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Grapevine rupestris stem pitting-associated virus: A Decade of Research and Future Perspectives

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ABSTRACT

Grapevine rupestris stem pitting-associated virus (GRSPaV), a recently identified virus, is classified as a member of the *Foveavirus* genus within the *Flexiviridae* family. The genome of GRSPaV is a single-stranded RNA of positive polarity and encodes five open reading frames (ORFs). ORF1 codes for a replicase polyprotein, which contains sequence domains conserved among Alphavirus-like superfamily of RNA viruses. GRSPaV also encodes three movement proteins, a feature distinct from most plant RNA viruses with a single movement protein. In this communication, we review the advancements that have been made on the virus over the past decade. GRSPaV has been demonstrated to comprise a family of molecular variants. Phylogenetic analyses reveal the presence of at least four distinct variant (lineage) groups. The genome of an isolate representing each of the viral variant groups has been sequenced. It is also demonstrated that commercial grape varieties are usually infected with mixtures of distinct viral variants, whereas rootstock varieties, at least those tested, are infected with a single variant. A specific relationship between some of the viral variant groups and distinct *Vitis* species seems to exist. Based on available information, a hypothetical model is proposed to explain the possible origin and evolution of different GRSPaV strains. The possible role of GRSPaV in the diseases Rupestris Stem Pitting and Vein Necrosis, as well as its economic importance are discussed. Lastly, we present our views on future directions for GRSPaV research.

Keywords: *Flexiviridae*, *Foveavirus*, genome structure, genetic diversity, evolution, *Vitis vinifera*, *V. sylvestris*, *V. rupestris*, *V. riparia*

CONTENTS

BACKGROUND

Grapevines (*Vitis vinifera* L., family *Vitaceae*) have been cultivated by humanity for about 5,000 thousand years (Reisch and Pratt 1996; This 2006). Today, grape is perhaps the most widely grown fruit crop in the world. Perhaps due to the prolonged history of cultivation, grapevines are known to be host to a large number of viruses from different taxonomic groups. So far, 55 distinct viral species belonging to 20 genera and seven families have been isolated from grapevines (Martelli 2003) and the number continues to rise. However, viral infections usually remain asymptomatic except for some viruses such as nepoviruses and closteroviruses. Consequently, the economic importance of many grapevine viruses has not been clearly recognized.

This situation took a sharp turn when the insect phylloxera (*Daktulosphaira vitifoliae*) was accidentally introduced into Western Europe in 1860's, which devastated grape growing within a few years (Goheen 1989). To combat phylloxera and other adversities, grapevine species that are native to North America were brought into Europe to be used as rootstocks. Since then, new viral diseases have been discovered. Thus, the recognition of many of these new diseases is attributable to the use of rootstocks in modern viticulture. As a result of the complexity of viruses that infect grapevines and the practice of grafting, a single grapevine may be infected with a mixture of viruses and viral strains. Mixed infections in turn lead to disease complexes, with symptoms of a specific grapevine varying according to the combination of viruses and viral strains infecting the plant. Consequently, the etiological role of many of the grapevine viruses has remained unknown. This situation is reflected in the names of many of the viruses, which contain the word "associated".

Grapevine rupestris stem pitting-associated virus (GRSPaV) is a good example of a virus that has been widely detected in grapes but its etiological role in a specific disease cannot be firmly established. Interests of research on this virus sprung from a graft-transmissible disease, Rupes-

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Invited Review

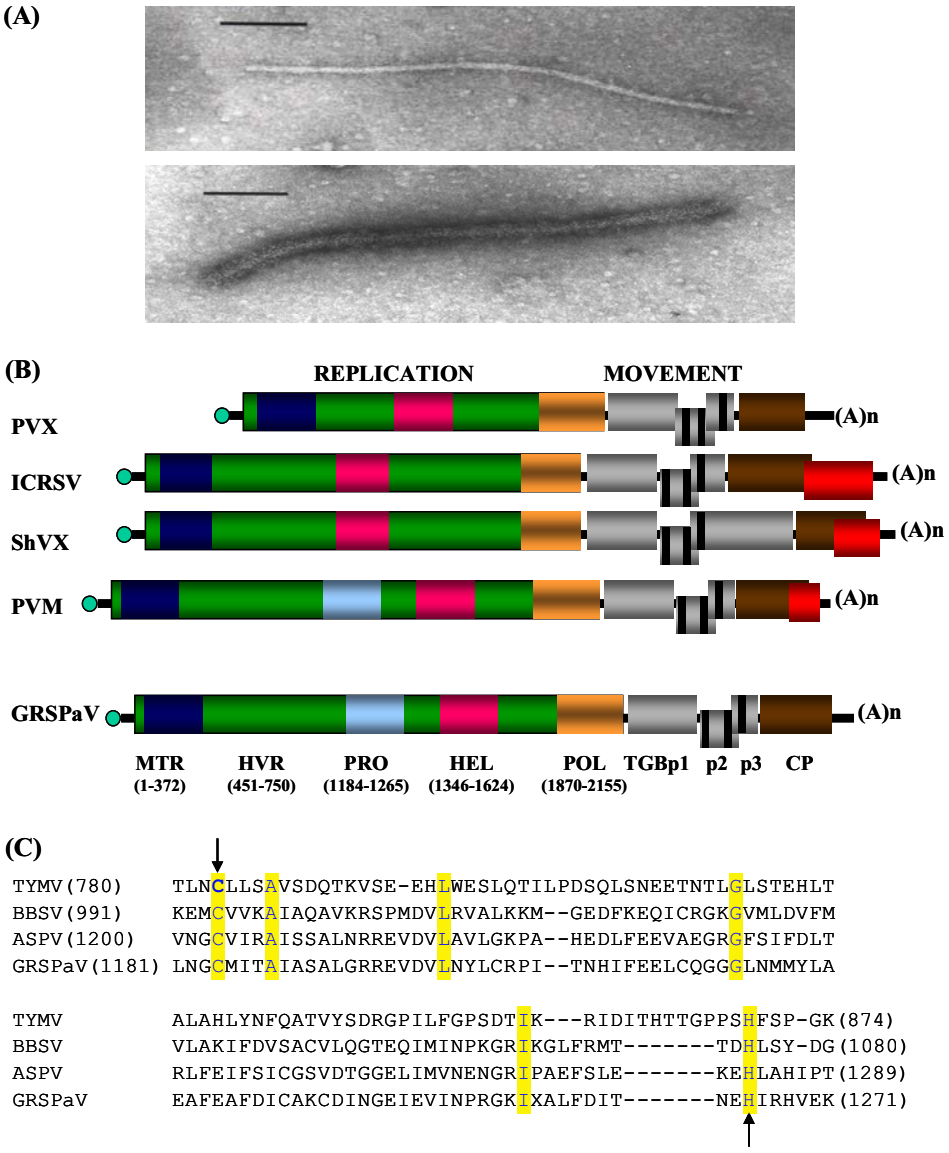

Fig. 1 (**A**) Virion morphology. A virion of *Grapevine rupestris stem pitting-associated virus* before and after decoration with polyclonal antibodies As7-276 raised against a recombinant coat protein is shown in the top and the bottom panels respectively. Bar represents 100 nm. (**B**) Genome structure of GRSPaV and viruses representing each of the five genera of the family *Flexiviridae*. PVX: *Potato virus X* (*Potexvirus*); ICRSV: *Indian citrus ringspot virus* (*Mandarivirus*); ShVX: *Shallot virus X* (*Allexivirus*); and PVM: *Potato virus M* (*Carlavirus*). MTR: methyl transferase; HVR: highly variable region; PRO: papain-like cysteine protease; HEL: RNA helicase; POL: RNA-dependent RNA polymerase; TGBp: triple gene block protein; CP: capsid protein. (**C**) Alignment of the amino acid sequences corresponding to the catalytic sites of the papain-like cysteine protease domains of GRSPaV and those of ASPV (*Apple stem pitting virus*, *Foveavirus*), TYMV (*Turnip yellow mosaic virus*, *Tymovirus*) and BBSV (*Blue berry scortch virus*, *Carlavirus*). The alignment was done using Jotun Hein (DNAStar). The catalytic diad composed of *cysteine* (C_{1184}) and *histidine* (H_{1265}) are depicted by arrows.

tris stem pitting (RSP), which was identified in the late 1970's in California by plant pathologist Austin C. Goheen. RSP is defined as a disease that produces a strip of small pits on the woody cylinder of the indicator *V. rupestris* cv. 'St. George' after graft-inoculation (Goheen 1989). An indicator indexing survey revealed that RSP was prevalent among grapevine selections imported from Europe and Australia. For example, 66% of 70 grapevine selections from France, 42% of 53 selections from Germany, and 67% of 33 selections from Australia were tested positive for RSP (Goheen 1989). For a long time, the only method to diagnose the disease was indexing using the woody indicator 'St. George', which takes two to three years to complete. The lack of information on the etiology of RSP precluded development of quicker and more sensitive diagnostic methods. Clearly, there was an urgent need for resolving the etiology of RSP.

The advent of recombinant DNA technology offered hope to tackle this problem. Through cloning of dsRNAs that were associated with RSP, two research groups independently sequenced the genomes of two GRSPaV isolates in 1998 (Meng *et al*. 1998; Zhang *et al*. 1998). This information has allowed researchers to pursue several lines of investigation on the virus, including detection using RT-PCR and serological methods, genetic diversity and population structure, and to a lesser extent transmission, and assessment of its economic impact. As a result, significant progress has been made in these areas. The purpose of this review is to summarize current knowledge on the genome structure, gene expression, and genetic diversity of GRSPaV. Based on available information, we propose a model to explain the possible origin and evolution of different GRSPaV strains. We also provide our perspectives for future research. The development and utilization of molecular detection methods have been discussed previously (Meng and Gonsalves 2003, 2007) and will not be covered here.

TAXONOMY AND NOMENCLATURE

GRSPaV contains a positive-strand RNA genome, which is encapsidated in a filamentous virion of 723 nm in length (**Fig. 1A**; Petrovic *et al*. 2003). GRSPaV is classified as a member of the *Foveavirus* genus that currently contains *Apple stem pitting virus* (ASPV) and *Apricot latent virus* (Martelli and Jelkmann 1998; Adams *et al*. 2005). The family *Flexiviridae* was erected in 2004 to contain *Foveavirus*, *Potexvirus*, *Carlavirus*, *Allexivirus*, *Mandarivirus* (**Fig. 1B**) and three other genera. Members of *Flexiviridae* share the following characteristics: (1) virions are flexuous filaments, 10-15 nm in diameter and 470-1,000 nm in length; and (2) their genomes are positive-strand RNA, 5.9-9.0 kb in length and encode 3-6 open reading frames (ORF) depending on the genus (Adams *et al*. 2005). The RNA-dependent RNA polymerases (POL) encoded by viruses of this family belong to the Alphavirus-like superfamily of RNA viruses (Koonin and Dolja 1993; Strauss and Strauss 1994). It is worth noting that five of the eight genera included in *Flexiviriade* possess the triple gene block (TGB) genetic module that encode three movement proteins (MP), whereas *Viti-*

virus, *Capillovirus* and *Trichovirus* lack the TGB structure but encode a single MP of the '30K' superfamily (Adams *et al*. 2005).

Since the identification of GRSPaV, two slightly different names have been used in the literature. The first is "Rupestris stem pitting-associated virus-1" (RSPaV-1) as proposed by Meng *et al*. (1998), while the second is "Grapevine rupestris stem pitting-associated virus" [GRSPaV, Zhang *et al*. (1998)]. The current official name designated by The International Committee for the Taxonomy of Viruses (ICTV) is "*Rupestris stem pitting-associated virus*" (Adams *et al*. 2005). To render the virus name more intuitive and considering precedence in the nomenclature of grapevine viruses, a proposal was put forth to ICTV to change the official name of the virus to "Grapevine rupestris stem pitting-associated virus". This proposal has been approved by the Executive Committee of the ICTV (Martelli, pers. comm.). Hence, we have used GRSPaV previously (Meng *et al*. 2005, 2006) as well as throughout this review.

GENOME STRUCTURE AND EXPRESSION

The genome of GRSPaV is composed of 8,725 nucleotides (nts) and encodes five ORFs. The 5′ most terminal nucleotide is a *guanosine* and is presumably capped. The 5′ noncoding region (NCR) consists of 60 nts. The 3′ NCR is 176 nts and is polyadenylated. The genome structure of GRSPaV resembles those of viruses of the genera *Potexvirus*, *Carlavirus*, *Allexivirus*, and *Mandarivirus* (**Fig. 1B**), all within the family *Flexiviridae* (Adams *et al*. 2005). ORF1 occupies three-fourths of the entire viral genome (nt positions 61-6546), and encodes a large polypeptide of 2,161 amino acids (aa) with a calculated Mr of 244 kDa (Meng *et al*. 1998; Zhang *et al*. 1998). The translation product of ORF1 contains all of the signature domains that are conserved among the replicase proteins of the Alphavirus-like superfamily of RNA viruses (Koonin and Dolja 1993; Straus and Straus 1994): a methyl transferase (MTR) (Rozanov *et al*. 1992), a superfamily I (SF-1) RNA helicase (HEL; Kadare and Hainni 1997), a papain-like cysteine protease (PRO, Gorbalenya *et al*. 1988), and an RNA-dependent RNA polymerase (POL) (Koonin 1991). Presumably, the MTR domain is responsible for the 5′ cap structure of both genomic and subgenomic RNAs, while the HEL domain is responsible for unwinding the replicative form dsRNAs during genome replication. Based on phylogenetic analysis using the POL domain, GRSPaV seems to be more closely related to members of *Carlavirus* (Zhang *et al*. 1998; Meng and Gonsalves 2003).

Papain-like cysteine proteases are encoded by a wide range of positive-strand RNA viruses, including *Carlavirus* (Lawrence *et al*. 1995), *Potyviridae* (HC-Pro), *Tymoviridae*, *Alphavirus* (nsP2, *Togaviridae*), as well as dsRNA-containing *Hypoviridae* (p29) (reviewed in Dougherty and Semler 1993). A high-resolution crystal structure of the nsP2 protease of *Venezuelan equine encephalitis virus* has been determined recently (Russo *et al*. 2006), which is the first viral cysteine protease whose molecular structure at the atomic level has ever been resolved. Sequence comparison reveals that the PRO domain of GRSPaV contains the catalytic dyad composed of *cysteine* (at amino acid position 1184) and *histidine* (at amino acid position 1265). Furthermore, the space between these two amino acid residues (80 amino acid residues) falls within the range for the cysteine proteases encoded by other RNA viruses (**Fig. 1C**). The presence of the PRO domain suggests that ORF1 of GRSPaV is translated into a polyprotein precursor that may undergo proteolytic processing to generate two or more mature protein products. Those cleavage products in turn interact with each other and likely with host factors to form a functional replication machinery. Preliminary results obtained from an *Escherichia coli* expression system seem to suggest that the PRO domain is functional (our unpublished data).

Located at the 3′ terminal region of the viral genome is ORF5, which encodes the capsid protein (CP) of 28 kDa. This was first suggested by the presence of the amino acid residues "R/QX—XFDF" (X represents any amino acid residue), a sequence motif that is conserved in the CPs of filamentous viruses with positive-strand RNA genomes. These amino acid residues were proposed to form a "salt" bridge (Dolja *et al*. 1991). This initial prediction has been confirmed with two lines of experimental evidence: polyclonal antibodies raised against a recombinant CP of GRSPaV clearly decorated particles of the virus (**Fig. 1A**; Petrovic *et al*. 2003); and the expected polypeptide of 28 kDa was consistently detected in GRSPaV-infected grapevines in Western blot using the polyclonal antibodies (Meng *et al*. 2000; Minafra *et al*. 2000; Meng *et al*. 2003). Interestingly, a smaller polypeptide of ca. 24 kDa was also detected in tissues of GRSPaV-infected grapevines. The nature of this smaller protein is unknown but it was proposed to be the degradation product of the full-length CP during sample processing (Meng *et al*. 2003).

Between ORF1 and ORF5 lies a genomic region that encodes three ORFs, commonly known as the Triple Gene Block (TGB). TGB is a conserved genetic feature identified in the genomes of viruses of five genera within *Flexiviridae* as described earlier. Similar genetic structures are also found in rod-shaped viruses with either bipartite (*Pecluvirus*) or tripartite (*Hordeivirus* and *Pomovirus*) RNA genomes. Recent research suggests that TGB encodes three polypeptides (referred to as TGBp1, TGBp2 and TGBp3) that are involved in the intra- and inter-cellular movement of ribonucleo-protein (RNP) complexes within infected plants (Davis *et al*. 1993; Lough *et al*. 1998; Santa Cruz *et al*. 1998; Verchot *et al*. 1998; Morozov and Solovyev 2003). TGBp1 is encoded by ORF2 and has a molecular mass of 24.4 kDa. Based on presence of the seven signature motifs conserved in SF-1 RNA helicases, TGBp1 is considered a SF-1 helicase (Morozov and Solevyev 2003). Thus, the genome of GRSPaV encodes two divergent copies of the SF-1 RNA helicase. However, the evolutionary relationship between TGBp1 and the HEL domain embedded in the replicase has yet to be determined. The conserved tripeptide "GKS" is located within Motif I (Meng *et al*. 1998; Zhang *et al*. 1998), which corresponds to the Walker A site of ATP-binding proteins and is presumably responsible for binding ATP and Mg^{2+} (Soultanas and Wigley 2001; Morozov and Solovyev 2003). Shortly after Motif I is the "DE" signature sequence of Motif II (the Walker B site), which is believed to be the catalytic site (Caruthers and McKay 2002). It is proposed that TGBp1 is involved in the translocation of itself and the newly synthesized viral RNP complexes across plasmadesmata, a process that likely requires energy generated by the ATPase function of TGBp1. Like other viral movement proteins, TGBp1 binds ssRNAs in a non-specific but cooperative manner. TGBp1 can also increase the size exclusion limit of plasmadesmata (Morozov and Solovyev 2003).

However, the mechanism with which TGBp1 helps translocate RNP complexes is unknown. Two possibilities exist: (1) TGBp1 may disrupt partial duplex structures that are present in nascent progeny RNAs, thus making them thin and linear molecules suitable for translocation through plasmadesmata; (2) TGBp1 may interact with and displace other viral and/or cellular proteins from viral RNAs during the process of translocation. It has been shown recently that TGBp1 binds to one end of the filamentous virions of PVX (Atabekov *et al*. 2000). It has also been shown that TGBp1 functions as a suppressor of the host RNA silencing, which is a pre-requisite for successful cell-to-cell movement of PVX (Bayne *et al*. 2005). Thus, TGBp1 is another example of a viral protein that possesses multiple functions.

TGBp2 is encoded by ORF3 and is 12.8 kDa in size. Based on *in silico* sequence analysis, TGBp2 contains two putative trans-membrane domains (TMD), one at each terminus. TGBp3 is encoded by ORF4 and has a molecular mass of 8.4 kDa. Similar to its homologues in other viruses, TGBp3 contains one putative TMD in its N terminus (Meng

et al. 1998; Zhang *et al*. 1998).

Recent studies on the intracellular localization of PVX suggest that TGBp1 is a cytosolic protein that increases the size exclusion limit of, and moves to adjacent cells through, plasmadesmata (Howard *et al*. 2004), while TGBp2 and TGBp3 together associate with internal membranes of the infected cell and translocate viral RNP complexes to, and across, plasmadasmata (Morozov and Solovyev 2003; Lucas 2006). Since the TGB proteins of GRSPaV resemble those of PVX in size and structure, we predict that they would function similarly as their counterparts in PVX. Based on computational analysis, TGBp3 seems to contain a signal that targets plasmadesmata, while TGBp2 helps recycle TGBp3 back for the translocation of additional viral RNP complexes. We have recently made protein expression constructs for each of the TGB proteins and for their fusions to auto-fluorescent protein tags. The sub-cellular localization of each individual protein and their combinations are being investigated in our laboratory using *Nicotiana tabaccum* BY-2 cells and fluorescence microscopy.

How these proteins are expressed from the GRSPaV genome during the virus replication cycle is virtually unknown. By default, ORF1 would be translated directly on the incoming genomic RNA to produce the replication-related polyprotein precursor. Co-translational proteolytic cleavage by the protease would cleave the polypeptide into two or more mature protein products. In line with this hypothesis, we recently detected the translation product of ORF1 in transfected BY-2 cells with immuno-fluorescence microscopy using polyclonal antibodies that were raised against a synthetic peptide derived from the POL domain (our unpublished data). By analogy to PVX (Verchot *et al*. 1998), we would predict that polypeptides corresponding to ORF2-ORF5 are likely expressed from three sub-genomic (sg) RNAs that share the 3′ terminal sequence with the genomic RNA. For example, TGBp1 is likely translated from the first sgRNA (which is approximately 2,149 nts in length), TGBp2 and TGBp3 are translated via the ribosome leaky scanning mechanism from the second sgRNA (which contains about 1,482 nts), while the CP is produced on the third sgRNA (which is approximately 956 nts in size). In addition to these five ORFs, Zhang *et al*. (1998) identified ORF6 at the 3′ terminal end of the viral genome. If proven to be functional, the polypeptide encoded by ORF6 should also be translated from another sgRNA. Nolasco *et al*. (2006) suggested that ORF6 might not encode a protein based on the lack of selection pressure on this potential gene. Of course, the above predictions need to be verified with experimental evidence.

HOST RANGE AND TRANSMISSION

Natural infection of GRSPaV is restricted to grapevines (*Vitis spp*.) (Martelli and Jelkmann 1998; Meng and Gonsalves 2007). So far, GRSPaV has been detected in many cultivated *V. vinifera* varieteis, *V. sylvestris*, *V. rupestris*, *V. riparia* and French-American hybrids. Despite repeated efforts to transmit GRSPaV into herbaceous experimental hosts through mechanical inoculation, it remains unknown whether it can infect and replicate in any experimental host species (our unpublished data).

Dissemination and transmission of GRSPaV are achieved primarily by human activities through the use of infected propagating materials and grafting between scion varieties and rootstocks. In fact, the frequent global exchange of propagating materials and grafting may have been responsible for the worldwide distribution of GRSPaV and mixed infections of common scion varieties that are used as wine grapes. Preliminary research seems to point to the possibilities that GRSPaV is transmissible through pollen (Rowhani *et al*. 2000) and seeds (Steward and Nassuth 2001). More recently, Lima *et al*. (2006b) detected GRSPaV in 0.4% of the seedlings obtained from seeds collected from GRSPaV-infected 'Cabernet Sauvignon'. Evidently, these aspects need to be further tested. It also remains to be investigated if GRSPaV is transmitted in nature by a yet to be identified insect vector.

GENOME SEQUENCES AND COMPARISON OF FOUR GRSPaV STRAINS

So far, the entire genomes of five GRSPaV isolates have been sequenced. The majority of the genome sequence of the first isolate was obtained from overlapping cDNA clones derived from pooled dsRNA preparations isolated from French-American hybrids 'Colobel 257', 'Seyval', 'Ravat 34', 'Couderc 28-112', 'Seyve Villard 14-287', 'Seyve Villard 3160', 'Bertelle Seyve 5563' and 'Bertille Seyve 3408'. The remaining gaps between these cDNA clones were bridged through RT-PCR using dsRNAs isolated from 'Colobel 257' (Meng *et al*. 1998). We have designated this isolate GRSPaV-1. In the same year, the genome sequence of the second isolate was determined based on dsRNAs isolated from *V. vinifera* cv. 'Cabernet Sauvignon' (Zhang *et al*. 1998). Surprisingly, these two isolates have an overall nucleotide sequence identify of 98%. As such, we consider these two isolates as the same strain. Meng *et al*. (2005) sequenced the genomes of two more isolates: GRSPaV-SG1 was sequenced from *V. rupestris* cv. 'St. George', while GRSPaV-BS was from French-American hybrid 'Bertille Seyve 5563'. Interestingly, GRSPaV-SG1 was obtained from 'St. George', which has been used in many countries as the standard biological indicator for indexing RSP as well as for two other diseases: Grapevine Fanleaf and Grapevine Fleck (Martelli 1993). It has also been used as one of the common rootstocks for growing wine grape varieties. More recently, the genome of another variant, GRSPaV-SY, was sequenced from *V. vinifera* cv. 'Syrah' (synonym. 'Shiraz'), which was exhibiting decline symptoms in California (Lima *et al*. 2006a).

As expected, the four strains have identical genome structures. When the entire genome sequences are compared, the four strains have nucleotide sequence identities ranging from 77.1 to 87.3%. GRSPaV-SY seems to be most divergent since it has the lowest level of sequence identities compared to the other three strains. The 5′ NCR is the most conserved among the four strains with 93.3-98.3% identities (**Table 1; Fig. 2A**). In contrast, the 3′ NCR seems to be

Table 1 Sequence comparison of four strains of *Grapevine rupestris stem pitting-associated virus* (GRSPaV).

	GRSPV-1/SG1		GRSPV-1/BS		GRSPV-1/SY		GRSPV-BS/SG1		GRSPV-BS/SY		GRSPV-SY/SG1	
	NT	AA	NT	AA	NT	AA	NT	AA	NT	AA	NT	AA
Overall	87.3	-	84.3	-	77.1	-	83.9	-	77.6	-	77.3	-
5′ NCR	98.3	-	96.7	-	93.3	-	98.3	-	96.7	-	95.0	-
3′ NCR	91.8	-	84.0	-	78.2	-	80.9	-	77.7	-	77.7	-
ORF1	86.5	92.2	85.5	92.7	75.6	85.2	85.0	91.6	76.3	85.1	76.0	85.1
ORF2	87.2	93.2	78.5	86.9	77.0	85.1	78.2	86.5	77.9	85.1	75.4	83.8
ORF3	91.2	98.3	80.8	89.8	80.2	85.6	79.9	88.1	78.8	86.4	78.5	83.9
ORF4	91.4	88.9	83.1	88.9	81.5	82.7	84.0	86.4	79.4	76.5	80.7	77.8
ORF5	90.6	96.2	82.3	92.7	84.5	92.7	81.7	90.8	83.8	92.3	84.5	91.2

GRSPaV-SG1: the major variant detected in the indicator *Vitis rupestris* "St. George" (Meng *et al*. 2005); GRSPaV-1: the first variant for which the genome was sequenced (Meng *et al*. 1998); GRSPaV-BS: the variant sequenced from French-American hybrid "Bertille Seyve 5563" (Meng *et al*. 2005); GRSPaV-SY: the variant sequenced from "Syrah" with declining syndromes (Lima *et al*. 2006a). Listed are percent identities of the entire genome sequences, their non-coding regions (NCR), and individual open reading frames (ORF). NT: nucleotide sequence; AA: amino acid sequence; –: not applicable.

(A)

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GRSPaV-1 5'NCR     GATAAACATAACAACAGAATTTGCATTGCA
GRSPaV-BS 5'NCR    GATAAACATAACAACAGAATTTGCATTGCA
GRSPaV-SG1 5'NCR   GATAAACATAACAACAGAATTTGCATTGCA
GRSPaV-SY 5'NCR    GATAAACATAACAACAGAAATTGCATTGCA

GRSPaV-1 5'NCR     GTAATATTCCTTGAATATAATTGCAACGCA
GRSPaV-BS 5'NCR    GTAATATTCTTTGAATATAATTGCAACGTA
GRSPaV-SG1 5'NCR   GTAATATTCCTTGAATATAATTGCAACGTA
GRSPaV-SY 5'NCR    GTAATATTCTTTGAATATAATTGCAACGTG
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Fig. 2 Alignments of the 5′ non-coding regions (NCR) (A) and the 3′ NCRs (B) of four GRSPaV strains. Note that the beginning region of the 3′ NCR is highly variable among the four strains. Shaded areas indicate positions where the nucleotide residues differ among isolates. The alignments were done using Clustal W (DNAStar). The boxed region represents the 21 nt residues also conserved in *Potato virus M* (*Carlavirus*) that was previously identified (Meng *et al*. 1998).

(B)

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GRSPaV-1 3'NCR     GGATGACGAAGTCAGCGACAATTCCGCAGTCC
GRSPaV-BS 3'NCR    GGTTGATGGTGGAAGCAATGCTCCTTCATCCA
GRSPaV-SG1 3'NCR   AGAGGATAAAGATGATGGCATTTCCGTAGTCC
GRSPaV-SY 3'NCR    GGTGCATAAAGGGGGCGGTGGCCCTCAGCGTT

GRSPaV-1 3'NCR     AATAATTCCCCGATTTCAAGGCTGGGTTAAGC
GRSPaV-BS 3'NCR    AATAATCACCCGATTTCAAGGCTGGGTTAAGC
GRSPaV-SG1 3'NCR   AATAATTCCCCGATTTCAAGGCTGGGTTAAGC
GRSPaV-SY 3'NCR    AATAATTCCCCGATTTCAAGGCTGGGTTAAGC

GRSPaV-1 3'NCR     CTGTTCGCTGGAATACCGTACTAATAGTATTC
GRSPaV-BS 3'NCR    CTGTTCGCTGGAATACCGTACTAATAGTATTC
GRSPaV-SG1 3'NCR   CTGTTCGCTGGAATACCGTACTAATAGTATTC
GRSPaV-SY 3'NCR    CTGTTCGCTGGAATACCGTACTAATAGTATTC

GRSPaV-1 3'NCR     CCTTTCCATGCTAAATCCTATTTAATATATAA
GRSPaV-BS 3'NCR    CCTTTCTGTGCTAAATCCTATTCAATATATAA
GRSPaV-SG1 3'NCR   CCTTTCCATGCTAAATCCTATTTAATATATAA
GRSPaV-SY 3'NCR    CCTTTCTATGCTAAATCCTATTTAATACATAA

GRSPaV-1 3'NCR     GGTGTGGAAAGTAAAAGAAGATTTGGTGTGTT
GRSPaV-BS 3'NCR    AGCATGGAAGGTTAAATAAATTTTGTGTGTTT
GRSPaV-SG1 3'NCR   GGTGTGGAAAGTTAAAGAAGCTTTGGTGTGTT
GRSPaV-SY 3'NCR    GGCATGGAAAGTAAAATAAATTTTGTGTGTT

GRSPaV-1 3'NCR     TTTATAGTTTTCATTCAAAAAAAAAAAAAAAA
GRSPaV-BS 3'NCR    TTATAGTTTTCACTCAAAAAAAAAAAAAAAA
GRSPaV-SG1 3'NCR   TTTATAGTTTTCACTCAAAAAAAAAAAAAAAA
GRSPaV-SY 3'NCR    TTTATAGTTTTCGCGCAAAAAAAAAAAAAAAA
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more variable, with 77.7-91.8% identities (**Table 1**). The majority of the variations are located in the beginning region of the 3′ NCR (**Fig. 2B**). The stretch of 21 nucleotides, previously identified to be conserved also in *Potato virus M* (*Carlavirus*) (Meng *et al*. 1998), was highly conserved among the four strains (**Fig. 2B**). As for the genomic regions that encode proteins, ORF5 (the CP gene) is the most conserved, whereas ORF4 is the least conserved (**Table 1**). A highly variable region is identified within ORF1, which is located at amino acid positions 451-750 flanking the more conserved MTR and PRO domains (**Fig. 3**; Meng *et al*. 2005). This region has amino acid identities of 49.5-72.9% and nucleotide identities of 60.6-76.4% among the four strains. In contrast, the region encoding the POL domain seems to be highly conserved not only among the fours strains of GRSPaV, or other members of the *Foveavirus* genus, but also among some members of the *Carlavirus* genus (our unpublished data).

GENETIC DIVERSITY AND POPULATION STRUCTURE

It is evident that GRSPaV exhibits a large degree of genetic variation, comprising a wide range of sequence variants. High levels of genetic diversity were initially recognized when the first GRSPaV isolates were being sequenced. The cDNA clones that were selected from the cDNA library after a single plaque hybridization experiment turned out to be different in nt sequence (Meng *et al*. 1998, 1999b). This heterogeneity in sequence hindered the progress of genome sequencing. Similarly, Zhang *et al*. (1998) reported that clones derived from the RT-PCR products were 82-99% identical among themselves. To dissect the composition of viral population present within a single grapevine, Meng *et al*. (1999b) used primers RSP9 and RSP10 in RT-PCR to amplify from dsRNAs isolated from *V*. *vinifera* cv. 'Cabernet Franc' and 'Chardonnay', and French-American hybrid 'Amania'. This pair of primers target a 498 nt region spanning the 3′ terminal part of ORF1 and the 5′ terminal part of ORF2. Resulting cDNA products were cloned and se-

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GRSPaV-1 HVR   ENVEEVMDNSWFGLGDLQFNRQRAPFFLGSSYWLNSKFSVEHKFSSTINS
GRSPaV-BS HVR  .....................................I.....GA..C
GRSPaV-SG1 HVR ..I.V......S....P.........L.................GA..F
GRSPaV-SY HVR  D.............SNA..D...C..S..P.H..G....I.....G.V.H

GRSPaV-1 HVR   QIMQVILSLIPFSDDPTFRPSSTEVNLALSEVKAALAATGQSKLFRFLVD
GRSPaV-BS HVR  ...........L.G.......PI.........RT..K.....R..S....
GRSPaV-SG1 HVR ..I.AV.....L.C........M......LK.....R........S....
GRSPaV-SY HVR  .VLHAA...F.L.S..S.....N.I.FV..GI.M..GVA.........I.

GRSPaV-1 HVR   DCVMREVRSSYKVGLFKHIKALTHCFNSCGLQWFLLRQRSNLKFLKNRAS
GRSPaV-BS HVR  ...IQ..Q..............AYF.....................G...
GRSPaV-SG1 HVR .G.....QK...........S..Y............KR........D.V.
GRSPaV-SY HVR  .IFIN.LKL..RSR.A..LR...KF.HF........K..I......GSET

GRSPaV-1 HVR   SFADLDCEVIKVYRFVTSQAILPEALLSLTKVFVRDSDSKGVSIPRLVSR
GRSPaV-BS HVR  .LV..N..A.RM..L......T.G.R....R..IWE..MENTFTHE.T.K
GRSPaV-SG1 HVR ...N.S.....A.KS.VL..T............I....LN.AFNSE.A.H
GRSPaV-SY HVR  ..VE.S.G.A.L.KS.IL.VSS.N..SG.AR.Y.DH.S.QLC.GSHFM.D

GRSPaV-1 HVR   DELNELAHPANSVLEEPQSVDCNAGRVQASVSSSQQLADTHSLSSVKSSI
GRSPaV-BS HVR  NDPTG.V...GPTP..S...A.DVSETPEGI..A.LP....L..D..LPV
GRSPaV-SG1 HVR ..SIR.VGS.G.TS.KS...S.E.S..LVNS..T.PP.HNLPV.N...P.
GRSPaV-SY HVR  ...PNP.LAD.ITS.ASL.E..QGFQTVEQAACAHPIN.ALCIDLAPP..

GRSPaV-1 HVR   ETANKAFNLEELRIMIRVLPEDFNWVVKNIGFKDRLRGRGASFFSKPGI
GRSPaV-BS HVR  ..TE.F...G.....V...........R....E...K............
GRSPaV-SG1 HVR K..E.V.D.G..K.AVK.....I.............K............
GRSPaV-SY HVR  KKIE.S.C.G..T.PVG...KP.D......S.N.K....R.........
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Fig. 3 Alignment of amino acid sequences of a highly variable region (HVR) of four GRSPaV strains. This region is located at aa positions 451-750 (corresponding to nt positions 1354-2250) of the replicase polyprotein, which is flanked by the conserved MTR and PRO domains. The amino acid sequence of GRSPaV-1 is shown as the top line. Identical amino acid residues are shown as dots while different residues are given as single letter symbols. Alignments were based on the Clustal W (DNAStar).

Table 2 Distribution of sequence variant groups of *Grapevine rupestris stem pitting-associated virus* (GRSPaV) in grapevine scion and rootstock varieties.

	GRSPV-1	GRSPV-SG1	GRSPV-BS	GRSPV-VS
Scion Varieties				
Niagara (Concord x Cassady)	+	+	+	+
Seyval (Seibel 4995 x Seibel 4986)	+	+	+	-
Ravat 34 (Chardonnay x *V. berlandieri*)	+	+	+	-
Pagadebit 2 (*V. vinifera*)	+	+	-	-
Trebbiano 12 (*V. vinifera*)	-	+	+	-
Merlot (*V. vinifera*)	-	+	+	+
Pinot Noir (*V. vinifera*)	-	+	-	+
Colobel 257 (Seibel 6150 x Seibel 5455)	+	+	-	-
Seyve Villard 3160 (Seibel 5163 x Seibel 2049)	+	+	-	-
Canino 9 (*V. vinifera*)	-	+	-	-
Pione (*V. vinifera* x *V. labrusca*)	+	+	?	+
Kyoho (*V. vinifera* x *V. labrusca*)	?	?	+	+
Rootstocks and Wild Grapevines				
Grande Glabre (*V. riparia*)	+	-	-	-
Kober 5BB (*V. berlandieri* x *V. riparia*)	+	-	-	-
Millardet 101-14 (*V. riparia* x *V. rupestris*)	+	-	-	-
Paulsen 1103 (*V. berlandieri* x *V. rupestris*)	-	+	-	-
St. George (*V. rupestris*)	-	+	-	-
V. sylvestris	-	-	-	+

Data were compiled from cDNA clones derived from RT-PCR with primers RSP13 and 14 and RSP21 and 22. Notice that ten isolates from ten 'St. George' plants were assayed. Adapted from Meng *et al*. (2006). Information for Japanese varieties "Pione" and "Kyoho" were from Dr. Nakaune (pers. comm.).

quenced. As a result, the cDNA clones were highly variable, with nt identities ranging from 76% to 98.4%. Also surprising was the discovery that each of the three isolates assayed harbored two distinct types of viral variants (Meng *et al*. 1999b).

Existence of distinct groups of viral variants was confirmed by several subsequent studies (Rowhani *et al*. 2000; Casati *et al*. 2003; Santos *et al*. 2003; Terlizzi and Credi 2003). For example, Rowhani *et al*. (2000) analyzed the genetic diversity of 17 GRSPaV isolates using primers RSP52 and RSP53 that target a genomic region of 905 nts flanking the CP gene. They identified three major groups of viral variants, which they tentatively named strains 1, 2 and 3. Nolasco *et al*. (2006) conducted an extensive analysis of GRSPaV genetic variation of 46 isolates obtained from Portugal and Slovenia. They revealed the presence of four groups of sequence variants, which they designated group 1, 2a, 2b, and 3. Importantly, they detected a novel viral variant (Group 1) in *V. sylvestris* as well as in some of the cultivated varieties of *V. vinifera* (Nolasco *et al*. 2006). This finding is important since it represented the first report of GRSPaV in *V. sylvestris*, the closest relative of domesticated grapevines. We would like to note that all of these sequence analyses conducted by these researchers used primers that target the CP gene. To compare the effectiveness of different primers that target the helicase domain (RSP13 and RSP14) or the central region of the CP (RSP21 and RSP22), Meng *et al*. (2006) conducted an analysis of the genetic diversity of GRSPaV in 24 isolates obtained from scion and rootstock varieties. They found that these two pairs of primers had a similar spectrum with regard to the types of viral variants that were detected. In line with the findings of Nolasco *et al*. (2006), four groups of viral variants were detected, which were designated groups I-IV

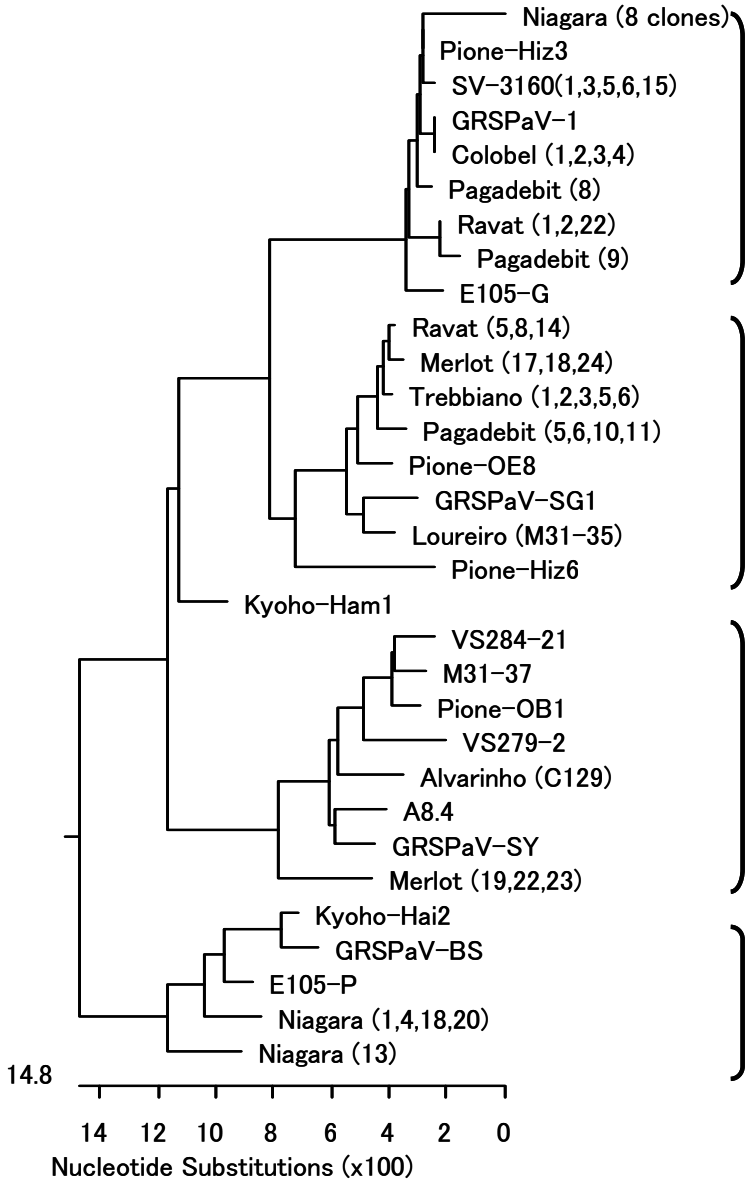

Fig. 4 Phylogenetic tree of sequence variants of GRSPaV obtained from grapevine scion and rootstock varieties. These sequence variants were derived from the replicative intermediate dsRNAs through RT-PCR using primers RSP21 and RSP22 targeting the central region of the coat protein. Sequences of corresponding regions from other isolates were obtained and extracted from the GenBank. Four distinct clusters of viral variants are identified. The reference isolates whose genomes have been completely sequenced fall into one of the four clusters and are marked in bold. The phylogenetic tree was constructed using the Neighbor-Joining method using Clustal W (DNAStar). GenBank accession numbers of representative isolates are: GRSPaV-1 (AF057136), GRSPaV-SG (AY881626), GRSPaV-BS (AY881627), GRSPaV-SY (AY268590), VS284-21 (AY927685), VS279-2 (AY927684), Pione-Hiz3 (AB277785), Pione-Hiz6 (AB277786), Pione-OE8 (AB277784), Pione-OB1 (AB277783), Kyoho-Ham1 (AB277788) and Kyoho-Hai1 (AB277787).

(Meng *et al*. 2006).

Another interesting finding was that isolates derived from scion varieties were composed of sequence variants that belonged to more than one viral variant groups. In some cases, a single isolate contains sequence variants from all four groups. In contrast, the isolates derived from rootstock varieties were homogeneous and composed of only a single type of sequence variants (**Table 2**; Meng *et al*. 2006). As far as we know, these rootstock varieties were derived from mother plants that had not been grafted.

In spite of the fact that these researchers used different primers and that the grapevine source materials were from different geographic regions, four groups of viral variants were identified. However, different researchers designated their viral variant groups differently, which may cause discord and confusion in the future. To prevent such a problem from occurring, Meng *et al*. (2006) proposed a classification system for a uniform grouping and designation of viral variants that have been or will be identified. They classified these viral variants into four "lineage" groups based on each of the reference isolates for which the complete genomes have been sequenced. These lineages are GRSPaV-1, GRSPaV-SG1, GRSPaV-BS, and GRSPaV-VS (**Fig. 4**; Meng *et al*. 2006). It seems that the GRSPaV-1 and GRSPaV-SG1 lineages are more prevalent, whereas the GRSPaV-BS and GRSPaV-VS lineages are less common. Concomitantly, Lima *et al*. (2006a) reported the complete genome sequence of GRSPaV-SY from 'Syrah' with decline syndromes in California. Phylogenetic analysis suggests that GRSPaV-SY belongs to the lineage of GRSPaV-VS.

Recent sequence data seems to support the grouping of GRSPaV variants as proposed above. For instance, using primers Sy9F and Sy8R that were designed to be specific to GRSPaV-SY, Habili *et al*. (2006) detected viral sequences that were 90% identical to the corresponding region in GRSPaV-SY. Also recently, the 3′ terminal 2.8 kb genomic region of GRSPaV was obtained from Japanese table grape varieties 'Pione' ('Kyoho' x 'CannonHall Muscat') and 'Kyoho' ('Ishihara Wase' x 'Centennial'). It is interesting to note that both varieties are hybrids between *V. vinifera* and *V. labrusca* with tetraploid genomes, and had been grafted on 'Kober 5BB' (Nakaune, pers. comm.). Phylogenetic analysis suggests that the four clones derived from cv. 'Pione' belong to GRSPaV-1, GRSPaV-SG1, or GRSPaV-VS (**Fig. 4**). In contrast, one of the two clones derived from cv. 'Kyoho' fell into the GRSPaV-BS lineage, while the other was more closely related to the GRSPaV-VS lineage (**Fig. 4**). We would like to mention that viral variants that are distinct from these four variant groups may exist in nature, and that additional distinct variants may be discovered later on. Indeed, using a pair of primers targeting the POL-encoding region, we recently identified a novel viral variant in 'Niagara' and 'Merlot' (our unpublished data).

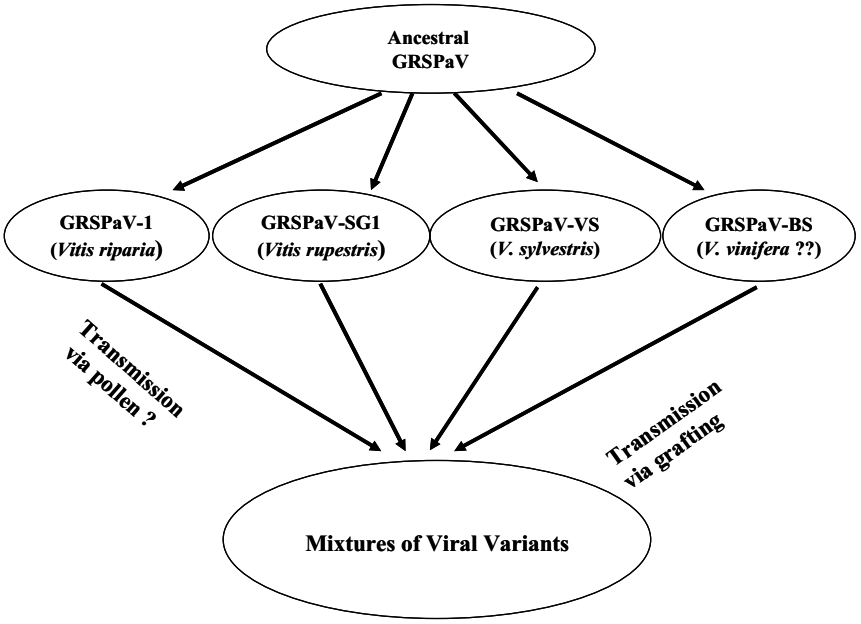

Fig. 5 Model for possible origin and evolution of GRSPaV. GRSPaV is perhaps an ancient virus and has been in co-existence with the grapevines for a long period of time. The ancestor of the present day GRSPaV gained entrance into different *Vitis* species at some point in the past. As a result of co-evolution and adaptation to the different grape species, the genome of the ancestral virus diverged significantly to produce the four major groups of GRSPaV. GRSPaV-1 is likely specific to *Vitis riparia*, while GRSPaV-SG1 is perhaps specific to *V. rupestris*, both grapevine species growing wild in North America. The third group is represented by GRSPaV-BS, whose specific *Vitis* species is yet to be established. The fourth group is represented by GRSPaV-VS, which was detected in wildly grown *V. sylvestris*. It is possible that viral variants belonging to the latter two lineages may have co-existed with *V. vinifera* grapevines. The practice of grafting between scion varieties and rootstocks commonly used in contemporary viticulture may have been responsible for the infection of grapevine scion varieties with mixtures of distinct sequence variants. In addition, GRSPaV might have been transmitted by pollens in nature. Adapted from Meng and Gonsalves (2007).

ORIGIN AND EVOLUTION: A POSSIBLE SCENERIO

Integrating all available information, we propose the following model to account for the origin and evolution of GRSPaV (**Fig. 5**). GRSPaV is a perhaps an ancient virus and has co-existed with grapevines since antiquity. The ancestor of the present day GRSPaV gained entry into different *Vitis* species at some point in the past. As a result of co-evolution and adaptation to different *Vitis* species, the genome of the ancestral virus diverged significantly, resulting in the four major groups of GRSPaV. For instance, GRSPaV-1 has likely long associated with *Vitis riparia* (River bank grape), while GRSPaV-SG1 has co-existed with *V. rupestris* (Sand grape). It is important to note that these two *Vitis* species are wild grapes native to North America. This notion seems to be supported by the detection of GRSPaV-SG1 from *V. rupestris* and its hybrids, and detection of GRSPaV-1 from *V. riparia* and its hybrids (Meng *et al*. 2006). Although speculative at the moment, the other two lineages may have co-evolved with *V. vinifera*. Indeed, viral variants of the GRSPaV-VS lineage have been detected in *V. sylvestris*, as well as in *V. vinifera* varieties including 'Syrah', 'Alvarihno', 'Merlot' and 'Pione'. Similarly, viral variants of the GRSPaV-BS lineage have been detected in *V. vinifera* varieties ('Merlot' and 'Trebbiano') as well as in interspecific hybrids including 'Seyval', 'Bertille Seyve 5563', 'Ravat 34', 'Niagara' and 'Kyoho' (**Fig. 4**; Meng *et al*. 2006).

The complex infection of commercial scion varieties with multiple viral variants cannot be explained merely as a result of accumulation of point mutations due to the error-prone nature of the RNA-dependent RNA polymerase encoded by the virus. Rather, it is more likely an outcome of independent introduction of distinct viral variants from different sources into the same grape variety. The practice of grafting of scion varieties unto rootstocks, commonly used in viticulture over the past century, may have been responsible for the infection of a grapevine scion variety with mixtures of distinct viral variants (Meng and Gonsalves 2007). In addition, potential transmission through pollen as reported by Rowhani *et al*. (2000) into other nearby grapevine plants may have also contributed to mixed infections.

The above hypothesis seems to be very different from that on the origin of other viruses such as nepoviruses that cause Grapevine Degenerations and closteroviruses that are associated with the Grapevine Leafroll Disease Complex. The origins of these later viruses could be traced to distinct but separate geographical regions (Martelli, pers. comm.). Naturally, the validity of our model needs to be ascertained. It is essential to verify the presence of GRSPaV in *Vitis* species that grow in the wild, which include *V. riparia*, *V. rupestris* and *V. berlandieri*. These three North American grapevine species have served as a source for rootstocks or for breeding French-American hybrids. Another missing piece of the puzzle that is absolutely required to support this model is detection and sequencing of GRSPaV variants from a *V. vinifera* grape that has never been grafted with American rootstocks. Such grapevines may still exist in remote areas in the Middle East, the likely origin of the domesticated grapevines. A major purpose of this model is to stimulate interests in a larger community of grapevine virologists around the world to test its validity.

DISEASES AND ECONOMIC IMPORTANCE

It remains an open question as to what diseases GRSPaV may cause. The close association between GRSPaV sequences and the disease RSP suggests that GRSPaV is the putative causal agent of RSP (Zhang *et al*. 1998; Meng *et al*. 1999a; Nolasco *et al*. 2000; Nakaune *et al*. 2006). Nonetheless, direct and unequivocal evidence for this causal relationship is still missing. The existence of multiple sequence variants of GRSPaV further complicates this matter. It is possible that these viral variants differ in their pathogenicity and the symptoms they induce. It has been experimentally demonstrated that GRSPaV-SG1 does not induce symptoms of RSP, while GRSPaV-1 induces, at most, very mild symptoms (Meng *et al*. 2005). Accumulating evidence seems to suggest that GRSPaV-SY and similar viral variants are responsible for the decline and graft-incompatibility problems observed on 'Syrah' and other *V. vinifera* varieties in recent years. For example, viral sequences that were highly similar to GRSPaV-SY were detected only from grapevines that manifested swelling at the graft union, pitting on the wood, and decline symptoms (Habili *et al*. 2006). Interestingly, these grapevines were free from 12 other viruses commonly targeted in virus testing. It remains to be elucidated whether GRSPaV-BS and similar variants would elicit disease symptoms.

As a further complication, recent research has revealed a close link between GRSPaV and Vein Necrosis, a latent and widespread disease of *V. vinifera* grapes characterized by necrotic veinlets on the underside of the leaves of graft-ino-

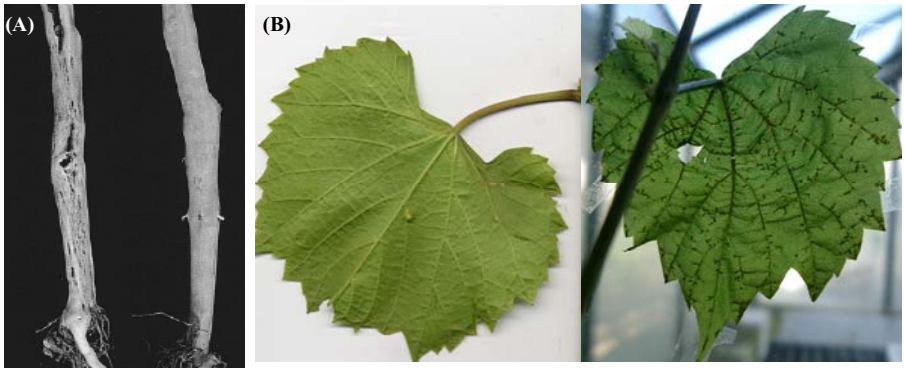

Fig. 6 Symptoms of two distinct diseases that are associated with GRSPaV. (**A**) Rupestris Stem Pitting, a disease that develops pits and grooves on the woody cylinder of the indicator *V. rupestris* 'St. George' after graft-inoculation. Note the smooth surface of the woody cylinder of healthy 'St. George' as shown on the right. (**B**) Vein Necrosis, a widespread disease of grapevines that induces necrosis of the veinlets on the downside of leaves of the indicator 'Richter 110R' after graft-inoculation. A healthy leaf of 'Richter 110R' is shown on the left.

culated 'Richter 110' (**Fig. 6**) (Bouyahia *et al*. 2005; Bouyahia *et al*. 2006; Mslmanieh *et al*. 2006). On the contrary, Borgo *et al*. (2006) concluded that GRSPaV was associated neither with RSP nor with Vein Necrosis. This conclusion was drawn from the low correlation between results of RT-PCR detection and those from indicator indexing. However, the data of Borgo *et al*. (2006) could be interpreted differently, since not all of the viral variants may induce the diseases.

Possibly, *V. vinifera* grapevines have carried certain strains of GRSPaV for a long period of time. However, these viral strains do not induce readily recognizable symptoms. Since these strains are new to other *Vitis* species that are native to North America, they would elicit symptoms once transmitted to the North America grapevines through grafting. Furthermore, it is also likely that GRSPaV infection may induce different types of symptoms on different genotypes of grapevines. Interestingly, 'Richter 110' (*V. berlandieri* x *V. rupestris*) is a hybrid and has *V. rupestris* as a parent. Given the complexity of infection with multiple viruses and viral strains, the ultimate proof that GRSPaV is the causal agent of RSP and/or Vein Necrosis has to be obtained via non-conventional means.

The impact of GRSPaV infection on grapevines also remains poorly understood. GRSPaV appears to be ubiquitous in most of the commercial grapevine varieties with a worldwide distribution. It is believed that GRSPaV itself does not cause much harm to grapevines. Based on these assumptions, GRSPaV was recently withdrawn from the list of viruses that are tested for in the certification programs in California and the European Union. This may prove to be an unwise decision since the actual impact of GRSPaV on viticulture and the wine industry may be far greater than commonly believed. The problem stems from the lack of information, which is due mainly to the complexity of viral infections in grapevines and the lack of knowledge about the biology and pathological aspects of different viral strains. Moreover, the actual damage caused by GRSPaV infection cannot be accurately assessed without direct comparison of the same grape variety growing side by side, with plants on one side being free from GRSPaV and those on the other side being infected with the virus. Recently, Fajardo and colleagues demonstrated that virus-free rootstocks that were grafted with GRSPaV-infected scion wood had a three to five-fold reduction in photosynthetic potential and an increase in dark respiration rate (Martelli 2006).

The detection of GRSPaV in declining 'Syrah' grapevines in California (Lima *et al*. 2006a) and Australia (Habili *et al*. 2006) suggests that GRSPaV may have been responsible for the decline and incompatibility problems that have been occurring in newly planted vineyards where certain combinations of scions and rootstocks were used. Based on personal observation, grapevines that are infected with GRSPaV exhibited much reduced vigor compared to those free of the virus. Recently, GRSPaV was also detected in the table grape 'Waltham Cross' that was afflicted with Shiraz Disease and Leafroll in South Africa (Prosser *et al*. 2007). The role that GRSPaV plays in these diseases needs to be determined.

It is well established that mixed infections with multiple viruses in general result in much more severe diseases (Credi 1997). GRSPaV may be a hidden problem that underlines many severe diseases including declines and graft-incompatibilities that are occurring in newly established vineyards across the world (Golino 1993; Bonfiglioli *et al*. 1998). Clearly, more efforts need to be made toward a better understanding of the genetic variability, pathogenicity, and interactions with other viruses.

CONCLUSIONS AND FUTURE PERSPECTIVES

Since its identification in 1998, significant progress has been made on GRSPaV. The complete genomes of four distinct strains of GRSPaV have been sequenced. GRSPaV is shown to be genetically diverse and is composed of at least four distinct sequence variant groups. Analysis of sequence variants obtained from a wide range of grapevine varieties suggested specific relationship between some of the variant groups and some species of grapes. Rootstock varieties are infected with a single variant whereas scion varieties are usually infected with mixtures of multiple and distinct viral variants. A model was proposed to account for the possible origin and evolution of GRSPaV. Moreover, highly sensitive and efficient methods based on RT-PCR and serology have been developed and successfully used for detecting the virus. These nucleic acid- and protein-based methods represent a major breakthrough in the detection of GRSPaV that could only be achieved through indicator indexing in the past.

Despite these advancements, much still needs to be learned about GRSPaV. Fortunately, these available information and technologies have laid the foundation for several lines of research on the virus, which include the creation and infectivity assays of infectious cDNA clones, mechanisms and sub-cellular localizations of virus replication and movement, and determination and mapping of sgRNAs. The viral cDNA clones would also be useful as a Virus-Induced Gene Silencing vector or for expressing valuable proteins in plants. Ultimately, Koch's postulates need to be fulfilled and this can only be achieved through delivery of the viral cDNA clone or the RNA transcripts into appropriate indicator plants. Moreover, the actual economic importance of GRSPaV will also need to be determined. The recently reported success in eliminating GRSPaV from infected grape varieties through somatic embryogenesis (Gribaudo *et al*. 2006) makes it possible to accurately assess the economic importance of GRSPaV.

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