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Molecular analysis of a California strain of *Rupestris stem pitting-associated virus* isolated from declining Syrah grapevines

Brief Report

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Summary. The sequence of the genome of a Rupestris stem pitting-associated virus (RSPaV) isolated from a declining Syrah grapevine in California, designated the Syrah strain (RSPaV-SY) was determined. The genome of this strain had an overall nucleotide identity of 77% in comparison with RSPaV sequences in GenBank; the coat protein was the most conserved gene among RSPaV sequences and the replicase was the least conserved gene. Phylogenetic analysis of partial coat protein and replicase gene sequences showed RSPaV-SY clustered independently from the majority of RSPaV isolates.

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Rupestris stem pitting (RSP) is one disease in the rugose wood disease complex of grapevines [2]. The other diseases are corky bark, Kober stem grooving, and LN33 stem grooving [4]. RSP is characterized by pitting symptoms below inoculum chips in St. George grapevines (*Vitis rupestris* Steele). Chronic infections produce stunting and slow decline [3]. The virus associated with the disease is Rupestris stem pitting-associated virus (RSPaV), which is a member of the genus *Foveavirus* [5] in the family *Flexiviridae* [1]. RSPaV is restricted to grapevines, is not mechanically transmissible [5], and is not known to spread naturally. The virus is found in pollen grains [9] and seeds [10], but these sources do not give rise to infected seedlings [6]. The objectives of this study were to sequence the genome of a strain of RSPaV isolated from a diseased Syrah grapevine (RSPaV-SY) and to use it for sequence comparisons with other RSPaV strains and for design of specific primers to assay field collections for the presence of this virus strain.

The source of RSPaV-SY was a field selection of *V. vinifera* L. variety Syrah exhibiting weak growth and red canopy with an enlarged scion trunk immediately above the graft union. Prior to sacrificing the grape plant, canes were collected and two-node cuttings were rooted and maintained in a greenhouse as source plants. Extracts of leaf petioles were tested for RSPaV, grapevine leaf roll-associated viruses -1, -2, -2 redglobe, -3, -4, -5, -7, and -9; grapevine viruses -A, -B, and -D; grapevine fanleaf virus, Arabis mosaic virus, and tomato ringspot virus in a one-step RT-PCR assay [9]; only RSPaV was identified.

Double-stranded RNA was purified from bark tissues and leaf petioles [11] and a dsRNA of ca. 8.7 kbp in size was detected, which was used to construct a cDNA library [12]. Complementary DNA clones were d(A) tailed [13] and cloned into the Topo TA cloning vector (Invitrogen-Life Technologies). Selected clones were sequenced on both sense and anti-sense strands using vector primers T7 and T3. Existing gaps were completed using specific PCR primers designed based on known nucleotide sequences. The 5′ and 3′ terminal sequences were obtained using a 5′ rapid amplification of cDNA ends kit (Invitrogen-Life Technologies) and RT-PCR with oligo d(T)-priming, respectively. Sequence data were analyzed using the BLAST program of the National Center for Biotechnology Information (NCBI). For primer design and ORF search, the DNAsis Max Program package version 2.0 (Hitachi software Engineering Co., Middlesex, UK) was used. The RSPaV-SY sequence was assigned the accession number AY368590 in GenBank.

The complete genome of RSPaV-SY was 8,725 nt long and had a genome organization similar to RSPaV with six ORFs ([12]; Fig. 1). The RSPaV-SY nucleotide sequence showed a genomic sequence identity of 77% to each of the previously published sequences of four other RSPaV isolates (RSPaV, RSPaV-1, RSPaV-BS, and RSPaV-SG1) [7, 8, 12]; these four isolates were more closely related to each other than to RSPaV-SY, sharing a genomic sequence identity varying from 83% between RSPaV-BS and RSPaV-SG1 [8] and 98% between RSPaV and RSPaV-1 [7, 8]. The coat protein gene (ORF 5; see Fig. 1) of RSPaV-SY

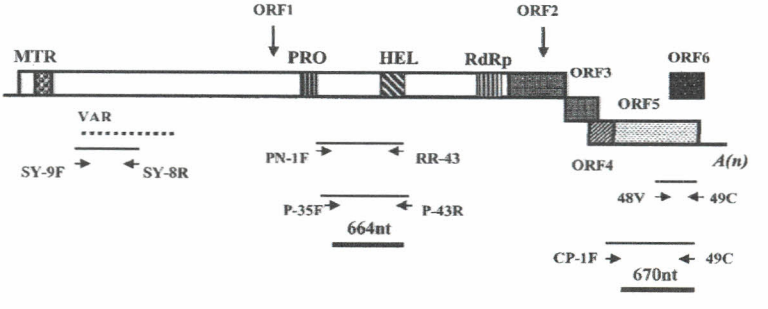

Fig. 1. Genome organization of the Rupestris stem pitting-associated virus, Syrah strain. Six open reading frames (ORF) were identified in the genome. The solid lines are the location of primers used in this report. Their respective sequences are given in Table 1. The thick lines show the size and location of the regions in the replicase and coat protein genes (ORFs 1 and 5) used for phylogenetic analysis. The broken line shows the location of the variable region (VAR) identified in the ORF1, which encodes for the replicase gene

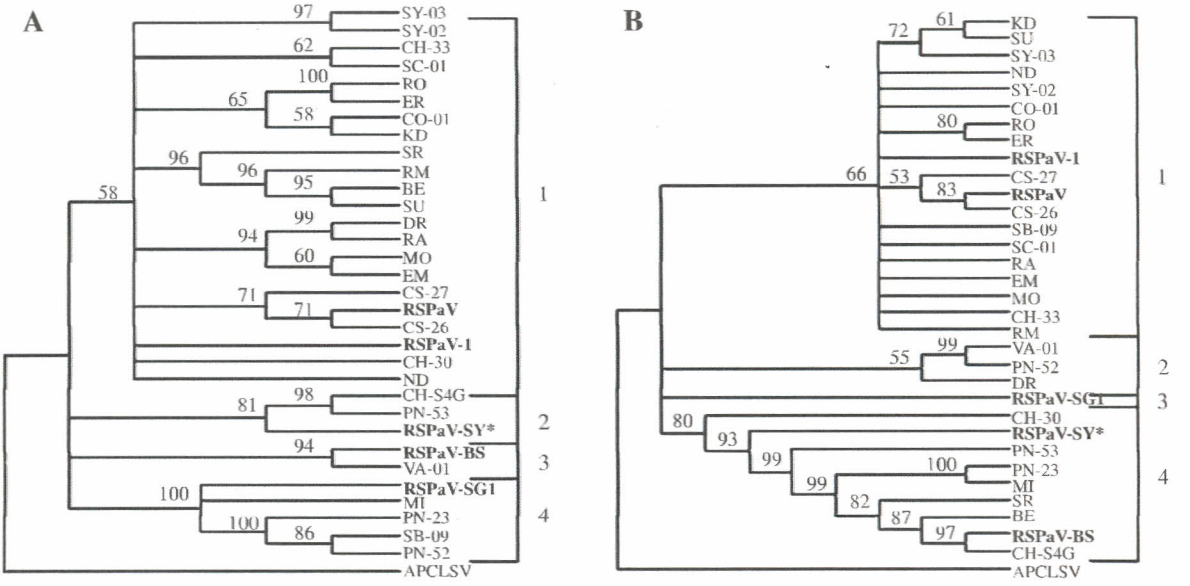

Fig. 2. Phylogenetic analysis showing the relationships among RSPaV-SY isolate (emboldened and marked with an asterisk) and other 31 RSPaV isolates including the four isolates (emboldened) for which their full-length sequences are available in the database [RSPaV (AF026278); RSPaV-1 (AF057136); RSPaV-SG1 (AY881626) and RSPaV-BS (AY881627)]. For the comparison, fragments of the replicase gene were amplified by RT-PCR using primers P-35F/P-43R or PN-1F/RR-43, and the overlapping 664-nt fragments were used in the phylogenetic analysis of ORF 1 (**A**). For the coat protein analysis, fragments were obtained with primers RSP-49C and CP-1F (Table 1). However, due to difficulties in sequencing this product in some isolates, an internal forward primer, CP-3F, was designed based on sequences of several isolates and used on the remaining isolates. Complete sequences of the resulting products (670 nt in length) were utilized in the phylogenetic analysis of the coat protein (**B**). Apricot pseudo-chlorotic leaf spot virus [APCLSV (AY713379)] in the genus *Trichovirus* was used as an out group. The phylogenetic trees were generated by the Mega 2.1 program, with assistance of the Clustal X and Genedoc programs. Horizontal distances are proportional to sequence distances. The dendrogram was bootstrapped 1000 times. Bootstrap scores are on relevant horizontal branches. Branches with less than 50% bootstrap support are presented as polytomies. RSPaV isolates used in this investigation were from the following grape selections and the corresponding database accession numbers (replicase, coat protein) are presented in parenthesis: *DR* = Damas Rose (AM180520, AM180426); *ER* = Emile Royal (AM180539, AM180438); *EM* = Emperor (AM180532, AM180419); *RM* = Red Malaga (AM180533, AM180424); *RA* = Rangspray (AM180534, AM180422); *RO* = Royal (AM180535, AM180427); *MI* = Murma Isium (AM180536, AM180423); *KD* = Kara Dzhidzhigi (AM180537, AM180437); *ND* = Noir D'automne (AM180531, AM180421); *BE* = Bellino (AM180538, AM180420); *MO* = Monukka (AM180540, AM180433); *SU* = Sultana (AM180541, AM180443); *SR* = Sultanina Rose (AM180542, AM180432); *CS-26* and *CS-27* = Cabernet Sauvignon-26 (AM180543, AM180440) and -27 (AM180521, AM180435); *CH-30* and *CH-33* = Chardonnay-30 (AM180522, AM180425) and -33 (AM180523, AM180439); *PN-23*, *PN-52* and *PN-53* = Pinot Noir-23 (AM180519, AM180418), -52 (AM180529, AM180436) and -53 (AM180524, AM180430); *SY-02* and *SY-03* = Syrah-02 (AM180525, AM180442) and -03 (AM180518, AM180431); *CO-01* = Coucoceira-01 (AM180526, AM180441); *VA-01* = Valdiguie-01 (AM180527, AM180428); *SB-09* = Sauvignon Blanc-09 (AM180528, AM180417); *SC-01* = Schiopp.-01 (AM180530, AM180434); and *CH-S4G* = Chardonnay-S4G (AM181038, AM180429)

shared the highest nucleotide and amino acid identities (83–84% and 91–92%, respectively) with other RSPaV sequences in the database [7, 8, 12], while the replicase gene (ORF1) shared the least sequence identity (75–76% and 85% at the nucleotide and amino acid levels, respectively). The most significant variation occurred in a region of 331 amino acid residues located between methyltransferase (MTR) and protease (PRO) conserved domains (See Fig. 1 – ORF1 dotted line). Amino acid sequence identity in this region was only 52–54% between RSPaV-SY and RSPaV/RSPaV-1, RSPaV-BS and RSPaV-SG1. The RSPaV-BS and RSPaV-SG1 variants shared 74 and 73% amino acid identity with RSPaV, respectively, in this region of ORF1. In contrast to the replicase gene, the 5′ and 3′ untranslated regions of the RSPaV-SY and these other RSPaV isolates shared 91–96% and 91–92% nucleotide identities, respectively. Further comparisons of the replicase gene between RSPaV-SY and the type virus of the genus *Foveavirus*, apple stem pitting virus (ASPV) [1] revealed 59% nucleotide and 42% amino acid identities.

Phylogenetic analyses of ~650-nt regions of both the replicase and coat protein genes (Fig. 1) were performed to determine the relationships between RSPaV-SY and 27 other RSPaV isolates from different grape varieties in a UC Davis collection (see legend Fig. 2). Analysis using the replicase gene sequence revealed that the RSPaV isolates segregated into four branches (Fig. 2A). The first branch was comprised of 22 isolates, including the previously sequenced RSPaV and RSPaV-1 isolates (which shared very close nucleotide identity of 98% of their complete genome). RSPaV-SY isolate, which has shown significant variability on the ORF1, clustered with only two other isolates, PN-53 and CH-S4G (branch 2). Branch 3 is formed by RSPaV-BS and VA-01 isolates, and RSPaV-SG1 with four other isolates was clustered on the fourth branch. The phylogenetic tree generated from the more conserved coat protein sequence differed to some extent in the groupings evident in the replicase sequence tree (Fig. 2B). Two main groups of isolates were identified in this analysis. One was formed by 17 isolates (branch 1) and the other by 9 isolates (branch 4). Most of the isolates in branch 1 were grouped together in both phylogenetic analyses. Branch 4 of this tree contained isolates from branches 2, 3 and 4 of the replicase gene tree. Overall, the RSPaV-SY sequence clustered with few of the other isolates in the collection and, in this regard, the previously reported genomic sequences also represent different phylogenetic groupings. Within either of the phylogenetic trees, the clustering reflected neither the geographic regions of isolation nor the grape varieties from which the isolates were made.

To investigate incidence of RSPaV-SY isolate in commercial vineyards, by RT-PCR, two pairs of primers targeting two different genes were used (Fig. 1; Table 1). One set of primers, RSP 48V/49C, was designed from a more conserved region (coat protein) and used as RSPaV-universal primers, which amplified a 331-bp fragment. The second pair, SY9F/SY8R, was designed from a more variable region (replicase gene) of the RSPaV-SY, which specifically detects this strain, and amplified a fragment of 628 bp. Among 383 plants tested, including 70 Syrah plants, 245 (64%) tested positive using RSPaV-universal primers (48V/49C); 59 of

Table 1. List of oligonucleotide PCR primers used in this study. Primers obtained from other sources are listed in the reference column

	Sequence	Location in the genome	Product size (nt)	Reference
48V	5′AGCTGGGATTATAAGGGAGGT3′	8,180–8,200	331	unpublished
49C	5′CCAGCCGTTCCACCACTAAT3′	8,509–8,528		
P-35F	5′ATGGTTGCATGATCACAGCCA3′	3,545–3,566	776	this work
P-43R	5′AGTGGCCAGCCTTCAATCC3′	4,300–4,319		
PN-1F	5′GATGGATACAAGTTACGGGC3′	3,442–3,462	868	this work
RR-43	5′ACATCCCACCCTTCCTTCTT3′	4,289–4,308		
CP-1F	5′GGTTTGAAGGCTTTAGGGGT3′	7,709–7,728	817	this work
49C	5′CCAGCCGTTCCACCACTAAT3′	8,506–8,526		unpublished
SY-9F	5′AGGATTCCAAACTGTAGAGCAA3′	2,083–2104	628	this work
SY-8R	5′TTGGTCGTCATCTTCCAGTT3′	2,689–2,710		
CP-3F	5′TGAAGAAATTGATTATC3′	7,741–7,757	–	this work

the positives were Syrah plants. Using the RSPaV-SY-specific primers (SY-9F/SY-8R), 36 (14.7%) of the RSPaV-positive samples were also positive for RSPaV-SY, including 29 of the positive specimens from Syrah plants. Thus, roughly half of the diseased Syrah plants, but very few of the other infected grape varieties (7/186), were infected with the SY strain. To confirm the specificity of the RSPaV-SY primers, twenty of the amplicons generated by these primers were sequenced and found to share about 90% nucleotide identity to RSPaV-SY and about 73% to RSPaV sequences in the database. Among the Syrah specimens in the collection tested, four asymptomatic plants (without any canopy symptoms) were included in these assays and all of the four plants tested positive for the 48V/49C universal and one for RSPaV-SY (SY-9F/SY-8R) specific primers. Research is continuing to investigate the correlation between the virus and the disease.

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