Serological Detection of Grapevine Virus A Using Antiserum to a Nonstructural Protein, the Putative Movement Protein

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ABSTRACT

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Grapevine virus A (GVA) is implicated in the etiology of the rugose wood disease. The coat protein (CP) and the putative movement protein (MP) genes of GVA were cloned and expressed in *Escherichia coli* and used to produce antisera. Both the CP and the MP were detected with

their corresponding antisera in GVA-infected *Nicotiana benthamiana*. The MP was first detected at an early stage of the infection, 6 to 12 h after inoculation, and the CP was detected 2 to 3 days after inoculation. The CP and MP were detected by immunoblot analysis in rugose wood-affected grapevines. The MP could be detected in GVA-infected grapevines that tested negative for CP, both with CP antiserum and with a commercially available enzyme-linked immunosorbent assay kit. The study shows that detection of the nonstructural MP may be an effective means for serological detection of GVA infection in grapevines.

Rugose wood is a complex disease of grapevines, characterized by modification of the woody cylinder of affected vines (17,21). There are four different disorders that show similar symptoms in the field, but that can be distinguished by indexing on specific *Vitis* indicators (14,35). The woody cylinder is typically marked by pits or grooves that may occur on the scion, rootstock, or both. Affected vines have impaired growth vigor and delayed bud opening, and they may decline and die within a few years (17,21). The control of rugose wood is based on the production of healthy propagation material (21). Detection of rugose wood in grapevines is difficult and expensive; budwood from tested plants is grafted onto sensitive *Vitis* indicators, and the appearance of symptoms is monitored for 3 years (21). Much effort has been invested in developing serological and nucleic acid-based methods for the detection of viruses implicated in the rugose wood complex (2-4,6,24,26).

The etiology of rugose wood is complex and has not yet been elucidated. Several elongated clostero-like viruses are consistently found in affected vines (5,7,29,30); one of them, grapevine virus A (GVA), is closely associated with Kober stem grooving, a component of the rugose wood complex (6,15). GVA has been tentatively allocated to a newly established genus of plant viruses, *Trichovirus* (22). Initially, serological detection of GVA was difficult, because of the low immunogenicity of the virus and the low concentration of particles in affected plants, but, recently, the method has been improved by the introduction of monoclonal antibodies (3). Polymerase chain reaction (PCR)-based detection protocols have been developed that have resulted in the more sensitive detection of GVA (24,25). Recently, Chevalier et al. (6) demonstrated a highly sensitive detection of GVA using an immunocapture-PCR protocol.

GVA has a single-stranded RNA genome whose complete nucleotide sequence has recently been determined (27,28). Based on this sequence information, we have cloned and expressed the coat protein (CP) and the putative movement protein (MP) of GVA in

Escherichia coli. Using antisera produced in response to these proteins, we investigated the expression of both proteins in GVA-infected plants. We found that the nonstructural movement protein may be a preferred antigen for serological detection of GVA in vines.

MATERIALS AND METHODS

Double-stranded RNA (dsRNA) isolation, cDNA synthesis, and DNA amplification, cloning, and sequence analysis. dsRNA was isolated by CF11 chromatography, followed by CC41 chromatography (11) from *Nicotiana benthamiana* plants infected by the GVA isolate PA3, kindly provided by A. Minafra and G. P. Martelli. cDNA was synthesized on a dsRNA template by the method of Wexler et al. (39).

Primers for cDNA synthesis and PCR were designed according to the GVA sequence (27). For cloning of the CP gene, two oligonucleotides were synthesized. Oligonucleotide I, used for cDNA synthesis, was 5'-GGGGGATCCATCTATATCTCAACAGCCTGC-3' (the underlined sequence represents introduced restriction enzymes sites). Oligonucleotide II was 5'-GGTTTGAAGACATATGGCAGACTACG-3'. Both oligonucleotides were used for PCR. cDNA corresponding to the MP gene was synthesized by means of oligonucleotide III, 5'-TCCTCAGTTGGCGTGGCTTTC-3', which was synthesized according to the overlapping sequence of the MP gene obtained from the CP cDNA clone. Oligonucleotide IV, which was used for second-strand synthesis and PCR amplification of the MP, was 5'-GGTGATTACACATATGTCGCAAGAAG-3'.

PCR was as described previously (39) using a UNO Thermoblock thermocycler (Biometra, Göttingen, Germany). Cycling conditions were one cycle of 94°C for 3 min, 65°C for 1 min, and 72°C for 2 min; followed by 35 cycles of 94°C for 1 min, 65°C for 1 min, and 72°C for 2 min. The amplification products were isolated after agarose gel electrophoresis and cloned in pCRII with the AT cloning system kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

DNA sequencing was performed with the Sequenase kit version 2 (United States Biochemical Corp., Cleveland) according to the manufacturer's instructions. Both strands of a single-PCR-generated clone were sequenced for each gene. Nucleotide sequences were

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compared with the published GVA sequence (27) using the UWGCG program (10).

Production of proteins in *E. coli* and generation of antisera. DNA fragments containing the CP or MP genes were subcloned in the T7 RNA polymerase expression vector pET3a (36) using the *Nde*I and *Bam*HI restriction sites. Protein production was induced in *E. coli* BL21(DE3)pLysE by the addition of 1 mM isopropyl thio- β -D-galactoside at 37°C for 4 h, as described by Studier et al. (36). Bacterial proteins were separated on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (20), and the protein bands corresponding to the viral CP or MP were excised from the gel, emulsified in complete Freund's adjuvant, and injected (1.5 to 2.0 mg) into rabbits. After two additional injections (in incomplete Freund's adjuvant) at 1-month intervals, antisera were collected and tested with Western blots of GVA-infected *N. benthamiana* plants.

Protein extraction, Western blotting, and enzyme-linked immunosorbent assay (ELISA). Total proteins from *N. benthamiana* and grapevine were extracted by pulverizing 1 g of tissue in liquid nitrogen and grinding in 5 ml of ESB buffer (9 M urea, 4.5% SDS, 7.5% β -mercaptoethanol, and 75 mM Tris-HCl, pH 6.8) (13). Extracts were boiled for 10 min, and insoluble material was discarded following 10 min of centrifugation at 10,000 $\times$ g and analyzed by Western immunoblotting using alkaline phosphatase-conjugated goat anti-rabbit antibodies (Sigma, Rehovot, Israel) (19). Leaf extracts were fractionated according to Deom et al. (9), except that the 2% Triton X-100 washes were pooled to give the Triton wash (Tw) fraction as described by Cooper and Dodds (8). Alternatively, subcellular extracts were prepared as described by Sato et al. (34). A 10- μ l sample of each protein fraction was analyzed by Western blotting (19). The Western blot membranes were scanned and analyzed with the B.I.S. 202D bio-imaging system (Dinco & Rhenium, Jerusalem, Israel), and the relative amount of MP in each fraction was determined as optical density units derived from 1 g of leaf tissue.

Fig. 1. Comparison of the amino acid sequences of **A**, the coat protein; and **B**, the movement protein of grapevine virus A (GVA) PA3 (upper sequence) and GVA Is 151 (26) (lower sequence). Alignment was generated using the UWGCG Gap program (10). Dashes indicate identical amino acids; dots indicate gaps introduced for optimal alignment.

ELISA detection of GVA was performed with a commercial kit (Bioreba, Renach, Switzerland). Plant tissue used for the ELISA test was extracted, as recommended by the manufacturer, in 0.14 M NaCl, 2% polyvinyl pyrrolidone-24000, 1% polyethylene glycol 6000, 0.02% NaN₃, 0.05% Tween 20, and 0.5 M Tris-HCl, pH 8.2.

RESULTS

Molecular cloning, sequence determination, and production of antisera to GVA CP and MP. GVA CP and MP were cloned by reverse transcriptase-PCR using oligonucleotide primers based on the sequence information published by Minafra et al. (27). The nucleotide sequences are accessible as accession number AF007415 at the GenBank data base. The deduced amino acid sequences are presented in Figure 1. Comparison with the Is 151 isolate of GVA (27) revealed 88 and 78% identity of the CP and MP nucleotide sequences, respectively. The amino acid sequences of the CPs of the two GVA isolates had 97% identity and 99% similarity of amino acid sequences, while the MPs shared only 80% identity and 92% similarity.

The expression of the CP and MP genes in *E. coli* resulted in the production of proteins of apparent molecular masses of approximately 26 and 35 kDa, respectively (data not shown), which are larger than expected from consideration of the nucleotide sequences (21.6 and 31 kDa for the CP and MP, respectively). Similar differences between molecular mass estimates derived from SDS-PAGE and sequence analyses are common (23,31).

Fig. 2. Time course of accumulation of grapevine virus A (GVA) proteins in the inoculated leaf of *Nicotiana benthamiana* plants. **A**, Immunoblot analysis using the coat protein antiserum. **B**, Immunoblot analysis using the movement protein antiserum. Leaf tissue was extracted before inoculation (lane 1) and 12 h (lane 2), 1 day (lane 3), 2 days (lane 4), 3 days (lane 5), 4 days (lane 6), 5 days (lane 7), 7 days (lane 8), and 11 days (lane 9) after inoculation. Numbers on the left indicate the positions of molecular mass markers. The movement protein was detected on the Western blot membrane as a faint band at 12 h (lane 2), which cannot be discerned in the photograph (**B**).

Antisera induced in response to the two *E. coli*-produced proteins reacted specifically with GVA CP and the MP in infected *N. benthamiana* plants as shown by Western blot analysis (Fig. 2).

Kinetics of accumulation and subcellular distribution of GVA proteins in infected plants. To monitor the accumulation of GVA in infected tissue, inoculated leaves of *N. benthamiana* were collected at different times after inoculation, and total proteins were

Fig. 3. Detection of movement protein in subcellular fractions prepared from grapevine virus A (GVA)-infected *Nicotiana benthamiana*. Proteins were extracted from inoculated leaves 11 days after inoculation and subjected to immunoblot analysis. Histogram shows the relative accumulation of movement protein in subcellular fractions (average of two separate extractions). CW, cell wall fraction; P30, 30,000 $\times$ g pellet fraction; S30, 30,000 $\times$ g supernatant; and Tw, Triton X-100 wash.

Fig. 4. Detection of grapevine virus A (GVA) in infected grapevines. Proteins were extracted from phloem scrapings of lanes 1 to 8, grapevines (cv. Superior) showing stem-pitting symptoms, collected in the Jordan Valley; lane 9, healthy vine used as control; and lane M, molecular mass markers. **A**, Immunoblot analysis using the coat protein antiserum. **B**, Immunoblot analysis using the movement protein antiserum.

extracted and analyzed by immunoblotting (Fig. 2). The MP was first detected 0.5 day after inoculation, whereas the CP was first detected 3 days after inoculation. The specific time after inoculation at which the GVA proteins were detected varied between experiments. In several experiments, the MP was detected 6 to 12 h after inoculation, while the CP was detected 1 to 3 days after inoculation.

To examine the subcellular distribution of the GVA MP, inoculated leaves were fractionated 11 days after inoculation into cell wall (CW), soluble proteins (S30), membrane- or organelle-bound proteins (P30), and Tw fractions. The GVA MP was detected in all fractions, but was consistently most abundant (per gram of fresh weight) in the S30 fraction (Fig. 3). Using an alternative method previously employed by Sato et al. (34) to study the subcellular distribution of apple chlorotic leaf spot trichovirus, protein extracts were fractionated to cell membrane, soluble, and cell wall fractions. Again, most of the GVA MP was detected in the soluble protein fraction (data not shown).

Detection of GVA CP and MP in rugose wood-affected grapevines. Phloem shavings of rugose wood-affected grapevines (cv. Superior) from the Jordan Valley were extracted with ESB buffer and subjected to immunoblot analysis. Both the MP and CP were detected in extracts of plants that showed symptoms (Fig. 4). In

Fig. 5. Detection of grapevine virus A (GVA) in infected grapevines. Lane 1, proteins extracted from GVA-infected *Nicotiana benthamiana* plants; and lane 2, proteins extracted from healthy *N. benthamiana* plants (used as positive and negative controls, respectively). Lanes 3 to 7, proteins were extracted from phloem scrapings of grapevines (cv. Thompson Seedless) showing corky rugose wood symptoms, collected in the Lachish region; and lane M, molecular mass markers. **A**, Immunoblot analysis using the coat protein antiserum. **B**, Immunoblot analysis using the movement protein antiserum.

each case in which GVA could be detected, the immunoreaction with the MP antiserum appeared as a stronger band relative to the detection signal obtained with the CP antiserum (Fig. 4). GVA was not detected in three out of the eight symptomatic vines tested (Fig. 4). This may have been because of uneven distribution of GVA in the vine, low concentration of the virus, or because the plants were not infected by GVA. In a similar test of corky rugose wood-affected vines (cv. Thompson Seedless) collected in the Lachish region, the MP could be detected in all five vines tested, while the CP could not be detected in the same extracts (Fig. 5). The same plants were also tested with a commercial ELISA kit for the detection of GVA (based on anti-GVA CP antiserum), and all five plants tested negative.

Figure 6 shows that phloem rich tissues—phloem shavings, petioles, and older leaf veins—were the best source of material for testing for the presence of GVA MP in grapevines. The MP was not detected in young leaves or in developing fruits.

To test whether detection of GVA by means of the MP antiserum is virus-specific, proteins were extracted from a grapevine (cv. Maressane) that had been tested by PCR and found positive for grapevine virus B (GVB) and negative for GVA (data not shown). These proteins were tested with GVA MP antiserum, which did not react with GVB (Fig. 7).

DISCUSSION

The single-stranded RNA genome of GVA encodes five open reading frames (ORFs) (27,28). ORF 4 was experimentally shown to code for the CP (27). ORF 3 protein was hypothesized to be the MP, because of its amino acid similarity to other viral MPs of the 30-kDa family (27). The MP was detected at an early stage of infection in the inoculated leaves of *N. benthamiana* plants. Early expression of MPs was reported for other viruses such as cucumber mosaic virus (CMV) (38), alfalfa mosaic virus (1), and tobacco necrosis virus (12). In the current study, the GVA MP was detected using two alternative cell-fractionation protocols in all fractions, but mainly in the soluble protein fraction (S30), whereas the MPs of several other plant viruses have been detected primarily in the cell wall fraction (9,16,32,34). Recently, it was demonstrated by means of the same extraction methods used in the current work that the distribution of CMV and tobacco mosaic virus MP in infected plants is complex: the distribution was found to differ among viruses, among leaf positions, and also between transgenic plants and virus-infected plants (8). More detailed study of GVA MP localization during different stages of infection is needed to determine whether GVA MP has a distinct subcellular distribution compared with other viral MPs.

Fig. 6. Immunoblot of total proteins and detection using antiserum to grapevine virus A (GVA) movement protein. Lane 1, petioles of greenhouse-grown, healthy vine used as a control. Lanes 2 to 7, extracts from field-grown, rugose wood-affected grapevine (cv. Superior): lane 2, phloem scrapings; lane 3, petioles; lane 4, veins of mature leaves; lane 5, young leaves; lane 6, cluster pedicels; and lane 7, developing fruits. Lane 8, petioles from greenhouse-grown vine used as a positive control. Lane M, molecular mass markers.

Comparison of the nucleotide sequence and deduced amino acid sequence of the PA3 isolate of GVA with those of the Is 151 isolate revealed a greater similarity between the CP genes than between the MP genes. The MP gene was also found to be more divergent in sequence than the CP in isolates of another *Trichovirus*, apple chlorotic leaf spot virus (33).

Immunoblot analysis showed that the detection of MP may provide a sensitive means of detection of GVA in infected grapevines. The MP antiserum proved to be superior both to the CP antiserum and to a commercially available ELISA kit based on anti-GVA antiserum. To our knowledge, this is the first demonstration that a nonstructural protein may be the preferred target for serological detection of a plant virus. While the CP of elongated plant viruses is present in several thousand molecules per genome, the nonstructural proteins are thought to be produced in much smaller quantities. There may be several reasons for the more efficient detection of the MP than of the CP: the GVA MP may be a more stable protein that accumulates in infected cells, while the CP concentration decreases as the virus titer decreases. Alternatively, the MP may be more immunogenic, resulting in better antiserum production in the vaccinated animal.

Serological relations between GVA and GVB were recently demonstrated using antisera to GVA and GVB in Western blots (18). Cross-reactivity was not detected by means of immunosorbent electron microscopy or ELISA (5,18). The finding that GVA MP antiserum did not detect GVB in infected vine indicated the additional advantage of using the MP as an antigen for detection of GVA infection.

Serological detection of GVA is difficult, because of low virus accumulation, uneven distribution of the virus in the host tissue, and poor immunogenicity of the virus (3). The production of monoclonal antibodies to GVA and the construction of an ELISA method based on protein A-sensitized plates provided more efficient serological detection of GVA (3). Recently, developed PCR and immunocapture-PCR detection methods for GVA ensure much more sensitive detection of GVA in infected vines (6,24,25). Although less sensitive than PCR, serological methods such as ELISA or dot-blot assays are more suitable for routine, large-scale indexing of plant viruses (37). Preliminary experiments indicate that a simple dot-blot procedure can be used for the detection of GVA MP in multiple samples of crude extracts (R. Gafny, *unpublished data*).

Fig. 7. Immunoblotting of proteins extracted from phloem scrapings of lane 1, grapevine virus A (GVA)-infected grapevine (cv. Superior); and lane 2, grapevine virus B (GVB)-infected grapevine (cv. Maressane). Detection was performed with antiserum to GVA movement protein. Numbers on the left indicate the positions of molecular mass markers.

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