Vitis **34** (2), 127–128 (1995)

Research Note

Detection of nepoviruses in ligneous grapevine material by using IC/PCR

SABINE BRANDT and G. HIMMLER

Key words: dormant tissues, immunocapture PCR, ArMV, GFLV, GCMV, diagnosis.

Introduction: Grapevine fanleaf virus (GFLV), arabis mosaic virus (ArMV) and grapevine chrome mosaic virus (GCMV) lead to great economical losses, as they can infect all cultivars, including rootstocks derived from North American *Vitis* species, *Vitis vinifera* and hybrids. The viruses affect productivity and longevity of the plants, causing their quick destruction or a decline over several years (BOVEY *et al.* 1980). GFLV, ArMV and GCMV are definite members of the nepovirus group with isometric particles about 30 nm in diameter. The encapsidated genome consists of two linear single stranded RNA molecules (RNA1 and RNA2) both needed to produce infection. The polyadenylated RNA2 contains only one long open reading frame. Whereas the 5´ region of RNA2 encodes a protein responsible for cell-to-cell transport, the coat protein gene is located at the 3´ end of RNA2 (MATTHEWS 1991).

Although DAS-ELISA seems sufficiently reliable for large scale testing, efforts were made to establish the polymerase chain reaction (PCR) for diagnosis in order to increase sensitivity. Since nucleic acid-based assays for plant virus detection in woody tissues are notoriously difficult, an immunocapture PCR (IC/PCR) assay (JANSEN *et al.* 1990; WETZEL *et al.* 1992) has been adapted to detect GFLV, ArMV and GCMV in ligneous grapevine material.

Materials and methods: Source of samples: Several cultivars of French Colombard, Welschriesling and 1217/3/21 VG x MO (Veltliner Grün x Muskat Ottonel) grown in the viticultural area of Klosterneuburg, Lower Austria, were testet serologically by DAS-ELISA (CLARK and ADAMS 1977). Whereas cultivar 1217/3/21 VG x MO proved non-infected, French Colombard was clearly infected by GFLV and the samples taken from Welschriesling were partly ArMV-, partly GCMV-positive. In the following, these plants were used to prepare negative and positive samples, respectively.

Preparation of samples: Grapevine shoots were freshly harvested from infected and healthy plants. After removing the bark, the shoots were scratched with a sharp blade. 0.25 g per sample of the obtained wood shavings were filled in plastic bags (Bioreba AG, Basel, Switzerland) together with 5 ml Tris-HCl (0.5 M pH 8.2) buffer containing 2 % (w/v) PVP K25, 1 % (w/v) PEG 6000, 0.14 M NaCl and 0.05 % Tween 20 and ground using an homogenizer-extractor (Homex 5, Bioreba) (GUGERLI 1984). Crude plant sap was then pipetted in reaction tubes (1.4 ml) and centrifuged for 5 min at 13,000 rpm. The supernatants were used for all further experiments.

For comparison of IC/PCR with DAS-ELISA, GFLV-infected plant sap was stepwise diluted with extract of healthy grapevine 1:20, 1:80, 1:320, 1:5120 and 1:20480 and tested simultanously by using both methods.

Immunocapture: Polypropylen microcentrifuge tubes (0.5 ml) (Multi Technology Inc., Salt Lake City, Utah) were coated with 120 µl of polyclonal anti-GFLV-, anti-ArMV- (Bioreba) and anti-GCMV-serum (Station de Pathologie Végétale, INRA, Pont de la Maye, France) diluted 1:500 (GFLV, ArMV) and 1:50 (GCMV), respectively, in coating buffer (0.1 M sodium carbonate buffer pH 9.6) and incubated 4 h at room temperature (RT). Then 100 µl aliquots of infected sap, healthy plant extract and distilled water were applied to the precoated tubes and incubated at 4 °C overnight.

Reverse transcription: The tubes were washed 5 x with PBS-Tween buffer (phosphate buffered saline and Tween 20, pH 7.2 - 7.4) and 2 x with PBS, then loaded with 10 µl of a prewarmed 1 % Triton X-100 solution, vortexed and incubated for 10 min at 65 °C in order to denature proteins. To disrupt the secondary structure of the released RNA, samples were treated with 10 mM methyl mercuric hydroxide for 10 min at RT. Following its neutralization by addition of 20 mM ß-mercaptoethanol (10 min at RT), the reverse transcription step was performed in the same tubes loaded with a mixture containing 50 mM Tris-HCl buffer pH 8.3 at 42 °C, 50 mM KCl, 7.5 mM $MgCl_2$, 1 mM dNTPs, 1 µM 3´primer, 10 U RNase inhibitor (Boehringer, Mannheim) and 22 U of AMV reverse transcriptase (Boehringer) in a final volume of 20 µl. The reaction mixture was then held at 42 °C for 45 min.

Polymerase chain reaction: For PCR, 10 mM Tris-HCl buffer pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 0.25 µM of each primer and 2.5 U of Taq polymerase (Boehringer) were added to a final volume of 100 µl/tube. The reaction mixes were overlaid with mineral oil and subjected to 35 thermal cycles of denaturation at 92 °C for 1 min, primer annealing at 54 °C for 1 min and DNA synthesis at 72 °C for 1 min. IC/PCR amplification products were analysed by electrophoresis in 1% agarose gel and visualised by ethidium bromide staining (SAMBROOK *et al.* 1989).

Oligonucleotides: The primers used for reverse transcription and subsequent PCR were selected according to published RNA2 sequences of GFLV strain F13 (SERGHINI *et al.* 1990), ArMV (STEINKELLNER *et al.* 1990) and GCMV (BRAULT *et al.* 1989). Respective positions are indicated in parenthesis:

GFLV 5´bine: 5´ - dATGTGGAAGAGGACGGAAGT - 3´ (position 2862 - 2881)

GFLV 3´bine: 3´ - dCCTAGACTGGGAAACTGGTTC - 3´ (position 3543 - 3563)

ArMV 5´550: 5´ - dCATCCCACCACTGGAATATG - 3´ (position 550 - 569)

ArMV 3´1352: 5´ - dGCTCAGTTTCACACCCCATG - 3´ (position 1333 - 1352)

GCMV 5´3140: 5´ - dCATGGTCTAGCCACTAGGAG - 3´ (position 3140 - 3159)

GCMV 3´3831: 5´ - dGTAGTGGCACACATGATGGC - 3´ (position 3812 - 3831)

Universität für Bodenkultur, Institut für Angewandte Mikrobiologie, Nußdorfer Lande 11, A-1190 Wien, Austria

DAS-ELISA: The double antibody sandwich ELISA was performed according to CLARK and ADAMS (1977). Coating of microtiter plates and incubation of samples was carried out as described above (immunocapture). Alkaline phosphatase conjugated antibodies (GFLV, ArMV: Bioreba; GCMV: INRA, Pont de la Maye) diluted 1:500 in PBS-Tween buffer pH 7.4 were incubated for 5 h at RT. Staining was performed by using p-nitrophenyl phosphate as substrate.

Results: Following antigen capture, cDNA synthesis was performed using the primers GFLV 3´bine, ArMV 3´1352 and GCMV 3´3831 for reverse transcription. For subsequent PCR, they were combined with primers GFLV 5´bine, ArMV 5´550 and GCMV 5´3140, respectively. According to primers, amplification products of 701 nt (GFLV), 802 nt (ArMV) and 691 nt (GCMV) were expected. The reaction yielded three cDNA fragments that were consistent with the anticipated sizes (Fig. 1). In order to compare IC/PCR and DAS-ELISA as to their sensitivity, both methods were simultaneously used for detection of GFLV in plant sap extracted from woody material and stepwise diluted (1:20 - 1:20,480) in virus-free extract. Whereas the DAS-ELISA detection limit was between dilutions 1:320 and 1:1,280, viral RNA could be detected until dilution 1:5,120 (Fig. 2).

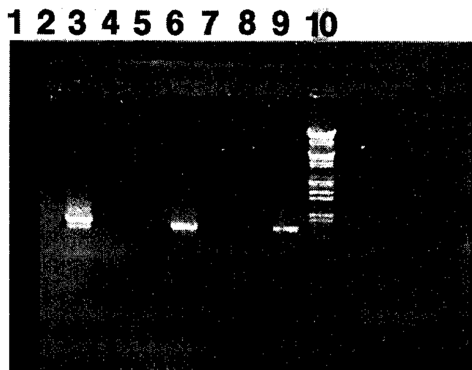

Fig.1: Detection of ArMV, GFLV and GCMV from ligneous grapevine material by using IC/PCR. Lanes 1, 4 and 7: distilled water (blank). Lanes 2, 5 and 8: healthy grapevine (negative control). Lane 3: ArMV cDNA (802 nt). Lane 6: GFLV cDNA (701 nt). Lane 9: GCMV cDNA (691 nt). Lane 10: molecular weight standard (Eco RI/Hind III cleaved lambda DNA; Boehringer Mannheim). Very weak side products probably result from not yet optimized annealing temperature.

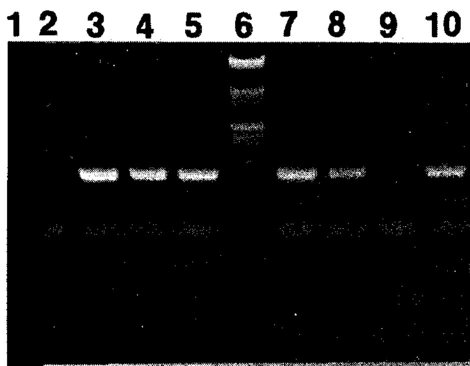

Fig. 2: Sensitivity of IC/PCR: The reaction was performed with samples of GFLV-infected plant sap of cultivar French Colombard diluted 1:20 (lane 3), 1:80 (lane 4), 1:320 (lane 5), 1:1,280 (lane 7), 1:5,120 (lane 8) and 1:20,480 (lane 9) in healthy extract. Except for dilution 1:20,480, PCR yielded 701 nt cDNA fragments. Lane 1: distilled water (blank), lane 2: healthy grapevine (negative control), lane 6: molecular weight standard (Eco RI/Hind III cleaved lambda DNA; Boehringer Mannheim), lane 10: cloned GFLV cDNA (positive control). Very weak small bands represent oligonucleotides remaining after PCR reaction.

Discussion: The results presented in this study are reproducible and demonstrate the utility of IC/PCR for detection of nepoviruses from naturally infected, dormant tissues, suggesting that this technique can be applied for all members of this group for which specific antibodies are available. Due to the fact that the specifity of an immunoaffinity capture step and the sensitivity of PCR are combined, IC/PCR meets the most important criteria required of a reliable diagnostic system. When compared to DAS-ELISA, the method proved to be at least 5 x more sensitive. Besides pathogen detection, IC/PCR can be used for characterization of viral genomes, since it renders a direct isolation of viral RNAs from naturally infected ligneous or herbaceous hosts possible (BRANDT *et al.* 1995) so that passage of the virus through an herbaceous host can be avoided. Since the entire procedure can be performed in one single tube, it is technically relatively simple and the risk of sample contamination is moderate.

We especially thank Dr. OLIVIER LE GALL of the Station de Pathologie Végétale, INRA, Pont de la Maye, France, for kindly providing GCMV antisera. This study was supported by the Forschungsförderungsfonds für die Gewerbliche Wirtschaft and the Federal State of Lower Austria.

BOVEY, R.; GÄRTEL, W.; HEWITT, W. B.; MARTELLI, G. P.; VUITTENEZ, A.; 1980: Virus transmis par des nématodes du sol. In: Maladies à Virus et Affectations Similaires de la Vigne, 10-14. Editions Payot, Lausanne.

BRANDT, S.; IBL, M.; HIMMLER, G.; 1995: Coat protein gene sequence of an Austrian isolate of Grapevine Fanleaf Virus. Arch. Virol. **140**, 157-164.

BRAULT, V.; HIBRAND, L.; CANDRESSE, T.; LE GALL, O.; DUNEZ, J.; 1989: Nucleotide sequence and genetic organization of Hungarian grapevine chrome mosaic nepovirus RNA2. Nucleic Acid. Res. **17**, 7809-7819.

CLARK, M. F.; ADAMS, A. N.; 1977: Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol. **34**, 475-483.

GUGERLI, P.; 1984: Une méthode simple pour le broyage de tissu végétal. Rev. Suisse Viticult. Arboricult. Horticult. **16**, 87-88.

JANSEN, R. W.; SIEGL, G.; LEMON, S. M.; 1990: Molecular Epidemiology of human hepatitis A virus defined by an antigene-capture polymerase chain reaction method. Proc. Natl. Acad. Sci. USA **87**, 2867-2871.

MATTHEWS, R. E. F.; 1991: Plant Virology. Academic Press Inc., San Diego, California.

SAMBROOK, J.; FRITSCH, E. F.; MANIATIS, T.; 1989: Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

SERGHINI, M. A.; FUCHS, M.; PINCK, M.; REINBOLT, J.; WALTER, B.; PINCK, L.; 1990: RNA2 of grapevine fanleaf virus: Sequence analyses and coat protein cistron location. J. Gen. Virol. **71**, 1433- 1441.

STEINKELLNER, H.; HIMMLER, G.; MATTANOVICH, D.; KATINGER, H.; 1990: Nucleotide sequence of AMV capsid protein gene. Nucleic Acids Res. **18**, 7182.

WETZEL, T.; CANDRESSE, T.; MACQUAIRE, G.; RAVELONANDRO, M.; DUNEZ, J.; 1992: A highly sensitive immunocapture polymerase chain reaction method for plum pox potyvirus detection. J. Virol. Meth. **39**, 27-37.