Serological detection of Grapevine rupestris stem pitting-associated virus (GRSPaV) by a polyclonal antiserum to recombinant virus coat protein

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Summary

The coat protein gene of Grapevine rupestris stem pitting-associated virus (GRSPaV) was amplified with primers based on the completely sequenced Californian GRSPaV isolate. The protein expressed in *Escherichia coli* was used to raise an antiserum in rabbit. This antiserum was successfully used to detect virus coat protein in infected grapevine extracts either spotted on polyvinyl difluoride membranes (dot immunobinding) or blotted on membranes after gel separation (Western blot). The antiserum titre was 1:5,000 in Western blot. GRSPaV was detected in leaf petioles and cortical scrapings from dormant canes during the whole vegetative season. Several accessions of *Vitis rupestris*, currently used as presumptive virus-free indicators of Rupestris stem pitting, were found to be infected by this virus. While the application of the antiserum in ELISA was ineffective, the availability of similarly simple and effective serological tools, such as dot immunobinding, may allow a wide survey for GRSPaV.

K e y w o r d s : Grapevine rupestris stem pitting-associated virus, recombinant coat protein, diagnosis, serology, Western blot.

Introduction

Rupestris stem pitting (RSP), a disease of the rugose wood complex with a worldwide distribution (Goheen 1988; Martelli 1993), is thought to be caused by a member of the recently established genus *Foveavirus* (Martelli and Jelkmann 1998), for which the name Grapevine rupestris stem pitting-associated virus (GRSPaV) has been proposed (Martelli and Jelkmann 1998; Zhang et al. 1998). This virus is also known as Rupestris stem pitting-associated virus 1 (Meng et al. 1998).

In infected *Vitis* species, GRSPaV occurs as a series of heterogeneous single-stranded RNA molecules, several of which have been sequenced in part or totally (Meng et al. 1998, 1999 a; Zhang et al. 1998). The availability of sequence data prompted the development of molecular tools (*i.e.* probes and PCR primers) (Meng et al. 1999 b; Petrovic et al. 2000; Rowhani et al. 2000; Soares et al. 2000) which have greatly expedited virus detection and identification. Up to a recent past, identification of RSP relied upon indexing on *Vitis rupestris*, which may require a couple of years or more for symptoms to develop (Goheen 1988; Martelli 1993).

GRSPaV is not mechanically transmissible and all attempts to recover particles from infected grapevine tissues have failed. Thus, GRSPaV virions have never been observed, and no antisera to the virus have been raised. This has impaired the use of serology for GRSPaV identification, a technique more readily available than nucleic acid-based detection methods to many laboratories.

Antisera to recombinant coat and movement proteins of Grapevine leafroll-associated virus 2 (Zhu et al. 1997) and Grapevine virus A (Rubinson et al. 1997), respectively, have recently been raised and successfully used for virus detection in infected grapevines. These works encouraged to synthesize a recombinant coat protein (CP) of a European GRSPaV isolate to be used as antigen source for antiserum production.

While this paper was being written, Meng et al. (2000) reported the production of an antiserum to recombinant CP from an American strain of RSPaV-1.

Material and Methods

V i r u s s o u r c e s f o r r e c o m b i n a n t p r o t e i n s y n t h e s i s a n d s e r o l o g i c a l t e s t s : A group of 12 RSP-affected *Vitis vinifera* accessions, from the collection of the University of Bari (Italy) was tested for the presence of GRSPaV by PCR amplification as described by Minafra et al. (1997) (Table). A highly reactive Portuguese wine grape accession (clone P2), was selected for CP gene amplification. Furthermore, in autumn 1999 a number of *V. rupestris* plants growing wild

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Table

Vitis vinifera accessions tested molecularly (RT-PCR) and serologically (Western blot)

Grapevine accession	Geographic origin	PCR primers (Replicase)[1]	(CP gene)[2]	Western blot
SV3	Italy	+	+	+
P1	Portugal	+	+	+
P2	Portugal	+	+	+
PA3	Italy	+	-	+
IL1	Israel	+	-	+
MP 5/3	Italy	+	-	+
M6	Italy	+	-	+
H36	Hungary	+	-	+
H9	Hungary	+	-	+
CY3	Cyprus	+	-	+
JOR1	Jordan	+	-	+
F6	France	+	-	+

[1] MINAFRA *et al.* (1997)
[2] This paper

along stone walls bordering plots that had hosted uprooted vineyards, were identified in the surrounding areas of Bari. These vines were relics of the rootstocks used for establishing vineyards at the beginning of the last century. Dormant canes were collected and tested by PCR and Western blot as described later.

S y n t h e s i s   o f   r e c o m b i n a n t   p r o t e i n   a n d   a n t i s e r u m   p r o d u c t i o n : The GRSPaV CP gene was amplified by a primer set (RSP-CP up: 5'-AAT TGG ATC CAT GGC AAA TTGG-3'; RSP-CP down: 5'-TTC AAA GCT TTC ATT CAT GTG TAAC) designed on the region immediately flanking ORF 5 in the sequence by ZHANG *et al.* (1998) (GenBank accession number AF026278). The amplified DNA fragment was directionally ligated in the pQE 30 expression vector (Qiagen, Germany), bearing a 6xHis residue linked to the N-terminus. The plasmid was transformed in *Escherichia coli* strain M15 and bacteria were grown on a medium containing 100 mg·l^{-1} ampicillin and 25 mg·l^{-1} kanamycin. Clones were screened by PCR, restriction mapping of extracted plasmids, and rapid small-scale cultures for protein expression upon induction. A fresh liquid culture (150 ml LB supplemented with the same antibiotics as above and 2 % glucose) of a selected clone denoted #8, was kept at 30 °C for 4 h, then induced by 1mM isopropylthiogalactopyranoside, incubated for additional 4-5 h and concentrated in a Beckman J2-21 centrifuge at 6,000 rpm for 5 min. Bacterial pellets were resuspended in sonication buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, pH 7.3), frozen in liquid nitrogen and briefly sonicated after thawing. Since preliminary tests had shown that the recombinant protein was almost insoluble, the sonicated protein suspension was centrifuged at 11,000 rpm for 15 min, the pellet was extracted with 1.5 % Na lauryl sarcosyne and 25 mM triethanolamine (GRIECO *et al.* 1992) and again centrifuged. The supernatant was collected

and gently stirred for 1 h at room temperature after addition of Ni-NTA resin (Qiagen), for allowing histidine tag binding. The resin was poured into a syringe, thoroughly washed with 0.1 M Na phosphate, 0.01 M Tris, pH 6.3 and the bound protein was eluted in a small volume of the same buffer at pH 4.5. Eluted protein was dialyzed, lyophilized and checked by SDS-PAGE.

A New Zealand rabbit was immunized with a subcutaneous injection of 500 mg recombinant protein in incomplete Freund's adjuvant followed by two additional injections at three-week intervals, each with 200 mg protein. Five bleeds were done at weekly intervals after the last injection. Titre and specificity of the antiserum were evaluated by Western blot.

E L I S A : DAS- and indirect-ELISA were essentially according to CLARK and ADAMS (1977). For DAS-ELISA, purified IgGs from the antiserum raised were directly conjugated by glutaraldehyde with alkaline phosphatase (Sigma Chemical Co., USA) and used as secondary antibodies. In indirect ELISA, grapevine sap, extracted in carbonate coating buffer (15 mM Na$_2$CO$_3$, 35 mM NaHCO$_3$ pH 9.6), was directly incubated in ELISA plates (Falcon, UK) and coated by primary antiserum (1:1500) and antirabbit IgG-AP conjugate (Sigma, USA) (1:5000), respectively. All sap incubations were kept overnight at 4 °C.

W e s t e r n   b l o t : 100 mg of tissue from mature leaf petioles or cortical scrapings from dormant canes were ground in 10 vol of extraction buffer (0.5 M Tris-HCl pH 8.8, 2 % SDS, 4 % 2-mercaptoethanol, 40 % sucrose). The extract was boiled for 10 min at 100 °C and centrifuged in a microcentrifuge at 14,000 rpm for 3 min. Aliquots of 30 ml of supernatant were loaded on 12 % SDS-PAGE slabs (Biometra, Germany). Gels were electroblotted on polyvinyl difluoride membranes (PVDF, Immobilon-P, Millipore) and directly incubated overnight at 4 °C with a 1:1500 dilution of the antiserum in blocking solution (1 % BSA, 5 % non-fat dry milk in TBS buffer, 0.05 % Tween-20). After 3 washes of 10 min each in TBS 0.3 % Tween-20, the membranes were incubated for 1 h at room temperature with a 1:2500 dilution in blocking solution of an antirabbit IgG-AP conjugate (Roche Diagnostics, Germany). Three sequential washing steps of 15 min each were followed by staining in nitroblue tetrazolium chloride-bromo chloro indolyl phosphate solution in AP buffer (Roche Diagnostics, Germany).

D o t   i m m u n o b i n d i n g : 100 mg of grapevine tissues (leaf petioles or cortical scrapings) were ground in 10 vol of a Tris buffer (0.3 M Tris-HCl, pH 8.2, 0.8 % NaCl, 2 % polyvinylpyrrolidone-40K, 1 % polyethylenglycol-6000, 0.05 % Tween-20) (POGGI-POLLINI *et al.* 1993). After a short centrifugation (8,000 rpm for 3 min) 30 ml of clarified sap were spotted on a prewetted PVDF membrane using a vacuum-blot apparatus (BioRad, USA). The membrane was let to dry for 10 min, rehydrated and incubated in blocking solution for 1 h at room temperature or overnight at 4 °C. The antiserum, diluted 1:1500 in blocking solution, was cross-absorbed overnight at 4 °C with sap of GRSPaV-free LN33 according to KNAPP *et al.* (1995). Membranes were incubated with primary antibod-

ies for 30 min at room temperature and thoroughly washed in TBS containing 0.5 % Tween-20. After a second incubation in a 1:2500 dilution of antirabbit IgG-AP conjugate in blocking solution for 30 min at room temperature, the membranes were washed and incubated for 5 min with CDP-Star solution (Roche Diagnostics, Germany) before a 30 min exposure to X-ray Fuji film.

Results

Purification of recombinant coat protein: Three M15 clones (Cl5, Cl8, and Cl18) showed correct insertion of the 780 bp GRSPaV-CP gene in pQE30. Small-scale bacterial cultures gave an overexpression of the expected 28 kDa recombinant protein (Fig. 1). Protein overexpression did not decrease by growing bacteria at 30 °C in a glucose-supplemented medium. The addition of a non-viral tag, such as the short 6xHis tail, to the recombinant protein in the expression system used did not avoid its insolubilization in bacterial cells. When bacteria were allowed to grow to large volumes, most of the expressed protein was associated with sonicated bacterial pellets, which required solubilization by washing with lauryl sarcosyne and triethanolamine. After elution, about 6.5 mg of purified protein were obtained per ml of culture.
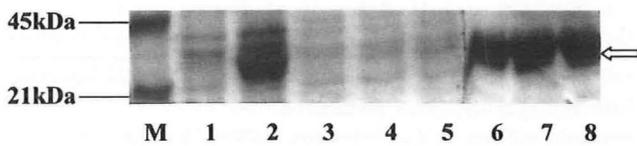

Fig. 1: SDS-PAGE of total protein extracts or Ni-NTA-eluted GRSPaV recombinant CP (arrow) from small scale culture in *E. coli*; M: molecular markers; lane **1**: induced pQE30 without insert; lane **2**: induced clone 8; lanes **3** to **5**: resin-bound proteins from clones 8, 5, 18, respectively; lanes **6** to **8**: resin-eluted recombinant protein from clones 8, 5, 18, respectively. Coomassie blue staining.

Although antisera from all bleedings reacted equally well in Western blot with extracts from clone P2 tissues, the antiserum from the fifth bleeding was routinely used in all tests. This antiserum had an estimated titre of 1: 5,000, as estimated by Western blot assays.

E L I S A : Detection of GRSPaV antigen by DAS- or indirect-ELISA using the antiserum to the recombinant protein was ineffective in repeated trials, regardless of the type of samples (*V. rupestris* and *V. vinifera* accessions) and tissues (leaf petioles, cortical scrapings) analyzed. Possibly, the antigenic structure of the bacterial-expressed viral CP was modified, thus the resulting antiserum was little efficient in recognizing native viral CP from tissue samples.

W e s t e r n b l o t : A band of ca. 28 kDa recognized by the antiserum to recombinant GRSPaV CP was consistently detected in all preparations from RSP-infected vines, regardless of whether the extracts were from leaf petioles or dormant canes (Fig. 2, lanes 1, 5, and 6). No

comparable bands were seen in grapevine sources, *i.e.* *V. vinifera* seedlings, healthy LN33 and vines regenerated from somatic embryos supposed to be RSP-free (Fig. 2, lanes 2, 3 and 4). The virus-specific band was still detectable when extracts from dormant canes were diluted up to the equivalent of 2.5 mg of tissue (not shown). The intensity of the signal was generally higher for extracts from canes than from petioles. Viral protein concentration apparently did not change appreciably in fresh tissues from early June to October, as judged from the intensity of the signal given by extracts obtained from the same amount of tissue (not shown).

D o t i m m u n o b i n d i n g : For these tests, cross absorption of the antiserum with healthy grapevine sap was necessary to reduce the background to an acceptable level. GRSPaV was detected in crude grapevine extracts clarified only by a short centrifugation (Fig. 3). It was confirmed that leaf petioles have a lower antigen concentration than cortical tissues, as there was virtually no reaction when petiole extracts were diluted 1:5 (Fig. 3, lane 2) while extracts from dormant canes were still reactive at a dilution of 1:20 (Fig. 3, lanes 3 and 4).
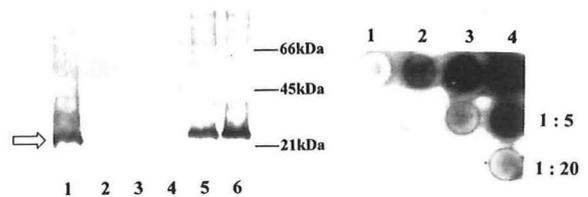

Fig. 2 (left): Western blot analysis for the detection of GRSPaV (arrow) in grapevine tissue extracts. Lane **1**: RSP-infected *V. vinifera* cv. Perricone; lane **2**: *V. vinifera* seedling (petioles); lane **3**: LN 33 (cortical scrapings); lane **4**: *V. vinifera* regenerated from somatic embryos (petiole); lane **5**: *V. vinifera* P2 (petioles); lane **6**: *V. vinifera* P2 (cortical scrapings). All extracts were 1:10 w/v in extraction buffer. GRSPaV was not detected in grapevine seedlings (lane 2), plantlets regenerated from somatic embryos (lane 4) and healthy control (lane 3).

Fig. 3 (right): Dot-immunobinding assay with chemiluminescent GRSPaV detection. Lane **1**: GRSPaV-free LN 33 (cortical scrapings); lane **2**: RSP-infected *V. vinifera* cv. Rossese (petioles); lane **3**: *V. vinifera* cv. Perricone (cortical scrapings); lane **4**: *V. vinifera* P2 (cortical scrapings). The highest virus concentration was in cortical scrapings from mature canes.

P C R d e t e c t i o n o f G R S P a V i n *V. r u p e s t r i s* : GRSPaV was detected by PCR in the selection of *V. rupestris* St. George currently used at Bari as an indicator, and in 16 of 20 *V. rupestris* vines from old vineyards. Western blots confirmed PCR results (Fig. 4). Comparable results were obtained with experiments carried out at Davis, California on *V. rupestris* St. George (A. ROWHANI and D. GOLINO, unpubl. results).

Discussion

The expression of GRSPaV CP gene in bacterial cells allowed the production of an antiserum that specifically recognized homologous antigen in infected grapevine ex-
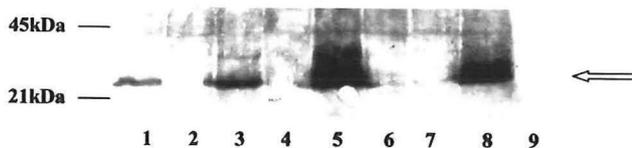
Fig. 4: Western blot analysis for detection of GRSPaV (arrow) in cortical scrapings from different *V. rupestris* accessions. Lane **1**: *V. rupestris* from Davis, California, used as RSP indicator; lanes **2-8**: *V. rupestris* accessions from Apulia (Southern Italy), three of which (lanes 3, 5 and 8) were infected; lane **9**: GRSPaV-free LN 33 (control).

tracts. Reproducible and sensitive responses were obtained using this antiserum in Western blot and dot-immunobinding, a test as simple and fast as ELISA, a technique with which our antiserum has given unreliable results. Dot-immunobinding, however, has the drawback of a high background which, if testing conditions are not carefully set, can obscure the reactions.

The concentration of GRSPaV antigen in infected fresh tissues was found to be constant in all tissues sampled from June through October. This tallies with the findings of Rowhani *et al.* (2000) based on molecular virus detection, and differs slightly from those of Meng *et al.* (2000), who observed an earlier decrease of virus antigen concentration in leaf tissues. Whether this may be due to the different environmental conditions of New York State and California/Southern Italy, remains to be established. In any case, the reproducible results obtained by our Western blot analysis of extracts from dormant canes, extends the period for serological detection of GRSPaV beyond the vegetative season. Interestingly, the consistent detection in Western blots from a range of grapevine samples of a single protein band with an apparently identical size, stands in favour of a basic homogeneity of CPs of different virus isolates, notwithstanding the molecular variability detected in GRSPaV populations (Meng *et al.* 1999 b; Rowhani *et al.* 2000; Soares *et al.* 2000).

Whether GRSPaV infection of the *V. rupestris* selection used as indicator in our indexing trials may have had a bearing in the outcome of the tests for RSP detection by inducing false negatives consequent to cross-protection, remains to be seen. The availability of an antiserum may help addressing this and other obscure aspects of the disease such as the ultimate definition of RSP aetiology, failure in finding virus particles, and extent of serological variability, if any, among molecular GRSPaV variants.

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