GENOMIC STUDY AND DETECTION OF A NEW VARIANT OF *GRAPEVINE RUPESTRIS STEM PITTING ASSOCIATED VIRUS* IN DECLINING CALIFORNIA PINOT NOIR GRAPEVINES

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SUMMARY

Declining cv. Pinot noir clone 23 plants on 3309 Couderc rootstock were observed in different vineyards in California exhibiting severe stunting, solid red leaves and rootstocks with stem necrosis-distortion. High molecular weight dsRNA of *ca.* 8.7 kbp was extracted from symptomatic vines, used as a template for cDNA libraries construction and sequencing and identified as a new strain of *Grapevine rupestris stem pitting-associated virus* designated as Pinot noir strain (GRSPaV-PN). Molecular characterization of GRSPaV-PN genome revealed that the complete nucleotide sequence was composed of 8,724 nt, excluding the poly-A tail. This strain shared nucleotide identity of 76% to 78% with other GRSPaV sequences present in the GenBank. Its genome organization included six open reading frames where its replicase gene had 76% and 85% nucleotide and deduced amino acid identities, respectively, to other GRSPaV sequences. The coat protein gene was the most conserved with nucleotide and amino acid identities of 81-88% and 91.8-95%, respectively. Limited field survey revealed that GRSPaV-PN was present mainly in Pinot noir clone 23. However, the causal nature of GRSPaV-PN and 3309C stem necrosis-distortion is not known.

Keywords: GRSPaV-PN, detection, RT-PCR, rugose wood disease complex.

INTRODUCTION

Rugose wood (RW), a complex of graft-transmissible disorders of grapevine that occurs worldwide (Martelli and Boudon-Padieu, 2006), was first reported in 1971 from California (Hewitt and Neja, 1971). Based on symptoms on different grapevine indicator hosts, four RW disorders have been recognized: corky bark (CB), Kober stem grooving (KSG), LN33 Stem grooving (LNSG) and Rupestris stem pitting (RSP) (Martelli, 1993).

RSP is of concern in California (Golino *et al*., 1992) after having been identified in imported selections from Western Europe and Australia (Goheen, 1988). The characteristic symptoms of RSP are distinct in rootstocks with *Vitis rupestris* Sheele parentage (Goheen, 1988). Commonly, basipetal pitting and grooving symptoms develop below inoculum buds grafted on plants of the indicator host *V. rupestris* cv. St George (Goheen, 1988; Martelli, 1993). In severe cases, pitting and grooving develop throughout the woody cylinder (Martelli, 1993). Diseased grapevines decline slowly (Goheen, 1988).

The etiology of RSP is not completely understood. The first association of *Grapevine rupestris stem pitting-associated virus* (GRSPaV) with the RSP disease was reported by Zhang *et al.* (1998) and Meng *et al.* (1998). Both isolates were very similar with nucleotide sequence differences of less than 2%. Additional strains of GRSPaV with significant sequence diversity were also identified and characterized (Lima *et al*., 2003, 2006; Meng *et al*., 2005, 2006; Habili *et al*., 2006)

GRSPaV belongs to the genus *Foveavirus* (Martelli and Jelkman, 1998) in the family *Flexiviridae* (Adams *et al.*, 2004). Viruses in this genus have filamentous particles *ca*. 800 nm long with *Apple stem pitting virus* (ASPV) as the type species. GRSPaV is a positive sense single-stranded RNA virus, 8.7 kb in size and polyadenylated at the 3' terminus (Martelli and Jelkman, 1998). The genome of the species in the genus *Foveavirus* is defined as composed of five open reading frames (ORFs) which encode for the replication-related proteins, the triple gene block, and the coat protein (ORF 5). However, two GRSPaV variants have been reported to have a sixth ORF, almost completely overlapping with the coat protein and the function of the expression product of which is unknown (Zhang *et al.*, 1998; Lima *et al*., 2006).

The only known hosts of GRSPaV are *Vitis* species. The dsRNA extraction technique (Walter and Cameron, 1991) has facilitated molecular study and has proven to be an efficient template for creating cDNA libraries (Jelkmann *et al.*, 1989; Valverde *et al.*, 1990; Zhang *et al.*, 1998;

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Meng *et al*., 1998; Lima *et al*., 2006). This approach was used to study molecular criteria of different strains of viruses associated with RSP disease (Zhang *et al.*, 1998; Meng *et al.*, 1998; Meng *et al*., 2005; Lima *et al*., 2006).

Our study in the possible cause of young vine decline of cv. Pinot noir/3309C grapevines lead to recovery of a new GRSPaV isolate, which was fully characterized. RT-PCR primers were designed for specific detection of the virus and used in limited field surveys.

MATERIALS AND METHODS

Virus sources and maintenance. The source of the GRSPaV isolate used in this study was a field selection of cv. Pinot noir clone 23 grafted on Couderc 3309 (3309C) rootstock. The source plant displayed solid red leaf canopy, poor vigor, vine decline, and severe stem necrosis and distortion of the rootstock (Figure 1). It was propagated by rooted cuttings and plants were maintained in the greenhouse and in the field. The vine source was tested by ELISA for Grapevine leafroll associated viruses (GLRaV-1, -2 , -3 and -6) and by RT-PCR for GLRaV -1, -2, -3, -4, -5, -7, and -9, GRSPaV, *Grapevine virus A*, *-B* and *-D* (GVA, GVB and GVD), *Grapevine leafroll associated virus 2* Red Globe strain (GLRaV-2RG), *Grapevine fanleaf virus* (GFLV), *Arabis mosaic virus* (ArMV) and *Tomato ringspot virus* (ToRSV).

RT-PCR tests. Total nucleic acids were extracted from leaf petioles and/or cambium scrapings of grapevine plants, using RNeasy Plant Mini kit (Qiagen, USA), according to MacKenzie *et al.* (1997) with some modifications. In this procedure 0.2 g of tissue were placed in a plastic bag (Habili *et al.*, 2006) with 2 ml of extraction buffer containing 4 M guanidine-HCl, 0.2 M sodium acetate, pH 5.0, 25 mM EDTA, 2% PVP-40 and 10 mg/ml sodium metabisulfite and homogenized using a Bioreba AG Homex 6 sap extractor (Bioreba, Switzerland). The homogenate (500 µl) was mixed with 60 µl of 20% sarcosyl solution and heated at 70°C for 10 min, before adding to the column. The total nucleic acids were eluted in 100 µl sterile water and stored at -80°C.

One-step RT-PCR (Rowhani *et al.*, 2000) was performed for virus detection. The RT-PCR mixture was prepared to yield final concentrations of 1x PCR Buffer [50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.1% Triton-X100], 0.2 mM each deoxynucleotide triphosphate, 5 mM dithiothreitol, 7 U/µl Superscript II Reverse Transcriptase (200 U/µl, Invitrogen-Life Technologies, USA), 4 U/µl RNasin Ribonuclease Inhibitor (40 U/µl, Promega, USA) and 1.25 U/µl Taq DNA Polymerase (5 U/µl, Promega, USA) per reaction. RT-PCR reactions were performed in an iCycler (Biorad, USA) with the following parameters: 30 min at 52ºC followed by 35 cycles of 30 sec at 94ºC, 45 sec annealing at the temperatures for the respective primer sets, and 1 min at 72ºC and a final extension of 7 min at 72ºC. PCR products (10 µl) were analyzed on a 1.5% agarose gel, stained with ethidium bromide and visualized under UV light.

Table 1. Comparative analysis of nucleotide (nt) and amino acid (aa) sequences of the five open reading frames (ORF) of the GRSPaV-PN genome and four full-length GRSPaV genome sequences available in the database. Comparison is shown as percentage identity. Analyses were performed with ClustalW from the European Bioinformatics Institute. Regions for the genome compared included: UTR=untranslated region; REP=replicase; TGB=triple gene block; CP=coat protein.

Encoded Gene	GRSPaV[1]		GRSPaV-BS		GRSPaV-SG1		GRSPaV-SY	
	nt	aa	nt	aa	nt	aa	nt	aa
Complete Genome	76	-	77	-	78	-	77	-
5'UTR	90	-	91	-	93	-	94	-
REP	75	84.7	75.6	84	74.8	84	76	84
TGB1	77.6	87	82.7	90.5	77	86	76	84
TGB2	78.8	83.7	82.7	87	80	82	74	78
TGB3	84	87.5	86.8	92	82	83.7	80	78
CP	82	93	88	95	81	91.8	83	93
3'UTR	93	-	93	-	95	-	91	-

[1]GRSPaV (Zhang *et al*., 1998); GRSPaV-BS and GRSPaV-SG1 (Meng *et al*., 1998); GRSPaV-SY (Lima *et al.,* 2006).

Double-stranded RNA extraction. Double-stranded RNA (dsRNA) was isolated from cambium scrapings or leaf petioles (30 g) of infected and healthy grapevine plants according to Valverde *et al.* (1990) and Dodds (1993) with modifications. A total of 30 g of grapevine cortical tissues were ground in liquid nitrogen and then mixed with: 90 ml 2x STE; 35 ml of 10% SDS; 35ml 2x STE saturated:phenol and 35 ml chloroform:isoamyl alcohol (24:1 v/v). The recovered dsRNA was resuspended in DEPC-treated water (1 µl /2 g of plant tissue) and used for constructing cDNA libraries. The integrity of the dsRNA was checked by electrophoresis in a 6% polyacrylamide gel.

cDNA library synthesis and sequencing. cDNA synthesis were performed using the SuperScript Choice System for cDNA Synthesis (Invitrogen-Life Technologies, USA), following the manufacturer's protocol with modification in the first-strand cDNA. Briefly, 8.7 µl of extracted dsRNA (~1.5 µg total) was mixed with 75 ng of random hexamers and 20 mM methylmercuric hydroxide (CH_3HgOH) for 10 min at room temperature, then chilled on ice as described by Zhang *et al.* (1998). The CH_3HgOH was neutralized by adding 2.0 µl 0.1 M DTT and processed for cDNA synthesis. The produced cDNAs were adenylated at the 3' termini according to Zhang and Rowhani (2000) before ligation to TOPO TA cloning vector (Invitrogen-Life Technologies, USA) following manufacturer's protocol. Transformation was performed using DH5-α *E. coli* electrocompetent cells (Invitrogen-Life Technologies, USA) by electroporation in a Biorad MicroPulser Electroporation Apparatus, according to manufacturer's instructions. Recombinants were selected on Luria-Bertani medium containing ampicillin (100 µg/ml) (Sambrook *et al.*, 1989). Selected clones were sequenced on both strands at the University of California, Davis, using vector primers T7 and T3. The gaps between clones were filled using downstream and upstream specific PCR primers designed carefully on obtained nucleotide sequences to generate overlapping cDNA fragments and always using dsRNA as a template.

The 5' and 3' terminal sequences were obtained from 10 µl (~1.5 µg total) of starting dsRNA using the 5' and the 3' RACE (Rapid Amplification of cDNA Ends, Invitrogen-Life Technologies, USA) and following the manufacture's procedure. The primers EGS-1 (5'GGAGAACAAAAATAGATGC3'), EGS-2 (5'CGAAAACTTGAGGATAGTCTAGC3'), EGS-3 (5'CCAATGCAGAAAAATCTTC3') and EM-RP3 (5'ATGTGGTGCGACTTACAACT3') were designed downstream from the 5´ end of the available sequences and used in a nested PCR to obtain the 5' end sequence (940 nt). The 3' terminal sequence was obtained using the upstream primer EM-TA4 (5'ACTGCTCCGATGTAGGTAG3'). The PCR products were ligated into pGEMT-easy cloning vector (Promega, USA) and transferred into *E. coli* (Invitrogen-Life Tech-

Table 2. RT-PCR results of grapevine samples collected from commercial vineyards in California using the GRSPaV universal (48V/49C) and GRSPaV-PN specific (PN-1F/2R) primers. Total RNA samples were prepared by Qiagene RNeasy kit and tested by one-step RT-PCR.

Scion	Samples tested (No.)	48V/ 49C	PN-1F /2R
Cabernet franc and Cabernet sauvignon	37	26	0
Chardonnay	27	17	0
Malbec	8	5	0
Merlot	21	13	0
Petit verdot	4	3	0
Pinot noir clone 23 and Pinot noir clone 4	81	71	43
Pomard	13	13	0
Primitivo	3	3	0
Syrah	85	79	2
Viognier	3	2	0
Zinfandel	38	21	0
Unknown	20	20	0
Total	340	272	45

nologies, USA) as previously described. Selected clones were sequenced using vector primers M13 and M13R. At least five recombinant plasmids were sequenced in both sense and antisense directions, to ensure the accuracy of the sequences.

The complete nucleotide sequence of the GRSPaV-PN isolate was deposited in the GenBank under the accession number AY368172.

Sequence analysis. Sequence data were analyzed and assembled using Sequence Analysis and Molecular Biology Data Management software from Invitrogen, Vector NTI AdvanceTM10 (InforMax, USA). Nucleic acid sequences were compared using BLAST (Altschul *et al.*, 1997) from the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov). Conserved domains on the amino acid sequences were identified by CD-Search on NCBI (Marchler-Bauer and Bryant, 2004). Amino acid sequences were analyzed with help of BLAST protein (Shäffer *et al.*, 2001). Multiple alignments of nucleotide and amino acid sequences were obtained using ClustalW (Higgins *et al.*, 1994) from European Bioinformatics Institute or DNAsis Max Program package version 2.0 (Hitachi software Engineering Co., UK). ORF search was performed with DNAsis Max Program package version 2.0 (Hitachi software Engineering Co., UK) and ORF Finder of the NCBI.

Primers design and virus detection. The variation among GRSPaV-PN and other GRSPaV sequences made it possible to identify variable regions in the genome sequence of this isolate and to design isolate-specific PCR primers. Multiple alignments were performed using the complete genome sequence of this isolate and those available in the GenBank including AF026278 (Zhang *et al.*, 1998), AF057136 (Meng *et al.*, 1998), AY881626 and AY881627 (Meng *et al.*, 2005) and AY368590 (Lima *et al.*, 2006) to design isolate-specific primers. The nucleotide sequences were aligned and specific primers were designed for the Pinot noir isolate and used in vineyard surveys.

The primer set PN1F (5'GATGGATACAAGTTACGGGC3') / 2R (5'TTCCCCAAACTTCCAACTTAC3') was designed based on the sequence of ORF1, in between the methyltransferase (MTR) and papain-like cysteine protease (P-PRO) conserved domains amplifying a fragment of 505 bp between nucleotide positions 3,032-3,537. These primers were evaluated for their specificity on different GRSPaV isolates using one step RT-PCR as described by Rowhani *et al.* (2000). PCR products were then checked by cloning and sequencing to ensure their specificity. These primers were then applied for virus detection to check the spread of GRSPaV-PN isolate in different regions in California (USA) as well as among popular grapevine varieties. A total of 340 samples were randomly collected from commercial vineyards in twelve counties in California and tested for GRSPaV-PN isolate using the specific primers (PN1F/2R) as well as for all other GRSPaVs using the universal primers RSP48V/49C (Table 2) designed within the highly conserved coat protein region to amplify a 331 bp amplicon. In addition, 48 samples from the US National Clonal Germplasm Repository at UC Davis which represented a geographical diverse grapevine collection were included. Total RNA was extracted from collected mature leaf petioles or cambium scrapings and tested by one-step RT-PCR as described previously. To confirm the specificity of the isolate-specific primers set, samples of PCR products were purified using a gel DNA recovery kit (Zymoclean DNA purification kit) and both strands sequenced and compared to sequence of GRSPaV-PN.

RESULTS

Double-stranded RNA extraction and sequence analysis. A high molecular weight dsRNA profile of *ca.* 8.7 kbp similar to those of GRSPaV (Martelli and Jelkman, 1998) was revealed from symptomatic tissues when electrophoresed on a 6% polyacrylamide gel (not shown).

The total GRSPaV-PN genome was determined to be 8,724 nt, excluding the poly-A tail. The nucleotide composition of this sequence was 27.5% A, 19.5% C, 29.4% T and 23.6% G, with a GC content of 43.1%. Six open reading frames (ORFs) were identified in the plus strand using the ORF finder of the NCBI Web server similar to the organization of GRSPaV isolates (Zhang *et al.*, 1998; Meng *et al.*, 1998, 2005; Lima *et al.*, 2006). Analytical study of the GRSPaV-PN genome showed that it comprises ORF 1 (nt 61-6,546) that encodes replicase-associated proteins, ORFs 2-4 (nt 6,577-7,242; nt 7,244-7,597; nt 7,518-7,760) constituting the triple gene block (TGB) that encodes the putative movement proteins, ORF 5 (nt 7,770-8,549) coding for the coat protein (CP), and a small ORF corresponding to nt 8,299-8,586 at the 3' end. This sixth ORF which is 288 nt, is almost 100% overlapping CP. Two untranslated regions of 60 nt and 138 nt long, respectively, were recognized at the 5' and 3'end of the sequence. The poly-A signal "AAUAA" was identified 28 nt upstream the 3' end of the viral sequence, at positions 8,691-8,695.

All conserved domains typical of the alpha-like supergroup of positive-sense RNA viruses (Rozanov *et al.*, 1992; Lawrence *et al.*, 1995; Gorbalenya *et al.*, 1988) were identified in the replicase gene. Analyses of the predicted amino acid sequence of this gene revealed the presence of four conserved domains, methyltransferase (MTR), papain-like cysteine protease (P-PRO), helicase (HEL) and RNA-dependent RNA-polymerase (RdRp). A fifth conserved motif, corresponding to 96 amino acid

residues (781-877) was identified in between the MTR and the P-PRO conserved domains of the replicase. This motif is called 2OG-Fe(II) oxygenase and belongs to the 2-oxoglutarate- and Fe(II)-dependent oxygenase superfamily (Bratlie and Drablos, 2005). Except for the MTR, which was identified by comparing its amino acid sequence against the database or aligning corresponding amino acid residues from other GRSPaV isolates, the other motifs were identified using CD-Search on NCBI (Marchler-Bauer and Bryant, 2004).

The deduced amino acid sequence of the replicase was approximately 84% identical to other GRSPaV sequences in the GenBank (Table 1). The variability in the GRSPaV-PN sequence was mainly found in a region of the replicase gene, which included more than 74.3% of the GRSPaV genome sequence (6,486 nt; 2,161 amino acid residues). The region located 189 amino acids downstream MTR IV and five amino acids upstream 2OG-FeII-Oxy conserved motifs, which corresponds to 331 amino acids, showed significant variability when compared to similar region of other GRSPaV isolates (Fig. 2).

In addition, the analysis of the deduced polypeptide of the replicase identified two putative proteases, the OTU-like cystein peptidase (125 amino acid residue) and the putative peptidase (89 amino acid residues) upstream the helicase conserved domain. Both proteinases are also present in the replicase gene of some members of the *Flexiviridae* family (Martelli *et al.*, 2007). Multiple alignment analyses of the nucleotide sequences of the replicase gene of GRSPaV-PN and the other sequences available in the database (not shown) revealed that the C-terminus of this gene is more conserved than the N-terminus.

The deduced amino acid sequences of TGB shared identities from 78-92% for TGB2 and 3 to GRSPaV-SY and for TGB3 to GRSPaV-BS, respectively (Table 1). The translated products of the CP which is considered to be the most conserved gene in the GRSPaV genome shared identity from 91.8% (GRSPaV-SG1) to 95% (GRSPaV-BS). The 5' terminus had sequence identities varying from 90% to 94% and the 3' end from 91% to 95% to corresponding regions of other GRSPaV isolates (Table 1). The complete genome sequence of the Pinot noir isolate shared nucleotide identity of 76% (GRSPaV; Zhang *et al.*, 1998) to 78% (GRSPaV-BS; Meng *et al.*, 2005).

Virus detection. Approximately 75% of samples collected from the Clonal Germplasm Repository at UC Davis tested positive for GRSPaV using primers RSP48V/49C and 8.3% by GRSPaV-PN primers (PN1F/2R). Among 340 randomly collected samples from commercial vineyards, the incidence of GRSPaV was 80% (272 vines, Table 2), and for GRSPaV-PN 13% (45 vines). However, the PN isolate was detected in 34 vines of Pinot noir 23 grafted on 3309C rootstock, 9 in own rooted Pinot noir and two in cv. Syrah on an unidentified rootstock. No amplification was obtained for healthy controls. Sequence analyses of RT-PCR products of positive samples using PN1F/2R confirmed virus strain as GRSPaV-PN.

DISCUSSION

A GRSPaV strain designated GRSPaV-PN was identified in a young declining grapevine of Pinot noir on 3309C rootstock that showed red canopy, poor shoot and berry development, and rootstock with severe necrosis and distortion of the woody cylinder (Fig. 1).

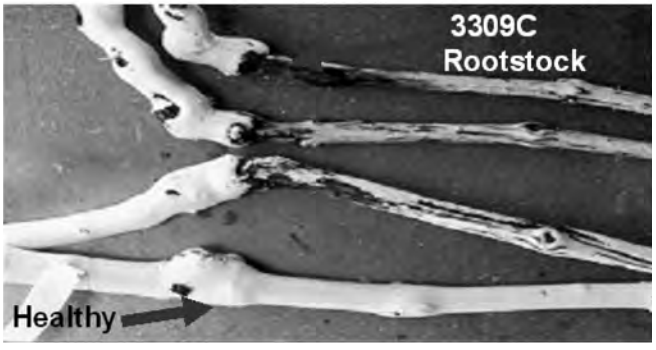

Fig. 1. Symptoms of pitting and stem necrosis-distortion on the rootstock of a Pinot noir clone 23 grafted on 3309C rootstock. Diseased Pinot noir grapevine showed solid red leaf canopy, poor vigor and decline.

Up to date efforts to experimentally reproduce these symptoms have failed. Also, GRSPaV has not been reported to incite reddening of the leaves on scions or cause vine decline on rootstock with *V. rupestris* parentage such as 3309C. However, field surveys of a few asymptomatic Pinot noir/3309C tested positive for GRSPaV-PN to suggest the possibility of involvement of a different biotic or abiotic agent or in combination with GRSPaV-PN in the disease syndrome

The full-length genome sequence of this isolate (GenBank accession No. AY368172) had an organization showing high similarity to other GRSPaV isolates (Zhang *et al.*, 1998; Meng *et al.*, 1998, 2005). The genome consisted of 8,724 nt and contained a large ORF that encodes the replicase gene and four smaller ORFs that encode the TGB and the CP. A sixth ORF was identified at the 3' end of the genome which significantly overlapped the CP gene.

The existence of the putative ORF 6 is controversial. Recent analyses (Nolasco *et al.*, 2006) based on the selection pressure operating in the superimposed ORF 5 concluded that ORF 6 does not exist. Meng *et al.* (1998, 2005) did not report the existence of ORF 6 in the genome of the GRSPaV-1, GRSPaV-BS and –SG1.

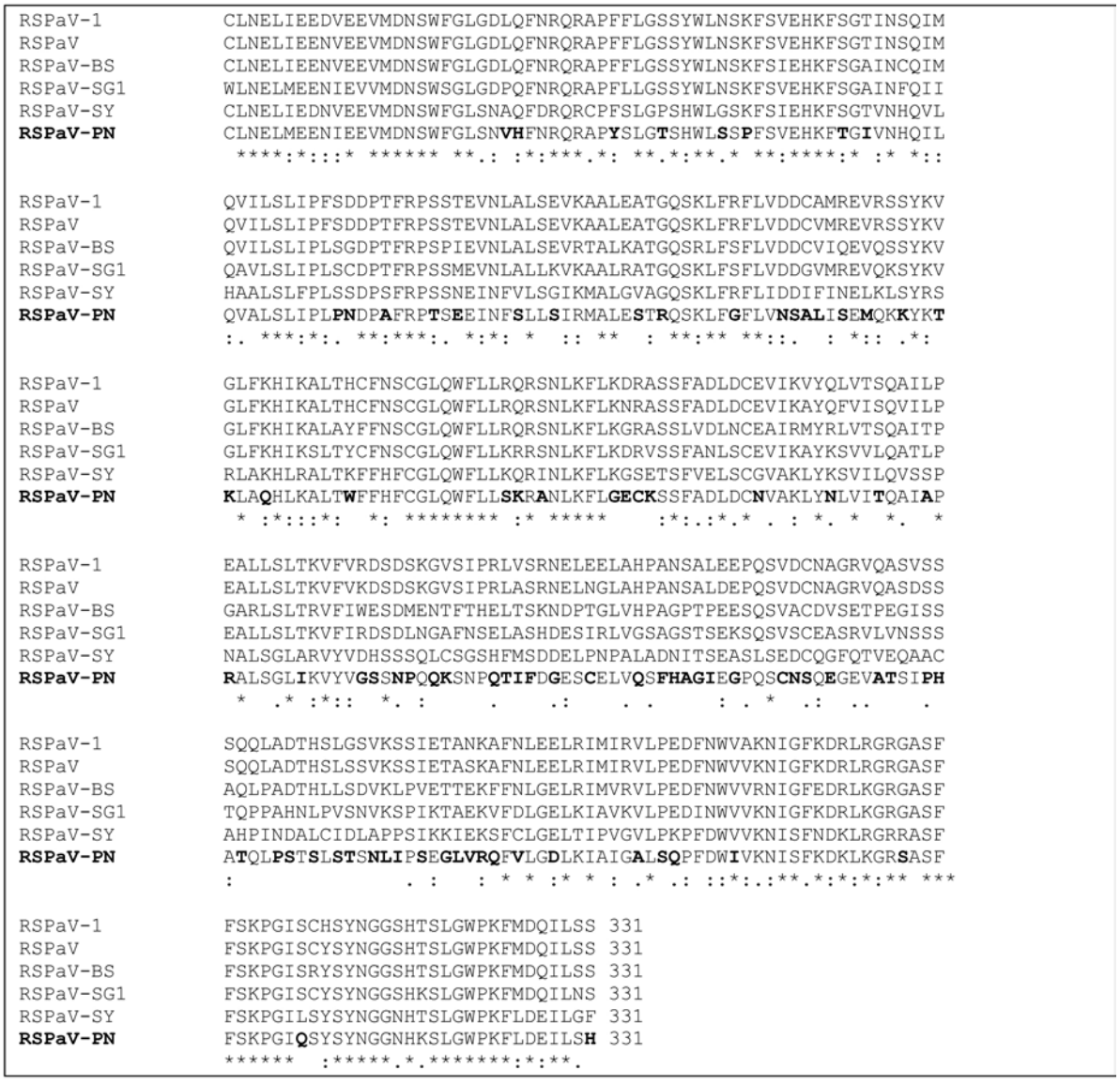

Fig. 2. Multiple sequence alignment of 331 amino acid residues located 189 amino acid downstream from MTR IV and 5 amino acid upstream from 2OG-FeII-Oxy conserved motif. This region showed significant variation when compared to similar region of other GRSPaV isolates. GenBank accession Nos. are: AF026278 (GRSPaV), AF057136 (GRSPaV-1), AY881626 (GRSPaV-BS), AY881627 (GRSPaV-SG1) and AY368590 (GRSPaV-SY).

However, ORF 6 was reported in the genome organization of GRSPaV isolates described by Zhang *et al.* (1998) and Lima *et al.* (2006) obtained from *V. vinifera* cvs Cabernet sauvignon and Syrah, respectively. In addition, the presence of a putative ORF superimposed on the CP gene was also reported for *Apricot latent virus* (ApLV) in the *Foveavirus* genus by Gentit *et al.* (2001).

Full-length genome analyses of GRSPaV-PN, revealed significant variations when compared to other GRSPaV sequences (Zhang *et al.*, 1998; Meng *et al.*, 1998, 2005) with percent nucleotide identity of 76-78% (Table 1). Comparative analysis of the five sequenced GRSPaV genomes among themselves revealed that four of them, GRSPaV (Zhang *et al.*, 1998), GRSPaV-1 (Meng *et al.*, 1998), GRSPaV-BS and GRSPaV-SG1 (Meng *et al.*, 2005) were more closely related to each other than to GRSPaV-PN. These sequences shared a sequence identity varying from 84% among GRSPaV-BS, GRSPaV-SG1 and GRSPaV to 98% between GRSPaV and GRSPaV-1. ORF1 which comprises approximately 74% of the total viral genome sequence was found to be more variable, due to sequence variation in the intervening region. It showed approximately 75% and 85% nucleotide and deduced amino acid identities, respectively, to database sequences (Table 1). Further analyses showed significant variation in a segment of 331 amino acid residues located between the conserved domains of MTR IV and 2OG-Fe(II) oxygenase (Fig. 2). Comparative analyses of this segment of GRSPaV-PN to corresponding regions of the replicase

of the reported GRSPaVs showed identities of approximately 50-60% to other known GRSPaV isolates. However, this segment is composed of 299 amino acid residues for GRSPaV-BS and -SG1 and 331 for GRSPaV-SY and -PN. Similar observations were also reported for other GRSPaV isolates (Meng *et al.*, 2005; Lima *et al.*, 2006). This variable region does not include any conserved domain and may have been more subjected to mutations (Meng *et al.*, 2005).

The domain 2OG-Fe(II) oxygenase (AlkB-like), which belongs to the 2OG-Fe(II) oxygenase superfamily, was identified in the replicase gene of GRSPaV-PN as previously reported for other isolates by Bratlie and Drablos (2005). However, using iterative database searches by Bratlie and Drablos (2005), it was found that AlkB-like domain is present in at least 22 different single-stranded positive-sense RNA viruses including GRSPaV (Zhang *et al.*, 1998), GRSPaV-1 (Meng *et al.*, 1998) and *Apple stem pitting virus* (ASPV; Jelkmann, 1994), three members of the genus *Foveavirus* in the *Flexiviridae* family.

The CP gene of GRSPaV was found to be the most conserved region in the genome sequence (Martelli and Jelkmann, 1998; Nolasco *et al.*, 2006) and the GRSPaV-PN shared amino acid identity of 91.8% with GRSPaV-SG1 to 95% with GRSPaV-BS. Multiple alignment of CP amino acid sequences showed that the N-terminus was more conserved. The motif "RR/QX-XFDF", which is conserved in the CP of all filamentous viruses (Dolja *et al.*, 1991), was identified in the CP amino acid sequence of the GRSPaV-PN sequence.

The 5' terminal sequence of RSPaV-PN was also highly conserved and may contain regulatory sequences involved in virus replication (Hull, 2002). Multiple alignments with GRSPaV-1, GRSPaV-BS and GRSPaV-SG1 revealed that 52 nt from total of 60 were identical.

In the limited field survey conducted to determine the spread of the virus, CP primers RSP 48V/49C identified GRSPaV in 80% of the tested vines and primers PN1F/2R detected GRSPaV-PN in just 13% (Table 2), showing that GRSPaV is widespread in the sampled vineyards. In addition, the high nucleotide identity between the GRSPaV-PN sequence and those resulting from sequencing PCR products with virus-specific primers indicated that these oligonucleotides were specific in identifying this viral isolate in grapevine.

The variation detected in the genome sequence of GRSPaV-PN compared to other GRSPaV isolates (Zhang *et al.*, 1998; Meng *et al.*, 1998, 1999, 2005; Lima *et al.*, 2006) and the presence of a very well defined AlkB domain, which has been suggested to counteract methylation, the host-defense mechanism, in some plant viruses (Aravind and Koonin, 2001), open to the possibility of the association of GRSPaV-PN isolate with the disease syndrome. Nevertheless, further research is needed to determine the role of GRSPaV-PN in disease syndrome and the possible involvement of another biotic agent or agents in disease development and symptoms expression.

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Received August 19, 2008
Accepted December 9, 2008