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Grapevine rupestris stem pitting-associated virus is linked with grapevine vein necrosis

H. Bouyahia, D. Boscia, V. Savino, P. La Notte, C. Pirolo, M. A. Castellano, A. Minafra and G. P. Martelli

Dipartimento di Protezione delle Piante e Microbiologia Applicata, Università degli Studi and Istituto di Virologia Vegetale del CNR, Sezione di Bari, Bari, Italy

Summary

Vein necrosis (VN), a virus-like disease latent in all European grapevine cultivars and in most American rootstock species and hybrids, induces necrosis of the veinlets of its specific indicator *Vitis rupestris* x *Vitis berlandieri* 110 R at the abaxial side of the leaf blade. VN is very common in southern Italy, *e.g.* 109 out of 218 of the putative grapevine clones selected during sanitary improvement programmes in the last few years indexed positive in 110 R. As assessed by ELISA, the same vines had a very low rate of infection (< 4 %) by major detrimental viruses (GFLV, GVA, GVB, GLRaV-1, GLRaV-2, GLRaV-3, GLRaV-7, GFkV) commonly looked for during selection. When the VN-positive 110 R indicators were checked by PCR and Western blot for the presence of *Grapevine rupestris stem pitting-associated virus* (GRSPaV) a strikingly high association (98 %) was observed between this virus and VN symptoms. Likewise, all 72 mother plants of *Vitis rupestris* used as indicators in indexing trials and recently discovered to be infected by GRSPaV, induced VN reactions after grafting onto 110 R. By contrast, no VN reactions developed in 110 R top-grafted on a single GRSPaV-free *V. rupestris*. Moreover, GRSPaV was consistently detected in the symptomatic lower leaves of the shoots of infected 110 R vines, but not in the symptomless upper leaves of the same shoots. These findings strongly suggest that GRSPaV is involved in the aetiology of VN.

K e y w o r d s: grapevine, vein necrosis, GRSPaV, *V. rupestris*, PCR, Western blot, *Foveavirus*.

Introduction

Vein necrosis (VN), a virus-like disease of grapevine, was first identified in France by Legin and Vuittenez (1973). Later it was found in several European countries, *i.e.* Ukraine (Milkus *et al*. 1978), Bulgaria (Martelli *et al*. 1978), Italy (Credi *et al*. 1985), Hungary (Lehoczky *et al.* 1986), Greece (Rumbos 1989), Malta (Martelli *et al.* 1992), as well as Turkey (Gursoy 1988), USA (Golino 1993), Australia (Woodham and Krake 1984), and Brazil (Kuhn 1994), but it is likely to have a much wider distribution (Martelli 1993). Although no vector is known, in countries it occurs VN has often a high level of incidence (Savino *et al.* 1985; Kuhn 1996; Kuniyuki *et al.* 1997), most likely because of its spread in infected propagating material.

VN is latent in all European grapevine cultivars and in most American *Vitis* species and hybrids except for the rootstock 110R (*V. berlandieri* x *V. berlandieri)* in which it induces necrosis of the veinlets at the abaxial side of leaf blades. The necrotic reactions develop first in leaves at the base of shoots, then, as shoots grow, on the younger, upper leaves. After some time, necrotic spots also appear at the adaxial side of leaf blades. Severe forms of VN induce necrosis of tendrils and dieback of green shoots, followed by an almost complete cessation of growth and, sometimes, death of the host (Martelli 1993). Symptoms appear 8-10 weeks after graft-inoculation in the field, or in less than 4 weeks following green-grafting (Walter *et al*. 1990). Mild forms of the disease may elicit positive reactions in the year after grafting. In all cases, VN symptoms persist throughout the vegetative season.

The agent of VN was thought to be an unknown virus. When, however, molecular (Meng and Gonsalves 2003) and serological (Minafra *et al.* 2000) tools became available for the detection of *Grapevine rupestris stem pitting-associated virus* (GRSPaV), this virus was found to be consistently present in VN-infected vines during our routine sanitary evaluation of grapevine clonal selections. We therefore checked in more detail the extent and significance of this finding.

Material and Methods

P l a n t m a t e r i a l : Object of the study were 218 putative clonal accessions of 35 wine and 20 table grape cultivars of *V. vinifera* and 5 rootstocks, that had been selected in the last few years in central and southern Italy, in the course of sanitary improvement programmes. All these putative clones had been checked serologically and/or molecularly for the presence of viruses associated with major detrimental diseases (fanleaf, leafroll, rugose wood, and fleck). Of these accessions, 108 had been heat-treated.

To establish their ultimate sanitary status, all selections were comparatively re-tested by biological (indexing) and laboratory methods.

I n d e x i n g : Indexing was initiated in 1999. Each putative clone was indexed on 4 replicates of each of the following indicators: (I) *V. vinifera* cv. Cabernet franc, for leafroll; (II) LN 33 (Couderc 1613 x *V. berlandieri*), for corky

Correspondence to: Dr. D. Boscia, Istituto di Virologia Vegetale del CNR, Sezione di Bari, Via Amendola 165/a, 70126 Bari, Italy. Fax: +39-080-544-2911; E-mail d.boscia@ba.ivv.cnr.it

bark and LN 33 stem grooving; (III) Kober 5BB (*V. berlandieri x V. riparia*), for Kober stem grooving; (IV) *V. rupestris* St. George, for fanleaf, fleck and Rupestris stem pitting; (V) 110 R (*V. rupestris x V. berlandieri*), for VN; (VI) *V. riparia* Gloire de Montpellier, for vein mosaic.

Seventy-two mother plants of *V. rupestris* St. George, currently used as indicators in our laboratory, were recently found to be infected by GRSPaV (MINAFRA *et al*. 2000); they were also submitted to indexing on 110 R together with a local accession of *V. rupestris* selected as GRSPaV-free.

ELISA: Crude extracts of cortical scrapings from mature canes of all 218 selections under study were tested by ELISA (CLARK and ADAMS 1977), to assess the level of association with VN, if any, of the following 8 viruses: *Grapevine fanleaf virus* (GFLV), *Grapevine leafroll-associated virus 1* (GLRaV-1), *Grapevine leafroll-associated virus 2* (GLRaV-2), *Grapevine leafroll-associated virus 3* (GLRaV-3), Grapevine leafroll-associated virus 7 (GLRaV-7), *Grapevine virus A* (GVA), *Grapevine virus B* (GVB), and *Grapevine fleck virus* (GFkV). The reagents used were monoclonal antibodies to GVA (BOSCIA *et al.* 1992), GVB (BONAVIA *et al*. 1996), GFkV (BOSCIA *et al*. 1995) and GLRaV-2 (ZHOU *et al.* 2000) and locally made polyclonal antisera to all the viruses mentioned above.

RT-PCR: Cortical scrapings from mature canes or leaf petiole extracts from inoculated 110 R plants were tested by RT-PCR for the presence of GRSPaV. Total nucleic acids (TNA) extracted according to FOISSAC *et al.* (2001) from cortical scrapings in winter or from leaf petioles in summer, were used as templates in a two-step RT-PCR protocol. After random primer cDNA synthesis, PCR was done using two different sets of primers: (I) forward primer RSP 48 (nt positions 8178-8199) and reverse primer RSP 49 (nt positions 8487-8507) designed by ZHANG *et al.* (1998) in the viral coat protein region; (II) forward primer 13 (nt positions 4373-4392) and reverse primer 14 (nt positions 4711-4692), designed by MENG *et al.* (1999 a) in the helicase-like domain of ORF-1. PCR products were analysed by polyacrylamide gel electrophoresis followed by silver staining.

Western blot: Leaf petioles from some of the inoculated 110R were tested by Western blot to confirm RT-PCR results, using the anti-GRSPaV polyclonal antiserum produced by MINAFRA *et al.* (2000) to recombinant virus coat protein. One hundred mg of petiole fragments from mature leaves were ground mechanically in 1 ml of extraction buffer (0.5 M Tris-HCl pH 8.8, 2 % SDS, 4 % 2-mercaptoethanol, 40 % sucrose). The extracts were boiled for 10 min at 100 °C and centrifuged in a microcentrifuge at 10,000 g for 3 min. Fifteen µl of the supernatant were mixed with 5 µl of 4 X Laemmli buffer and loaded in 12 % SDS-PAGE slabs (Biometra, Germany). Gels were immersed for 30 min in transfer buffer (25 mM Tris-HCl pH 8.3, 20 % methanol, 129 mM glycine), electroblotted on polyvinyldifluoride membranes (PVDF, Immobilon-P, Millipore) and directly incubated overnight at 4 °C with a 1:1,000 dilution of the antiserum in a blocking solution composed by 1 % BSA, 5 % non-fat dried milk in TBS buffer (20 mM Tris-HCl pH 7.5, 0.5 M NaCl), 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 1:2,500 dilution in blocking solution of antirabbit IgGs-AP conjugate (Sigma, Germany). Three sequential washings of 15 min each were followed by staining with BCIP/NBT Alkaline Phosphatase Substrate (Sigma, Germany).

Results

Indexing: One hundred-and-nine of 218 (50 %) accessions, including 57 of 108 (53 %) previously submitted to heat therapy, induced clear-cut VN symptoms in 110 R. Necrotic reactions developed first in leaves at the base of shoots then, progressively, in upper leaves. Necroses were also observed inside leaf petioles and shoots (Fig. 1). Leaf symptoms began to appear in June, reached a peak in mid-July, and persisted until the end of the season (late autumn).

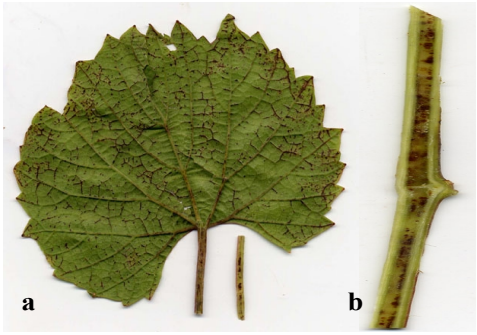

Fig. 1: Symptoms of vein necrosis in the indicator 110 R: necrosis of leaf veinlets and petioles (**a**) and of the pith (**b**).

The high rate of VN infection (50 %), despite the selected nature of the accessions, confirmed both the alleged high incidence of the disease in Italian grapevines (CREDI *et al.* 1985; SAVINO *et al.* 1985) and the low efficiency of VN elimination by heat treatment.

ELISA: Only 16 of 218 accessions (7 %) were positive to at least one of the 8 viruses they were tested for. All the others (93 %) were negative (virus-free). The incidence of single viruses was: 0.9 % (2/218) for GFLV; 3.6 % (8/218) for GVA; 2.7 % (6/218) for GLRaV-1; 0.4 % (1/218) for GLRaV-2; 1.8 % (4/218) for GLRaV-3; 1.8 % (4/218) for GFkV. GVB and GLRaV-7 were not detected. The low infection rate determined by ELISA confirmed the efficiency of field selection and the satisfactory sanitary status of the putative clones.

Of the 16 ELISA-positive accessions, only 8 (50 %) were affected by VN as shown by indexing. We therefore concluded that the low infection rate (7 %) by very common grapevine viruses, such as GFLV, GVA, GVB, GLRaV-1, GLRaV-2, GLRaV-3, GLRaV-7 and GFkV and their equal distribution in accessions with and without VN, excludes their involvement in the aetiology of VN.

RT-PCR: The presence of GRSPaV was checked by RT-PCR in 174 110R indicators that had been grafted each with a different selected grapevine accession. Of these vines, 93 showed VN symptoms (VN+), whilst 81 were symptomless (VN-).

Preliminary comparative tests on 50 VN(+) samples had given diverging results in that all samples amplified by primers RSP 48/RSP 49 were also amplified by primers 13/14, but

the number of amplifications obtained with primers 13/14 was higher than that given by the first couple of primers (RSP 48/RSP 49). This was in agreement with the notion that a high variability exists in the GRSPaV coat protein sequence on which RSP 48/RSP 49 had been designed (Meng *et al.* 1999 b). Therefore, it was decided to carry out RT-PCR with primers 13/14, which appeared more suitable for detecting GRSPaV variants missed by the other set of primers.

Amplification products were observed in PCR gels of 58 % (101 of 174) of the samples. However, of the 101 samples, 44 yielded only the expected specific amplicon of 339 bp, 24 gave the 339 bp amplicon plus additional unidentified products (Fig. 2, pattern A), and 33 gave amplicons with a higher molecular weight than the expected product (Fig. 2, pattern B).

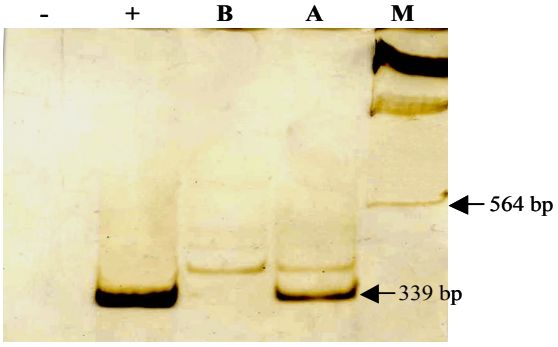

Fig. 2: Patterns of PCR products amplified by the set of primers designed by Meng *et al.* (1999 a). M = Marker; + = typical positive sample yielding a single amplicon of 339 bp; - = negative sample; A and B = atypical patterns showing multiple bands (**A**) or an amplicon with a size higher than the expected product (**B**).

To determine the nature of the diverging RT-PCR amplicons of patterns A and B, amplification products from three different accessions with patterns A (A1, A2 and A3) and one with pattern B were recovered with QIAquick PCR purification Kit (Invitrogen Life technologies, Carlsbad, USA), cloned in pGEMT Easy vector (Promega, Madison, USA), sequenced (MWG Biotech, Ebersberg, Germany), and their nucleotide and amino acid sequences were aligned using the default options of Clustal W multiple sequence alignment programme. The analysis of these sequences showed that the three PCR products from pattern A had a homology at the nucleotide level of 88.7, 89.3 and 98.5 % and a homology at the amino acid level of 98.2, 99.1 and 98.2 % with a GRSPaV sequence by Zhang *et al.* (1998; GenBank accession number AF026278) (Fig. 3). By contrast, the PCR product from pattern B did not show significant homology with sequences of GRSPaV or any other virus in database (not shown). Therefore, samples with pattern A were classified as positive, while samples with the undetermined pattern B were retained as negative.

In summary, the global result of RT-PCR processing of the 174 grafted 110R vines was that 68 were GRSPaV (+) and 106 were GRSPaV (-). Interestingly, all positive samples, except for one (67 of 68 samples), were comprised in the group of 93 accessions that had been identified as VN(+) by indexing. Thus, only one out of 81 accessions recorded as VN(-) by indexing, was GRSPaV (+) by RT-PCR.

Since, as shown by these results, about 1/3 of the VN(+) samples (26/93) were negative for GRSPaV, the association GRSPaV/VN was clearly incomplete, thus no tenable conclusions could be drawn in support of a possible cause-effect relationship between the virus and the disease. How-

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A1              ..........G........C........T........G..T..A...G.C.....C...
A2              ..........G..................T.....CT.G..T..A...G.C.....C...
A3              ..........G.....................................................
AF026278        GATGAGGTCCAATTGTTTCCTCCTGGATACATCGATCTATGCTTGCTTATTATACGTAGT

A1              ......................T...........C..G..............A..G......
A2              ......................T...........C..G............A.A..G......
A3              ..............................C.....................................
AF026278        GATGCTTTCATTTCACTTGCCGGTGATCCATGTCAAAGCACATATGATTCGCAAAAGGAT

A1              ..A...........A..............T.....C...........A..A.....A...
A2              ..A...........A......................C...........A..A.....A...
A3              ...............................................................G.....
AF026278        CGGGCAATTTTGGGCGCTGAGCAGAGTGACATACTTAGACTGCTTGAGGGCAAGACGTAT

A1              .....C..............AC.T..................T.....T.....A......
A2              .....C..............AC.T..................T.....T............
A3              ..........T..............................G.....................
AF026278        AGGTATAACATAGAAAGCAGGAGGTTTGTGAACCCAATGTTCGAATCAAGACTGCCATGT

A1              ..........A..T..A........A..C..T.....................C......
A2              ..........A..T..A........A..C..T.....................C......
A3              ...............................................................
AF026278        CACTTCAAAAAGGGCTCGATGACTGCCGCTTTCGCTGATTATGCAATCTTCCATAATATG

A1              ........C..............................
A2              ........C..T.....A.....................
A3              ......................................
AF026278        CATGACTTTCTCCTGGCGAGGTCAAAAGGTCCTTTGGAT
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Fig. 3: Nucleotides sequence alignments of three RT-PCR products with pattern A (A1, A2, A3) from different 110 R accessions and a published GRSPaV sequence (GenBank accession number AF026278).

ever, keeping in mind that sampling for PCR was made in winter, usually from one of the 4 110 R vines on which each accession was indexed, it cannot be excluded that the material to be assayed was collected by chance from an indicator that had not been infected due to graft failure.

When the indexing positions of the 26 PCR-negative accessions that were recorded as VN(+) in 2003 were visually re-examined in summer 2004, we found that in 24 cases there was the contemporary presence of symptomatic and symptomless 110R indicator in the same position. RT-PCR from leaf petioles of vines with and without symptoms, showed that the totality (24/24) of symptomatic 110 R were GRSPaV-positive whereas the reverse was true for the 24 symptomless indicators. As a consequence of this additional set of PCR assays, the level of association GRSPaV/VN rose to a striking 98 % (Table).

Table

Association of GRSPaV to vein necrosis disease

No. of accessions	Indexing for vein necrosis	GRSPaV +	GRSPaV -
174	VN+ (93)	91/93 (98 %)	2/93 (2 %)
	VN- (81)	1/81 (1 %)	80/81 (99 %)

Western blot: When 93 samples (58 VN(+) and 35 VN(-)) were comparatively tested by RT-PCR and Western blot, using extracts from leaf petioles, the results showed that 57 of the 58 PCR-positive samples were also positive in Western blot (Fig. 4), while all 35 PCR-negative samples remained negative in Western blot. We concluded that Western blot assays seem to be as good as RT-PCR (nearly 99 % of agreement) for GRSPaV detection in grapevine tissues.

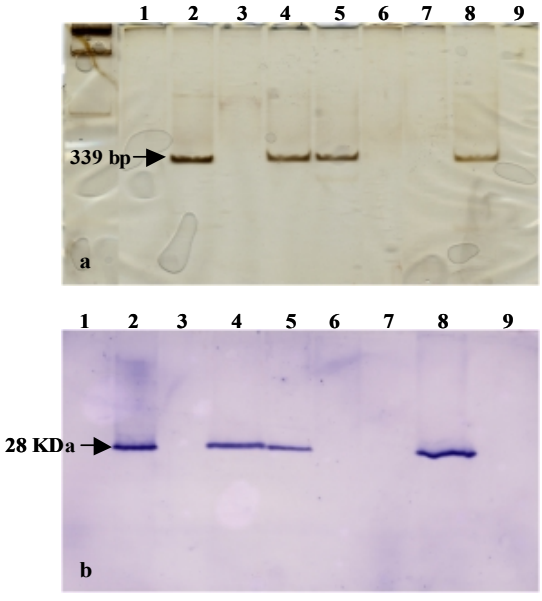

Fig. 4: Comparative analysis for GRSPaV from 110 R extracts by RT-PCR (**a**) and Western blot (**b**): in both gels lanes 2,4,5,8 contain infected samples, whereas lanes 1, 3, 6, 7 and 9 contain healthy samples.

Graft transmission of GRSPaV from naturally infected *V. rupestris* to 110 R: Seventy-two field-grown *V. rupestris* vines known to be naturally infected by GRSPaV (Minafra *et al.* 2000) were top-grafted in February 2004 with budsticks from a 110 R source ascertained to be GRSPaV-free by RT-PCR and Western blot. The negative control consisted of a GRSPaV-free *V. rupestris*. Graft take was 100 % and by July 2004 all 72 GRSPaV-infected accessions had induced clear-cut VN responses in 110 R. By contrast, no symptoms developed in the 110 R vine top-grafted on GRSPaV-free *V. rupestris*.

Correlation between VN symptoms and the presence of GRSPaV: Petioles collected from symptomatic basal and symptomless apical leaves of shoots of 10 110 R plants affected by VN (Fig. 5) were tested for the presence of GRSPaV by RT-PCR and Western blot. The results of both types of assays tallied, disclosing that the virus could only be detected in symptomatic samples. This was taken as an indication that GRSPaV moves relatively slowly in infected 110 R, and is restricted to symptomatic tissues.

Fig. 5: Symptomatic (**S**) and symptomless (**A**) leaves of the same shoot of a graft-inoculated 110 R. Only lower symptomatic leaves reacted positively to GRSPaV by RT-PCR and Western blot.

Discussion

With this study, a very high association (98 %) of GRSPaV with VN was experimentally ascertained by RT-PCR and Western blot, and confirmed by the fact that 99 % of VN(-) accessions were shown to be GRSPaV-free. The association was further confirmed by the VN responses elicited in 110R by 72 *V. rupestris* plants naturally infected by GRSPaV, while no symptoms developed when a GRSPaV-free *V. rupestris* was indexed.

This strongly supports that GRSPaV is responsible for the appearance of VN symptoms in 110 R and opens the possibility that 110 R may be used as an indicator in order to reduce the time of indexing for Rupestris stem pitting (RSP) of 2-3 years, as required by the current indicator *V. rupestris*, to a few weeks or months. This, provided that the aetiological relationship between GRSPaV and RSP is ultimately demonstrated. In fact, current records report occurrence of GRSPaV in a substantially high percentage of grapevine sources that index negative for RSP in *V. rupestris*, ranging from 22 to 30 % (Meng *et al.* 2003; Meng *et al*. 2000 a), to 50 % (Meng *et al.* 1999a), up to 66 % (Nolasco *et al.* 2000). The reasons for this lack of consistency are still unknown, although the presence of latent GRSPaV infections in the *V. rupestris* sources widely used by many laboratories for routine indexing trials (Minafra *et al.* 2000; Meng *et al*. 2000 b; Petrovic *et al.* 2000) may interfere with symptom expression due to a sort of cross-protection mechanism.

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