Genetic diversity analyses of grapevine *Rupestris stem pitting-associated virus* reveal distinct population structures in scion versus rootstock varieties

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Grapevine *Rupestris stem pitting-associated virus* (GRSPaV) is a member of the genus *Foveavirus* within the family *Flexiviridae*. GRSPaV is closely associated with the disease Rupestris stem pitting and is frequently detected in grapevines worldwide. Previous research in several laboratories suggests that GRSPaV consists of a family of sequence variants. However, the genetic composition of GRSPaV variants in viral isolates from scion and rootstock varieties has not been studied extensively. In this report, the genetic diversity and population structure of GRSPaV isolates from scion and rootstock varieties were analysed using two pairs of primers targeting two different genomic regions encoding the helicase domain of the replicase and the capsid protein. In total, 190 cDNA clones derived from 24 isolates were sequenced and analysed. At least four major groups of GRSPaV variants were found to exist in grapevines. Interestingly, the majority of the scion varieties (9/10) that were analysed, regardless of their genetic background and geographical origin, harboured complex viral populations composed of two to four distinct viral variants. In contrast, the viral populations in isolates from rootstock varieties were homogeneous and comprised a single variant. The practice of grafting between scion and rootstock varieties commonly used in modern viticulture, coupled with the frequent regional and international exchange of propagating materials, may have played a major role in the ubiquitous distribution and mixed infections of distinct GRSPaV variants among scion varieties. The possible origin and evolution of GRSPaV are also discussed.

INTRODUCTION

Grapevines are perhaps the most widely grown fruit crop across the world. Grape growing and winemaking have long been associated with, and played an important role in, the civilization in the Middle Eastern and Mediterranean regions before the Christian era (Reisch & Pratt, 1996). Perhaps as a result of the prolonged history of cultivation and the more recent practice of grafting in viticulture, grapevines are known to be host to a great number of viruses. At present, 55 distinct RNA viruses belonging to seven families and 20 genera have been identified in grapevines (Martelli, 2003). It is likely that some of the viruses have co-existed with grapevines since ancient times, whilst others might have been introduced more recently from other plant hosts.

Grapevine *Rupestris stem pitting-associated virus* (GRSPaV) is a member of the genus *Foveavirus* (Martelli & Jelkmann, 1998) within the newly erected family *Flexiviridae* (Adams et al., 2004). GRSPaV is closely associated with the disease Rupestris stem pitting (RSP) (Goheen, 1988), a component of the rugose wood disease complex, which are among the most widespread and devastating disease syndromes affecting grapevines (Martelli, 1993). GRSPaV is perhaps the most prevalent virus of grapevines and is commonly detected in cultivated grapevines (Meng & Gonsalves, 2003, and references therein). Nonetheless, the aetiological role of GRSPaV in RSP has not been established.

The genomes of four isolates of GRSPaV have been sequenced completely. Two nearly identical isolates of GRSPaV were sequenced independently in two laboratories (Meng et al., 1998; Zhang et al., 1998). Recently, the genome sequences of two additional GRSPaV isolates were determined (Meng et al., 2005). The first isolate, GRSPaV-SG1, was detected in the majority of the ‘St George’ plants that had been used as the standard indicator for RSP, whilst the other, GRSPaV-BS, was obtained from ‘Bertille Seyve 5563’, a French–American hybrid wine grape. The nucleotide sequence identities among these two new isolates and the first sequenced isolate GRSPaV-1 are 87.3 % between GRSPaV-1...
and GRSPaV-SG1, 84±3 % between GRSPaV-1 and GRSPaV-
BS and 83.9±9 % between GRSPaV-SG1 and GRSPaV-BS
(Meng et al., 2005). Based on data obtained from a biological
indexing experiment, the authors demonstrated that infec-
tion with GRSPaV-SG1 was asymptomatic, since it did not
induce RSP symptoms on graft-inoculated ‘St George’
plants (Meng et al., 2005).

Previous work has suggested that GRSPaV is genetically
diverse, consisting of numerous sequence variants (Meng
et al., 1999b; Rowhani et al., 2000; Casati et al., 2003; Santos
et al., 2003; Terlizzi & Credi, 2003). The initial discovery of
genetic diversity of GRSPaV was based on identification of
viral clones with different sequences and on the detection
of mixtures of viral variants from three grapevine varieties
(Meng et al., 1999b). This finding was confirmed by several
recent reports. For example, Rowhani et al. (2000) reported
the existence of three ‘strains’ of GRSPaV. Santos et al.
(2003) identified four groups of GRSPaV variants by using
a pair of broad-spectrum primers. Similarly, Casati et al.
(2003) detected three tentative groups of sequence variants
from isolates obtained from Italy and California. Using
single-strand conformation polymorphism of cDNA clones
and sequence comparison, Terlizzi & Credi (2003) also
detected three groups of GRSPaV variants.

Most of the studies on the genetic diversity of GRSPaV
conducted so far have involved only the genomic regions
encoding the whole or part of the capsid protein (CP) and
the isolates assayed were from Eurasian grapevine varieties
belonging to the species Vitis vinifera. Since many of the
sequence data were reported as meeting abstracts, they were
not required to be deposited into the public database and are
thus unavailable. As a result, a general picture of the genetic
variability of GRSPaV could not be obtained.

The objectives of this investigation were: (i) to compare the
efficacy of two pairs of primers targeting different genomic
regions for assessing genetic diversity of GRSPaV; (ii) to
compare the population structures of GRSPaV from grape-
vine scion varieties (including V. vinifera and interspecific hybrids) with those from rootstock varieties; and (iii) to
obtain an overall picture of the genetic diversity and popula-
tion structure of GRSPaV. We demonstrate the existence of
at least four sequence variant groups of GRSPaV. We also
reveal that the population structures of GRSPaV in scion
varieties differ drastically from those in rootstock varieties.

METHODS

Viral isolates. The genetic background and the origin of the
samples assayed are given in Table 1. In total, 24 isolates were assayed.
Each of the isolates was derived from a single plant and only one
isolate was analysed for each of the following varieties: five V. vinifera
varieties (‘Grande Glabre’ (Vitis riparia), ‘Kober 5BB’, ‘Paulsen 1103’ and
‘Millardet 101-14’). Furthermore, 10 isolates each from an individual plant of Vitis rupestris ‘St George’
collected from Sidney, British Columbia, and Geneva, New York,
were also assayed. ‘Pagadebit 2’, ‘Canino 9’ and ‘Trebbiano 12’ were
from the Foundation Vineyard of the Dipartimento di Colture
Arboresce, University of Bologna, Italy, and were kindly provided
by Dr R. Credi. ‘Merlot’ was from the Casa Larga Vineyard at Fairport,
New York, while ‘Pinot Noir’ was from J. Doolittle’s vineyard at
34’, ‘Seyval’ and ‘Grande Glabre’ were kindly provided by P. Forsline
of the US Department of Agriculture-Plant Genetic Resources Unit
at Geneva, New York. The remaining samples were from the
Grapevine Breeding Collection Vineyard of the University of Guelph
at Vineland, Ontario, Canada. All but two of the isolates were
collected from the germplasm collections where they were grown
on their own roots. The exceptions were ‘Pinot Noir’ and ‘Merlot’,
which were collected from commercial vineyards in New York.
However, there was no information about the rootstock varieties
used for these two isolates.

Isolation of viral RNAs. DsRNAs were isolated from cambium
scrapings of dormant grapevine cuttings using the methods described
by Meng et al. (1998). Viral genomic ssRNAs were isolated from
‘Niagara’, ‘Kober 5BB’, ‘Paulsen 1103’ and ‘Millardet 101-14’ using
leaves collected in early August following the method of Steward

RT-PCR. Resulting ssRNAs or dsRNAs were reverse transcribed into
cDNAs at 42 °C with Moloney murine leukemia virus reverse transcriptase (Promega) following the manufacturer’s instructions. First-
strand cDNAs were amplified via PCR using Taq DNA polymerase
(New England Biolabs). Primers used were RSP13 and RSP14, which
amplify a region within the helicase domain of ORF1 (nt 4373–
4711) resulting in a DNA fragment of 339 bp (Meng et al., 1999a)
and RSP21 and RSP22, which target the central region of the CP
gene (nt 7917–8357) resulting in a DNA fragment of 441 bp (Meng
et al., 2003).

Cloning, sequencing and sequence analysis. RT-PCR products were cloned into pGEM-T (Promega) and the products of ligation
reactions were used to transform JM109 cells (Promega). Recombinant clones containing inserts of the expected size were quick-
screened using PCR, purified using the Miniprep kit (Qiagen) and
sequenced on an ABI 373 sequencer (Applied Biosystems). The
sequences of cDNA clones derived from different viral isolates were
aligned using CLUSTAL_X (Thompson et al., 1997). Phylogenetic trees
were constructed using the Bayesian method (MrBayes version 3.01;
Ronquist & Huelsenbeck, 2003). The parameters for the evolution-
ary model were determined by the maximum-likelihood ratio test
(Goldman, 1993) and calculated using MODELTEST version 3.06
(Posada & Crandall, 1998). Four Markov chains were used and the
dataset was run for 4 × 10^6 generations to allow for adequate time
of convergence. The default ‘prior’ settings were used. Trees were
sampled every 100 generations. We designated the first 30 000
sample trees as ‘burn in’ and used the last 10 000 sample trees to
estimate the consensus tree and the Bayesian posterior probabilities.
Two separate runs, which included a total of four independent tree
searches, were conducted and the resulting trees were compared and
pooled.

RESULTS

Genetic analysis of viral isolates from scion
varieties: evidence for mixed infections

In a previous study, we demonstrated that each of the three
isolates of GRSPaV obtained from scion varieties ‘Aminia’,
‘Chardonnay’ and ‘Cabernet Franc’ comprised two distinct
sequence variants (Meng et al., 1999b). To determine whether mixed infection with GRSPaV is a general phenomenon for scion varieties, we initiated this study to analyse further the genetic diversity and population structure of 10 isolates, each from a different grapevine scion variety. Five of the isolates were from *V. vinifera* varieties (‘Pagadebit 2’, ‘Trebbiano 12’, ‘Canino 9’, ‘Merlot’ and ‘Pinot Noir’) and the other five were from interspecific hybrids (‘Seyve Villard 3160’, ‘Colobel 257’, ‘Ravat 34’, ‘Seyval’ and ‘Niagara’) (Table 1).

The viral populations within these 10 isolates were dissected through cloning and sequencing of RT-PCR products amplified with primers RSP13 and RSP14. This primer pair targets the helicase-encoding region of ORF1 (Fig. 1a) and was designed based on the consensus sequence of multiple viral variants (Meng et al., 1999a). Between four and 12 clones were sequenced for each isolate. In total, the nucleotide sequences of 69 clones were determined. Fig. 1(b) presents one of the final phylogenetic trees generated from the Bayesian analysis with the Bayesian posterior probability marked on the tree. Due to the large number of clones that were compared relative to the small number of informative sites for each of the clones, some of the branches had posterior probability values below 50%. However, four major groups of sequence variants could be discerned (Fig. 1b). To confirm the validity of the clustering, we conducted a phylogenetic analysis using the neighbour-joining method, which resulted in an identical clustering pattern (data not shown). Furthermore, a similar clustering pattern could clearly be seen in the phylogenetic tree of clones derived from the CP region (Fig. 1c). Group I contained the reference isolate GRSPaV-1 and 17 cDNA clones derived from five isolates: ‘Seyve Villard 3160’, ‘Seyval’, ‘Ravat 34’, ‘Canino 9’ and ‘Falstaff’. Viral variants within this group had nucleotide sequence identities of 91–99%.

**Table 1.** Origin, parentage (genotypes) and usage of the grapevines used to assay genetic diversity of GRSPaV

<table>
<thead>
<tr>
<th>Viral isolates (grapevine genotypes)</th>
<th>Location</th>
<th>Grouping (no. of clones)</th>
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<tbody>
<tr>
<td></td>
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<tr>
<td>Scion varieties</td>
<td></td>
<td>RSP13/RSP14 RSP21/RSP22</td>
</tr>
<tr>
<td>Canino 9 (<em>V. vinifera</em>)</td>
<td>Italy</td>
<td>II (4)</td>
</tr>
<tr>
<td>Pagadebit 2 (<em>V. vinifera</em>)</td>
<td>Italy</td>
<td>I (1), II (4) I (2), II (4)</td>
</tr>
<tr>
<td>Trebbiano 12 (<em>V. vinifera</em>)</td>
<td>Italy</td>
<td>II (1), III (4) II (5)</td>
</tr>
<tr>
<td>Seyve Villard 3160 (Seibel 5163 x Seibel 2049)</td>
<td>USA</td>
<td>I (1), II (4) I (5)</td>
</tr>
<tr>
<td>Colobel 257 (Seibel 6150 x Seibel 5455)</td>
<td>USA</td>
<td>I (2), II (4) I (4)</td>
</tr>
<tr>
<td>Niagara (Concord x Cassady)</td>
<td>Canada</td>
<td>II (1), IV (5) I (7), III (5)</td>
</tr>
<tr>
<td>Pinot Noir (<em>V. vinifera</em>)</td>
<td>USA</td>
<td>II (7), IV (1) NA</td>
</tr>
<tr>
<td>Merlot (<em>V. vinifera</em>)</td>
<td>USA</td>
<td>III (5), IV (3) II (3), IV (3)</td>
</tr>
<tr>
<td>Ravat 34 (Chardonnay x V. berlandieri)</td>
<td>USA</td>
<td>I (2), II (7), III (1) I (3), II (3)</td>
</tr>
<tr>
<td>Seyval (Seibel 4995 x Seibel 4986)</td>
<td>USA</td>
<td>I (7), II (1), III (4) NA</td>
</tr>
<tr>
<td>Rootstock varieties</td>
<td></td>
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</tr>
<tr>
<td>Grande Glabre (<em>V. riparia</em>)</td>
<td>USA</td>
<td>I (8)</td>
</tr>
<tr>
<td>Kober 5BB (*V. berlandieri x V. riparia)</td>
<td>Canada</td>
<td>I (6)</td>
</tr>
<tr>
<td>Millardet 101-14 (*V. riparia x V. rupestris)</td>
<td>Canada</td>
<td>I (4)</td>
</tr>
<tr>
<td>Paulsen 1103 (*V. berlandieri x V. rupestris)</td>
<td>Canada</td>
<td>II (7)</td>
</tr>
<tr>
<td>St George 990-IA11 (*V. rupestris)</td>
<td>Canada</td>
<td>II (4)</td>
</tr>
<tr>
<td>St George 990-IA14 (*V. rupestris)</td>
<td>Canada</td>
<td>II (6)</td>
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<tr>
<td>St George 990-IA15 (*V. rupestris)</td>
<td>Canada</td>
<td>II (5)</td>
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<tr>
<td>St George 990-IA28 (*V. rupestris)</td>
<td>Canada</td>
<td>II (6)</td>
</tr>
<tr>
<td>St George 990-IA29 (*V. rupestris)</td>
<td>Canada</td>
<td>II (2)</td>
</tr>
<tr>
<td>St George C1-2-9 (*V. rupestris)</td>
<td>USA</td>
<td>II (3)</td>
</tr>
<tr>
<td>St George C1-2-10 (*V. rupestris)</td>
<td>USA</td>
<td>II (4)</td>
</tr>
<tr>
<td>St George C1-2-6 (*V. rupestris)</td>
<td>USA</td>
<td>II (6)</td>
</tr>
<tr>
<td>St George C1-2-30 (*V. rupestris)</td>
<td>USA</td>
<td>II (5)</td>
</tr>
<tr>
<td>St George C1-2-3 (*V. rupestris)</td>
<td>USA</td>
<td>II (11)</td>
</tr>
</tbody>
</table>

NA, Not assayed.
‘Pinot Noir’, ‘Merlot’ and ‘Niagara’ (Fig. 1b). The variants within this group were 92.9–97.6 % identical in nucleotide sequence.

In terms of within-isolate genetic diversity, with the exception of ‘Canino 9’ (which comprised only one type of sequence variant), all of other isolates comprised sequence variants that belonged to two or three sequence variant groups (Table 1). For example, the isolates ‘Ravat 34’ and ‘Seyval’ each contained viral variants from groups I, II and III. Among the 12 clones derived from ‘Seyval’, seven belonged to group I, one belonged to group II, and four belonged to group III, with sequence identities ranging from 87.3 to 98.5 % among themselves. The composition of variants in isolate ‘Ravat 34’ was slightly different; among the 10 clones sequenced, two belonged to group I, seven belonged to group II, while the other belonged to group III. The clones from ‘Ravat 34’ had 87.9–93.5 % sequence identities among themselves. The remaining seven isolates each contained two distinct types of viral variant. However, the types of sequence variants detected in these isolates varied. For example, among the eight clones from the isolate ‘Pinot Noir’, seven belonged to group II and one belonged to group IV, with nucleotide sequence identities ranging from 76.7 to 99.7 %. Isolates ‘Pagadebit 2’, ‘Seyve Villard 3160’ and ‘Colobel 257’ each contained viral variants from groups I and II (Table 1).

To verify that RSP13 and RSP14 were effective in covering the spectrum of sequence variants of GRSPaV, primers RSP21 and RSP22 were used in RT-PCR to obtain cDNA clones from seven of the above 10 isolates. This primer pair targets the central region of the CP gene (Fig. 1a) and was designed based on the consensus sequence of three viral variants (Meng et al., 2003). Again, between four and 12 clones were sequenced for each of the isolates and a total of 44 clones was sequenced (Table 1). To link our data with those from others, we also included sequences of clones representing each of the four groups recently identified by Nolasco et al. (2006) (sequences were kindly provided by Dr Nolasco prior to their release in the GenBank). The phylogenetic relationships of these clones are presented in Fig. 1(c). Similar to data obtained using primers RSP13 and RSP14, four groups of viral variants were again identified (Fig. 1c). Group I contained the reference isolate GRSPaV-1 and 22 clones from ‘Colobel 257’, ‘Niagara’, ‘Seyve Villard 3160’, ‘Ravat 34’ and ‘Pagadebit 2’. Clone E105-G representing the group 2b of Nolasco et al. (2006) fell within this group. Group II contained the reference isolate GRSPaV-SG1, as well as 15 clones from ‘Ravat 34’, ‘Trebbiano 12’, ‘Pagadebit 2’ and ‘Merlot’. Clone M31-35 representing the group 2a of Nolasco et al. (2006) belonged to this group. Group III contained the reference isolate GRSPaV-BS and five clones from ‘Niagara’, as well as clone E105-P representative of the group 3 of Nolasco et al. (2006). Lastly, group IV contained three clones from ‘Merlot’ and several representative clones from the group 1 of Nolasco et al. (2006) (Fig. 1c).

After compiling the data obtained, four groups of viral variants were identified by using the two pairs of primers targeting two different regions of the GRSPaV genome. Based on the presence of the reference isolates, we concluded that the first three groups of viral variants identified by both primer pairs were the same. However, the relationship of group IV identified using primers RSP21/RSP22 compared with that identified using primers RSP13/RSP14 could not be determined at the present time due to the lack of a completely sequenced reference isolate.

Primers RSP13 and RSP14 seemed to be equally effective (in the case of ‘Pagadebit 2’) or more effective (in the case of ‘Trebbiano 12’, ‘Seyve Villard 3160’, ‘Colobel 257’ and ‘Ravat 34’) than primers RSP21 and RSP22, as the former primer pair detected more sequence variants (Table 1). In the case of ‘Merlot’, two types of sequence variants were detected by using each pair of the primers. However, only one type of sequence variant was detected in common for both primer pairs. For example, primers RSP13 and RSP14 detected viral variants belonging to groups III and IV, whilst RSP21 and RSP22 detected viral variants belonging to groups II and IV (Table 1). For ‘Niagara’, although two types of sequence variant were detected with both primer pairs, those detected with RSP13/RSP14 belonged to groups II and IV, whilst those detected by primers RSP21/RSP22 belonged to groups I and III (Table 1).
Genetic analysis of viral isolates from rootstock varieties: evidence for single infections

To examine the genetic diversity and population structure of GRSPaV in rootstock varieties, we assayed 14 isolates derived from five grapevine rootstocks. A single isolate was obtained from each of the following rootstock varieties: ‘Grande Glabre’ (V. riparia), ‘Kober 5BB’, ‘Paulsen 1103’ and ‘Millardet 101-14’. The other 10 isolates were obtained from 10 individual plants of V. rupestris ‘St George’ that were maintained as indicator selections at Sidney, British Columbia, Canada, or at Geneva, New York. All of these rootstocks were grown on their own roots. Based on the finding that primers RSP13/RSP14 were more effective than primers RSP21/RSP22, only the former primers were used to analyse the genetic diversity of isolates from rootstocks.

In sharp contrast with the isolates from scion varieties, each of these rootstock varieties was infected with only a single type of sequence variant, either group I or group II (Table 1 and Fig. 1b). No viral variants belonging to groups III or IV were detected in any of these rootstock varieties. Furthermore, the type of viral variant detected in a particular rootstock seemed to correlate with the genotype of the rootstock. For example, the eight clones obtained from ‘Grande Glabre’ were 100 % identical to each other and belonged to group I. It is interesting to note that ‘Grande Glabre’ is a selection of wild V. riparia and has been used as a parent for breeding several rootstock varieties. Similarly, the six clones derived from ‘Kober 5BB’ were 99-4-100 % identical to one another and were 97-9 % identical to GRSPaV-1. Interestingly, ‘Kober 5BB’ is a selection of seedlings derived from a cross between Vitis berlandieri (the female parent) and V. riparia (the male parent). Moreover, the four clones derived from ‘Millardet 101-14’ were 100 % identical to one another and were 98-8 % identical to GRSPaV-1.

On the other hand, the seven clones derived from ‘Paulsen 1103’ were 98-2-100 % identical amongst themselves and were 97-9 % identical to GRSPaV-SG1; all belonged to group II. ‘Paulsen 1103’ is a selection of seedlings derived from a cross between V. berlandieri (the female parent) and V. rupestris (the male parent). For the 10 isolates from ‘St George’ plants, all 52 cDNA clones fell into group II, although slight variation was observed in the composition of sequence variants among these isolates. For example, seven isolates contained GRSPaV-SG1, one isolate contained GRSPaV-SG2, one isolate contained both GRSPaV-SG1 and -SG2, and the last contained GRSPaV-SG1, -SG2 and -SG3. The nucleotide sequences of GRSPaV-SG1, -SG2 and -SG3 differed by 3-5-9-8 % (Meng et al., 2005).

DISCUSSION

Grapevines are perhaps one of the most ancient plants cultivated by humans and the most widely grown fruit crop around the world (Reisch & Pratt, 1996). Like most crop plants, grapevines were always grown on their own roots until the 1860s when Phylloxera, a devastating root-attacking insect, was accidentally introduced from North America into the vineyards in Western Europe, resulting in the mass death of grapevines. To combat this problem, Phylloxera-resistant Vitis species that are grown in the wild in North America were introduced into Europe to be used as rootstocks (Pongracz, 1983; Howell, 1987; Reisch & Pratt, 1996). Since then, rootstocks have been used increasingly in viticulture. Today, the vast majority of grapevines throughout the world are grown on rootstocks. As a result, grapevines are known to be infected by a large number of viruses. GRSPaV is perhaps one of the most prevalent viruses that infect grapevines (Meng & Gonsalves, 2003).

Despite the recent efforts towards understanding the genetic diversity of GRSPaV (Meng et al., 1999b; Rowhani et al., 2000; Casati et al., 2003; Santos et al., 2003; Terlizzi & Credi, 2003), an extensive analysis and an overall picture of the genetic diversity and population structure of GRSPaV are still lacking. After this study was completed, Nolasco et al. (2006) reported on the genetic variability of GRSPaV isolates collected from Portugal. The present study involved extensive analyses of the genetic diversity and population structure of GRSPaV, since it was based entirely on direct analysis of nucleotide sequences derived from GRSPaV-infected grapevines belonging to three Vitis species and various interspecific hybrids. Furthermore, the isolates assayed included a number of commonly used scion and rootstock varieties. Several important findings were made. First, in agreement with the report of Nolasco et al. (2006), we identified four distinct groups of GRSPaV variants. Second, we demonstrated that the scion varieties assayed were mostly infected with a mixture of distinct viral variants (Table 2). Third, we revealed, for the first time, that the viral population in GRSPaV-infected rootstocks, or at least the ones assayed here, consists of a homogeneous population of identical or nearly identical variants from the same lineage (Fig. 1b; Table 2). Last, we revealed specific associations between two of the variant groups with two of the North American Vitis species as well as their progeny, shedding light on the possible origin and evolution of GRSPaV.

Analyses of cDNA clones obtained by using two pairs of primers targeting the viral genomic regions encoding the CP and the helicase domain revealed nearly identical phylogenetic relationships. Four major groups of sequence variants were obtained. To standardize the nomenclature used for grouping GRSPaV variants so as to avoid confusion, we propose the following nomenclature scheme using four lineages for classifying GRSPaV variants. Designation of these lineages is based on the presence of the specific reference isolates within each of the sequence variant groups. The viral variants from our group I and those from group 2b of Nolasco et al. (2006) should be designated ‘GRSPaV-1’ lineage; those from our group II and group 2a of Nolasco et al. (2006) should be designated ‘GRSPaV-SG1’ lineage; the viral variants from our group III and group 3 of Nolasco et al. (2006) should be designated ‘GRSPaV-BS’ lineage;
Table 2. Distribution of sequence variant groups (lineages) of GRSPaV in grapevine scion and rootstock varieties

Data were compiled from sequences of cDNA clones derived from RT-PCR with primers RSP13/RSP14 and RSP21/RSP22. Note that 10 isolates from ten ‘St George’ plants were assayed.

<table>
<thead>
<tr>
<th>Variety</th>
<th>Group I (GRSPaV-1)</th>
<th>Group II (GRSPaV-SG1)</th>
<th>Group III (GRSPaV-BS)</th>
<th>Group IV (GRSPaV-VS)</th>
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<tbody>
<tr>
<td>Scion varieties</td>
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<tr>
<td>Niagara</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Seyval</td>
<td>+</td>
<td>+</td>
<td></td>
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<tr>
<td>Ravat 34</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Pagadebit 2</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Trebbiano 12</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Merlot</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Pinot Noir</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Colobol 257</td>
<td>+</td>
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<td>Seyve Villard 3160</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Canino 9</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Rootstock varieties</td>
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</tr>
<tr>
<td>Grande Glabre</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<td>Kober 5BB</td>
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<td>Millardet 101-14</td>
<td>+</td>
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<td>Paulsen 1103</td>
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<td>St George (10 isolates)</td>
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whilst those from group IV based on primers RSP21/RSP22 and those from group I of Nolasco et al. (2006) should be designated ‘GRSPaV-VS’ lineage. The last lineage is so named in recognition of detection of similar viral sequences from wild Vitis sylvestris by Nolasco and associates. We recommend that sequences obtained in the future also be treated in a similar fashion.

As shown in Table 2, sequence variants of the GRSPaV-SG1 lineage were the most prevalent, as they were detected in all scion varieties assayed, as well as in two rootstock varieties. Sequence variants of the GRSPaV-1 lineage were the second most prevalent and were detected in six of the scion varieties and three rootstock varieties. Sequence variants of the GRSPaV-BS lineage were less common and were detected in five of the scion varieties. Sequence variants of the GRSPaV-VS lineage seemed to be the least prevalent, as they were detected only in three of the scion varieties. Moreover, the latter two lineages were not detected in any of the rootstock varieties assayed.

Interestingly, very different patterns of population structure were identified for isolates derived from scion varieties versus those from rootstock varieties. All of the isolates from rootstock varieties contained a homogeneous population of a single type of viral variant of either the GRSPaV-1 or the GRSPaV-SG1 lineage. In contrast, nine of the 10 isolates from scion varieties comprised sequence variants that belonged to two, three and possibly even four lineages. The isolates derived from two of the French–American hybrid scion varieties, ‘Ravat 34’ and ‘Seyval’, clearly comprised variants of three lineages. The interspecific hybrid scion variety ‘Niagara’ seemed to be infected with variants from all four lineages. However, each of the two pairs of primers detected only two groups of variants. Further research is needed to determine whether the isolate derived from ‘Niagara’ indeed comprises four distinct sequence variants. Mixed infections with divergent GRSPaV variants have been reported recently by others (Rowhani et al., 2000; Casati et al., 2003; Nolasco et al., 2006).

What is the explanation of the phenomenon that scion varieties are infected mostly with mixtures of divergent sequence variants, whilst rootstock varieties are infected with a homogeneous population of a single variant? Based on the seemingly specific association between the type of sequence variants and the genotypes of the host plant, we propose the following scenario. GRSPaV-1 may have co-existed with V. riparia for a long time. Likewise, GRSPaV-SG1 may have been in co-existence with V. rupestris since ancient times. As V. riparia and V. rupestris grapevines or their hybrids have been used commonly as rootstocks on to which different scion varieties are grown, viruses carried in these rootstocks could have been transmitted into the scion varieties through grafting. The same scion variety may have been grafted on to different rootstocks at different times and in different geographical regions. As a result, different viral variants accumulated in the scion varieties, leading to the mixed infection of many of the commonly used scion varieties with multiple sequence variants of the virus. This more
recent viticultural practice of grafting, coupled with the asymptomatic infection of GRSPaV and frequent regional and international exchange of grapevine propagation materials, may have played a major role in the commonly observed mixed infection of scion varieties.

It is worth noting that the genetic variability detected may encompass mutations resulting from the polymerases used in RT-PCR. In general, RT-PCR produces less than 0.5% errors (Teycheney et al., 2005). Nonetheless, such low levels of errors should not have much impact on the validity of our conclusions. Nor can the mixed infections be explained by accumulation of point mutations within the same plant over time. Despite the fact that viral RNA-dependent RNA polymerases possess a higher mutation rate due to their lack of proofreading capacity, an RNA virus may not change much with time unless it adapts to a new host. As stated by Gibbs et al. (1999), ‘a potential to vary need not result in variability, as the latter depends on the balance between mutation and selection’. Relative genetic stability has been demonstrated for other plant RNA viruses. For example, isolates of Turnip yellow mosaic virus from Europe and Australia differ by only 3–5% in nucleotide sequence, despite their isolation for at least 13 000 years (Gibbs et al., 1999). Thus, it is more likely that the mixed infection of scion varieties with diverged variants of GRSPaV has resulted from separate introduction of distinct sequence variants into the same plants.

Grapevines are host to a large number of taxonomically distinct viruses. Several recent studies have demonstrated that mixed infections of grapevine scion varieties with divergent variants of several other viruses, including Grapevine fanleaf virus (Vigne et al., 2004), Grapevine leafroll-associated viruses 1 and 3 (Little et al., 2001; Turturo et al., 2005), Grapevine virus A (Goszczyński & Jooste, 2003) and Grapevine virus B (Shi et al., 2004). Mixed infections have also been reported for citrus with Citrus tristeza virus (Ayllón et al., 2001; Rubio et al., 2001) and for apple with Apple chlorotic leaf spot virus (Candresse et al., 1995). It will be interesting to examine whether rootstocks also play a role in these situations.

It remains an open question as to how the wild Vitis species became infected with different variants of GRSPaV in the first place. It is possible that the ancestor of GRSPaV was with Vitis from the start. As different Vitis species diverged, the virus also co-evolved and diverged. As a result, GRSPaV-1 adapted to V. riparia, whilst GRSPaV-SG1 adapted to V. rupestris and it is only within the past one and a half centuries that these two viral variants were brought into V. vinifera varieties through grafting. Alternatively, both viral variants may have existed in V. vinifera varieties and been transmitted horizontally to V. riparia and V. rupestris rootstocks by insect vectors or by humans. Testing of wild-grown species of Vitis that are in geographical separation from V. vinifera will probably provide clues that are important in answering this question.

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Genetic diversity and population structure of GRSPaV


