 2 µl total nucleic acid extract or isolated virus RNA (0.6—1 µg RNA) in TE buffer and 2.5 µl H_2O were incubated for 10 min at 70 °C and quick-chilled on ice (JELKMANN 1991).

Reverse transcriptase reaction: 4 µl HRT buffer (5 ×, Gibco-BRL), 2 µl dithiothreitol (0.1 M., Gibco-BRL), 1 µl of each dNTP (10 mM), 1.5 µl H_2O, 1 µl M-MLV reverse transcriptase (200 U/µl, Gibco-BRL) were added to the 7.5 µl annealing mix, overlaid with mineral oil, incubated for 10 min at 23 °C, 60 min at 37 °C, 5 min at 95 °C and quick-chilled on ice (INNIS *et al.* 1990).

PCR amplification

10 µl of the heat-treated reverse transcriptase reaction were added to 5 µl Taq DNA buffer (10 ×, Promega), 5 µl $MgCl_2$ (25 mM), 1 µl of each dNTP (10 mM), 0.5 µl Taq-polymerase (Promega), 21.5 µl H_2O, 2 µl of each primer S418 and A871 (10 µM) and overlaid with mineral oil.

Amplification conditions were as follows: 5 min at 95 °C, followed by 30 cycles consisting of 60 s at 94 °C, 30 s at 60 °C and 90 s at 72 °C; increment time: 3 s/cycle.

Gel electrophoresis

10 µl of the amplified PCR product were mixed with 10 µl loading buffer I (MANIATIS *et al.* 1982) and separated on a 2.0 % agarose gel in TBE buffer (DAVIS *et al.* 1986) with ethidium bromide (1 µg/ml buffer) at 7 °C and 4 V/cm. The 123 base pairs (bp) DNA Ladder (Gibco-BRL) was used as size marker. According to DAVIS *et al.* 1986, it is possible to estimate the length of unknown DNA fragments by means of DNA sequences of known length with an exactness of ±5 %.

Results

PCR with purified ArMV-RNA and total nucleic acids from ArMV-inoculated *Ch. quinoa*

RT/PCR with purified ArMV-RNA (Fig. 1) or total nucleic acid extracts from ArMV-inoculated leaves of *Ch. quinoa* (Figs. 2 + 3) leads to the same results.

Using primers A871 (RT), A871/S418 (PCR), a principal amplification product i.e. a DNA sequence with a length of 450 ± 25 bp calculated from the 123 bp DNA ladder was obtained. A product of the same size resulted when RT was carried out with primer A871 and PCR with primer A871 or primer S418. In this case, the band always showed a lower intensity than if both primers were simultaneously used in the PCR (Figs. 1 + 2).

Amplification with the primer combination S418 (RT) and A871/S418 (PCR) yielded a different picture (Figs. 1 + 3). An intensive principal band size 450 ± 25 bp appeared as well as two different bands with 700 ± 35 and 1000 ± 50 bp.

When primer A871 was omitted from PCR, all virus isolates examined had two amplification products (220 ± 10 bp and 1000 ± 50 bp). If only A871 was used as a primer in PCR, all samples provided a band of 450 ± 25 bp. With all primer combinations, but depending on the virus isolate, several additional bands with lower intensity may appear which can be longer or shorter, and their intensity is reduced if a part of the PCR reaction is used as a template for another amplification.

When total nucleic acid extracts from healthy plants of *Ch. quinoa* were used as templates, only short amplification products with a low intensity were obtained but never bands with 450 ± 25 bp or 1000 ± 50 bp.

PCR with other nepoviruses-RNA

To test the specifity of the primers, RNA from purified GFV and RRV isolates and the total nucleic acids from GFV and RRV-inoculated leaves of *Ch. quinoa* were used as templates for PCR (Figs. 2 + 3).

No amplification products with RRV-RNA were found. With total nucleic acids from RRV-inoculated leaves only weak bands identical to those from healthy plants, were obtained. The amplification products from GFV-inoculated leaves reached a maximum length of 700 ± 35 bp and generally were low in intensity. This fact also proved to be true for the band with 450 ± 25 bp. This very weak band was the only one found when using the RNA of one of the GFV isolates. In contrast, the above mentioned amplification product appeared as a very intensive principal band when ArMV-RNA was used as a template.

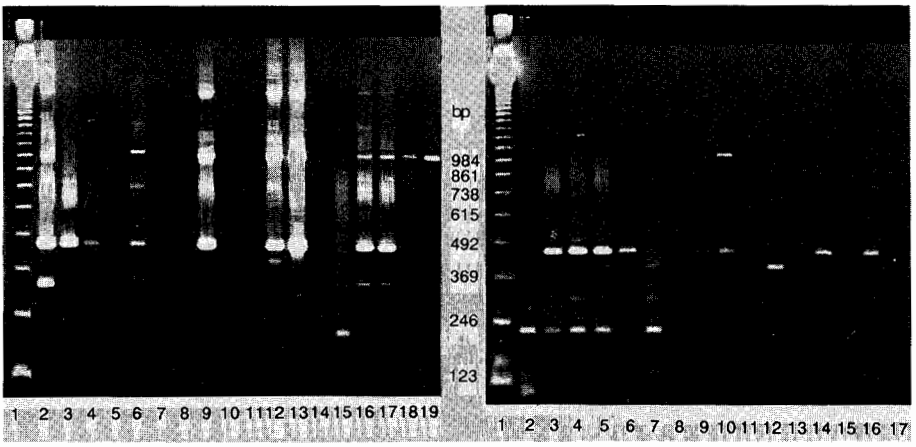

Fig. 1 (left): RT/PCR with purified ArMV-RNA (lanes 2, 3, 6, 7, 9, 10, 12, 13, 16—19), GFV-RNA (lanes 4, 8, 11, 14) and RRV-RNA (lanes 5, 15). Lane 1: 123 bp DNA ladder as size markers. Primer combinations: lanes 2—5: A871 (RT), A871/S418 (PCR), lanes 6—8: A871 (RT and PCR), lanes 9—11: A871 (RT), S418 (PCR), lanes 12—15: S418 (RT), A871/S418 (PCR), lanes 16, 17: S418 (RT), A871 (PCR), lanes 18, 19: S418 (RT and PCR). 2 % Agarose gel stained with ethidium bromide.

Fig. 2 (right): RT/PCR with nucleic acid extracts from leaves of *Ch. quinoa*, healthy (lane 2) or infected with ArMV (lanes 3—6, 9, 10, 14), GFV (lanes 7, 11, 12, 15, 16), RRV (lanes 8, 13, 17). Lane 1: 123 bp DNA ladder as size markers. Primer combinations: lanes 2—8: A871 (RT), A871/S418 (PCR), lanes 9—13: A871 (RT and PCR), lanes 14—17: A871 (RT), S418 (PCR). 2 % Agarose gel stained with ethidium bromide.

PCR with total nucleic acids from ArMV-infected grpevines

Only the primer combination A871 (RT), A871/S418 (PCR) was tested with total nucleic acids from ArMV-infected grapevine leaves. Amplification yielded a band size 450 ± 25 bp which was weaker than that with total nucleic acids from ArMV-infected *Ch. quinoa* (Fig. 4). However, the intensity increased if a part of the PCR reaction was used as a template for a second amplification. When total nucleic acids from ArMV-infected grapevines were used together with those from ArMV-infected *Ch. quinoa*, the intensity of the above mentioned band was also weaker compared with that obtained with total nucleic acids from ArMV-infected *Ch. quinoa* alone. The intensity of the amplification product depended on the grapevine variety, the location of the plants (greenhouse or field) and the timing of sample collection.

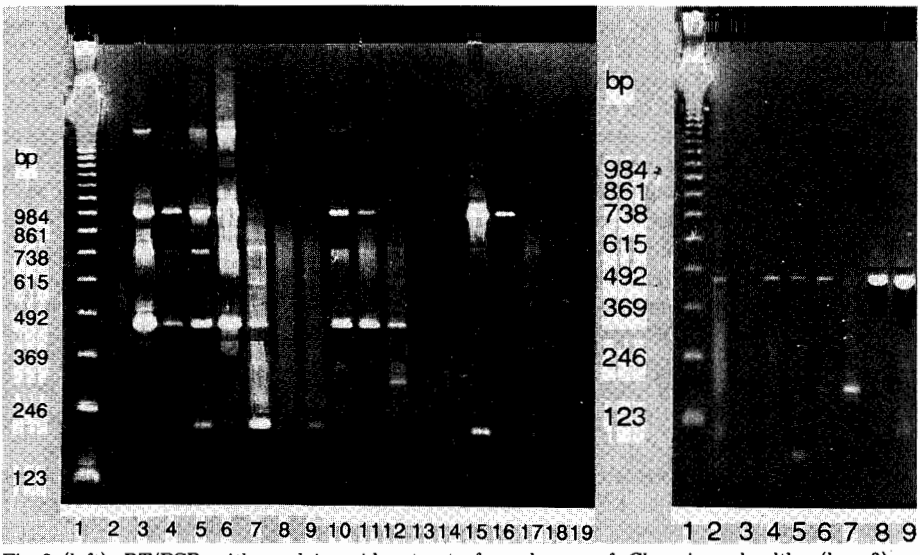

Fig. 3 (left): RT/PCR with nucleic acid extracts from leaves of *Ch. quinoa*, healthy (lane 2) or infected with ArMV (lanes 3—6, 10, 11, 15, 16), GFV (lanes 7, 12, 17) and RRV (lanes 8, 9, 13, 14, 18, 19). Lane 1: 123 bp DNA ladder as size markers. Primer combinations: lanes 2—9: S418 (RT), A871/S418 (PCR), lanes 10—14: S418 (RT), A871 (PCR), lanes 15—19: S418 (RT and PCR). 2 % Agarose gel stained with ethidium bromide.

Fig. 4 (right): RT/PCR with nucleic acid extracts from leaves of ArMV-infected *Ch. quinoa* (lanes 8, 9) and ArMV-infected *Vitis vinifera* (lanes 2—7). Lane 1: 123 bp DNA ladder as size markers. Primer combination: A871 (RT), A871/S418 (PCR). 2 % Agarose gel stained with ethidium bromide.

Discussion

The ArMV isolate, sequenced by BERTIOLI *et al.* 1991, was derived from lilac. When primers A871 and A871/S418 were used for RT and PCR, the amplified DNA sequence was supposed to show a length of 454 bp, provided that the primer sequences were present in ArMV isolated from other origins. During our investigations, one principal band with 450 ± 25 bp, corresponding exactly to the expected length, was found with all ArMV isolates used.

ArMV, GFV and RRV all belong to the nepovirus-group, however only ArMV and GFV are closely 'related' (NIENHAUS 1985). A comparison of the total amino acid sequences of both coat proteins revealed that they resemble by 72.2 % (BERTIOLI *et al.* 1991). The primer-corresponding amino acid sequences situated inside the coat protein

gene are the same for ArMV and GFV. When comparing the nucleotide sequences (FUCHS *et al.* 1989; SERGHINI *et al.* 1990), it was found that the position of the antisense primer A871 was 86 % similar, whereas that of the sense primer S418 had only 71 % similarity. The primers can only partly anneal to the GFV-RNA so that only a weak amplification of the 454 bp sequence may be obtained.

The sense primer S418 can also be successfully applied in the reverse transcriptase reaction for the cDNA synthesis from ArMV-RNA. The complete RNA of the ArMV consists of two linear molecules, the first one being RNA-1 with a length of 6960 bp and the second RNA-2 with 4060 bp (JELKMANN *et al.* 1988). Currently, only RNA-2 has been partially sequenced (BERTIOLI *et al.* 1991), therefore no details are known about the annealing position of primer S418. Primer combination S418 (RT), A871/S418 (PCR) yields two principal amplification products with 450 ± 25 bp and 1000 ± 50 bp, while the latter never appeared with GFV-RNA. To establish whether the band with 450 ± 25 bp is really the same or by chance has the same length as that with A871 (RT) and A871/S418 (PCR), they should be cut with restriction enzymes.

The detection of ArMV directly from grapevine leaves using PCR is more difficult than using leaves of *Ch. quinoa*. Especially grapevine leaves which are rich in phenols and quinones as they can complicate the isolation of nucleic acids (REZAIAN and KRAKE 1987), reduce the yield of amplification products or stop the PCR totally. The best method to isolate nucleic acids from grapevine was that according to REZAIAN *et al.* 1988, but it was still not optimal. There remained some inhibitory substances in the extracts since the amplification of added nucleic acids from *Ch. quinoa* was reduced also.

By using other primers it might be possible to carry out a 'DNA amplification fingerprinting' analysis (DAF) for characterizing the nepoviruses genomes as already described by CAETANO-ANOLLES *et al.* (1991 a, 1991 b) and WELSH *et al.* (1991) for the analysis of bacteria and different eucaryonts. The main difficulty with RNA viruses is the fact that the RNA must first be transformed into a cDNA before amplification can be started.

The results demonstrate that certain ArMV-specific primer combinations (A871 for RT, A871/S418 for PCR and S418 for RT) permit the detection of ArMV infection with the PCR technique. Secondly it can be used to rapidly distinguish an ArMV from a GFV or RRV infection. Nucleic acids from 1 mg leaves of *Ch. quinoa*, 5 mg grapevine leaves or 0.6 µg purified virus RNA were sufficient for these tests.

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