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BRIEF REPORT

A genome-wide diversity study of grapevine rupestris stem pitting-associated virus

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Abstract

Over the last decade, many scientific disciplines have been impacted by the dawn of new sequencing techniques (HTS: high throughput sequencing). Plant pathology and more specifically virology have been greatly transformed by this 'metagenomics' paradigm shift. Such tools significantly facilitate disease diagnostics with tremendous sensitivity, providing invaluable information such as an exhaustive list of viruses being present in a sample as well as their relative concentration. In addition, many new plant viruses have been discovered. Using RNAseq technology, *in silico* reconstruction of complete viral genome sequences is easily attainable. This step is of importance for taxonomy, population structure analyses, phylogeography and viral evolution studies. Here, after assembling 81 new near-complete genome sequences of grapevine rupestris stem pitting-associated virus (GRSPaV), we performed a genome-wide diversity study of this ubiquitous virus of grapevine worldwide.

Grapevine rupestris stem pitting-associated virus (GRSPaV), classifiable in the genus *Foveavirus* (Order: *Tymovirales*, Family: *Betaflexiviridae*) [16], is restricted to grapevines and is considered as one of the most prevalent graft-transmissible viruses infecting the *Vitis* species [28]. Since its discovery less than two decades ago, this virus has been detected in most grapevine growing regions, from Europe to the American continent, Australia, as well as in Asia [28]. So far, no vectors have been linked with the spread of GRSPaV [25] and its dispersion relies mainly on the exchange of infected materials, vegetative propagation and grafting. The real etiological role of GRSPaV is still unclear, but the virus has been associated with rupestris stem pitting (RSP) disease, a disorder of the rugose wood (RW) complex [25]. Lately, GRSPaV has been tentatively connected with other diseases of grapevine such as Syrah decline [1], vein necrosis [6] or the vein-clearing complex on Chardonnay [21]. In those studies, GRSPaV was always detected in a mixed infection with a cocktail of other viruses, preventing the drawing of any firm conclusions [12]. No etiological evidence fulfilling the Koch's postulates has been shown to date. While some rootstocks can display symptoms of small pits and grooves on the stem coinciding with reduced vigor and probable graft-incompatibility, GRSPaV causes limited symptoms on most cultivars and is generally considered as a latent virus [32]. The majority of grapevines are symptomless carriers of the virus, speculated to be due to a long co-existence with its sole host, resulting from adaptation and evolution of less severe viral strains. Nonetheless GRSPaV infection induces transcriptome changes in *Vitis vinifera* L., indicating interactions between the plant and the virus [9]. The GRSPaV virion is a non-enveloped, flexuous rod-shaped particle about 725 nm long and 12 nm in diameter [31], containing a linear positive sense single stranded RNA genome of about 8.7 Kb in size with a capped 5' end and a poly-adenylated 3' terminus [24]. The genome harbors 5 open reading frames (ORF, Fig. 2A) with ORF1 encoding the replication-associated protein, ORFs 2-4 encoding the triple gene block proteins (TGBp1-3) involved in intra- and inter-cellular movement [22] and ORF5 encoding the coat protein (CP). A putative ORF6 has been reported [36], but

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its potential expression and role in the virus life cycle remain unclear.

In the last decade, many efforts have been devoted to describe the genetic diversity of GRSPaV, looking mostly at only parts of the genome [2, 20, 26, 29, 35] but rarely looking at the whole genome level [10, 14]. All aforementioned studies established that GRSPaV displays a high level of genetic diversity, with a wide range of sequence variants clustering in four main phylogroups.

Here, we report 81 new complete or near-complete (*i.e.* covering at the minimum all ORFs) genome sequences of GRSPaV assembled from 39 leaf samples collected from different vineyards of France (Table 1). All samples were flash-frozen in liquid nitrogen and kept at -80 °C before being processed. Briefly, total RNA was extracted from 100 mg of leaf tissue using the RNeasy Plant mini kit (Qiagen, Hilden, Germany), as per the manufacturer's recommendations. After a poly(A) selection, cDNA libraries preparations for Illumina sequencing were performed at the GeT-Genotoul platform facility (INRA-Toulouse, France) or as otherwise mentioned for the P70 accession grapevine only [4]. Experiments were performed on an Illumina Hiseq 3000 (Illumina, San Diego, CA, USA) using a paired-end read length of 2x150pb with the Illumina Hiseq3000/4000 SBS sequencing kits. Dataset analyses and *de novo* assembly protocol were previously detailed [13].

The phylogenetic analysis of 103 GRSPaV genomes encompassing the ORFs [22 available at NCBI (https://www.ncbi.nlm.nih.gov/, January 2018 and 81 new sequences (GenBank# KX034981-KX035006, MG938294-MG938348)] confirmed that GRSPaV isolates segregate into the four previously described clades [14] (Fig. 1). The inter-clade nucleotide (nt) diversity, estimated around 21-23%, confirms the high genome-wide genetic diversity reported in previous studies. At the clade level, Clade 4 is the most genetically homogenous, with only 4% nt diversity. However, this clade comprise only three members. For the other three clades, consisting of at least 27 genomes, the intra-clade diversity is much higher with sequences showing up to 18.1% nt diversity (Clade 3). Also, Clade 2 can be further divided into 3 sub-clades 2a, 2b and 2c. When using an arbitrary 95% similarity cut-off, 25 sub-groups were separated, with twelve of them comprising a single isolate, confirming the high genome diversity (as shown, see Letters in Fig. 1). From this full-length genome analysis, no particular phylo-geography was supported, as previously suggested by analyses focusing on particular GRSPaV genome regions from cultivated (*V. vinifera*) and Sicilian wild (*V. sylvestris*) grapevines [2, 26, 30]. This is reinforced by the fact that the previously unique GRSPaV isolate from China defining group 4 is now joined with two sibling isolates from France. It is interesting to notice that for all sequences within Clades 3 and 4, except for AMME-GRSPaV-1, the additional ORF6 was not predicted (Fig. 1, grey boxes). The opposite was true for all sequences of Clade 1 and 2 and 34-GRSPaV-1, GRSPaV-BS (AY881627), GRSPaV-SK30 (KX274277). All 32GRSPaV sequences lacking ORF6 contained a single nucleotide point mutation, switching the canonical AUG start codon to an ACG. Such a high percentage of sequences lacking ORF6 further questions the potential expression and role of this particular ORF [36].

From this study, we can conclude that most grapevine varieties were infected by multiple variants (Table 1), with up to four variants being *de novo* assembled within a single sample (*e.g.* P70, B47, EVC60…). These variants were generally affiliated to different phylo-groups (Table 1), confirming previous observations [3, 26, 29]. In addition, we could not link the presence of some GRSPaV phylo-groups to particular grapevine varieties. However, Table 1 might be misleading regarding clade distribution and the presence of a particular variant within a sample. It is important to emphasize the fact that the presence of a 'checkmark' describes the presence of complete [or near complete] GRSPaV genomes being assembled. The absence of a 'checkmark' does not necessarily means the absence of a variant in a sample, but simply that a near complete sequence of the variant from that particular clade was not obtained. This potentially underestimates the number of variants within a sample. Nevertheless, after a thorough analysis, we can confirm that all rootstock samples (all coming from the same 41B variety clone 231) were infected by a single variant, belonging to the same phylogenetic clade (Table 1). These findings are consistent with the model proposed by Meng and Gonsalves on the possible origin of GRSPaV [27]. Also, it is worth mentioning that, in our particular dataset, only one sample, named 32 (Table 1), did not display any sequence (nor any reads) corresponding to GRSPaV. This particular clone, 110R rootstock E39, was actually the result of a successful sanitation therapy via microshoot tip culture [11].

The overall nucleotide (nt) diversity index (π) between the 103 aligned coding sequences is 0.177. It is interesting to note that, when comparing genomes obtained from samples collected in France versus genomes from samples collected around the world (Fig. 2A), we could not see any major variations in the nucleotide diversity along the genome. This observation confirms the lack of geographical structuration of GRSPaV populations. The hypervariable region (HVR) of the polymerase gene is clearly defined when looking at the genetic diversity along the genome of all available sequences (Fig. 2A). This stretch of 300 nt, adjacent to the AlkB-like coding portion of ORF1 surrounding position 2050 exhibits up to 3x more ($\pi = 0.457$) the average level of diversity found along the genome. This AlkB-like domain, putatively involved in viral RNA methylation repair, is found in *Eukarya* and bacteria but also in certain positive-stranded plant RNA viruses [34] and is thought to have been recently

Table 1 General information about the plant material from which the 81 new genome sequences of grapevine rupestris stem pitting-associated virus (GRSPaV) were assembled

Sample name	Sampling date	Location	Cultivars	Clones scion/rootstock	GRSPaV genomes assembled	Clade			
						1	2	3	4
P70	2012, March 27	France: Colmar/Savigny-lès-Beaune	Pinot Noir	nd	4	√	√	√	√
28	2015, June 9	France: Bagneux la Fosse	Chardonnay	nd	2	√		√	
29	2015, June 9	France: Bagneux la Fosse	Chardonnay	nd	3	√	√	√	
34	2015, June 25	France: Colmar/Volnay	Chardonnay	nd	2	√	√		
35	2015, June 25	France: Colmar/Volnay	Chardonnay	nd	4	√	√	√	√
AMCF	2010	France: Colmar/Bordeaux	Cabernet Franc	nd	3	√	√	√	
AMME	2010	France: Colmar/Bordeaux	Merlot	nd	2	√		√	
30	2015, June 25	France: Colmar/ Chateauneuf du Pape	Grenache	E173/E39	2		√	√	
31	2015, June 25	France: Colmar/ Chateauneuf du Pape	Grenache	E173/E39	1		√		
33	2015, June 25	France: Colmar/ENTAV	Grenache	E173	1		√		
B34	2016, May 17	France: Colmar	Gewurztraminer	Gw643/K5BB259	1			√	
B52	2016, May 17	France: Colmar	Gewurztraminer	Gw643/K5BB259	3	√	√	√	
B53	2016, May 17	France: Colmar	Gewurztraminer	Gw643/K5BB259	2	√		√	
B47	2016, May 17	France: Colmar	Gewurztraminer	Gw643/K5BB259	4	√	√	√√	
C39	2016, May 17	France: Colmar	Gewurztraminer	Gw643/K5BB259	2	√		√	
C40	2016, May 17	France: Colmar	Gewurztraminer	Gw643/K5BB259	2	√		√	
EVC53	2013, sept 5	France: Colmar	Gewurztraminer	Gw643/K5BB259	2			√√	
EVB47	2013, sept 5	France: Colmar	Gewurztraminer	Gw643/K5BB259	3	√	√	√	
EVC42	2013, sept 5	France: Colmar	Gewurztraminer	Gw643/K5BB259	3	√		√√	
EVC60	2013, sept 5	France: Colmar	Gewurztraminer	Gw643/K5BB259	4	√	√	√√	
EVC56	2013, sept 5	France: Colmar	Gewurztraminer	Gw643/K5BB259	2	√		√	
SWT1	2015, May 28	France: Colmar	Pinot Meunier	PM817/41B231	2	√√			
SWT2	2015, May 28	France: Colmar	Pinot Meunier	PM817/41B231	2	√√			
SWT3	2015, May 28	France: Colmar	Pinot Meunier	PM817/41B231	1	√			
SWT4	2015, May 28	France: Colmar	Pinot Meunier	PM817/41B231	2	√√			
SWT5	2015, May 28	France: Colmar	Pinot Meunier	PM817/41B231	2	√√			
SWT6	2015, May 28	France: Colmar	Pinot Meunier	PM817/41B231	2	√√			
SGM1	2015, May 28	France: Colmar	Pinot Meunier	PM817/41B231	1		√		
SGM4	2015, May 28	France: Colmar	Pinot Meunier	PM817/41B231	2	√	√		
SGM5	2015, May 28	France: Colmar	Pinot Meunier	PM817/41B231	2	√	√		
MBE8	2013, July 22	France: Colmar	Pinot Meunier	PM817/41B231	4	√√√	√		
MBE10	2013, July 22	France: Colmar	Pinot Meunier	PM817/41B231	2	√√			
WTR1	2015, May 28	France: Colmar	41B rootstock	41B231	1	√			
WTR2	2015, May 28	France: Colmar	41B rootstock	41B231	1	√			
WTR3	2015, May 28	France: Colmar	41B rootstock	41B231	1	√			
WTR4	2015, May 28	France: Colmar	41B rootstock	41B231	1	√			
WTR5	2015, May 28	France: Colmar	41B rootstock	41B231	1	√			
WTR6	2015, May 28	France: Colmar	41B rootstock	41B231	1	√			
MBE9	2013, July 22	France: Colmar	41B rootstock	41B231	1	√			
32	2015, June 25	France: Colmar/ENTAV	110 R rootstock	E39	0				

The number of near-complete genomes (*i.e.* covering at the minimum all ORFs) assembled with CLC Workbench 8.5.1 software from each sample (GRSPaV genomes) and the clade in which they cluster (see Fig. 1) are shown by a check mark. If two location names are given (*i.e.* Colmar/Bordeaux), the former indicates location of the replicate being kept and sampled from, while the latter indicates the origin of the mother grapevine

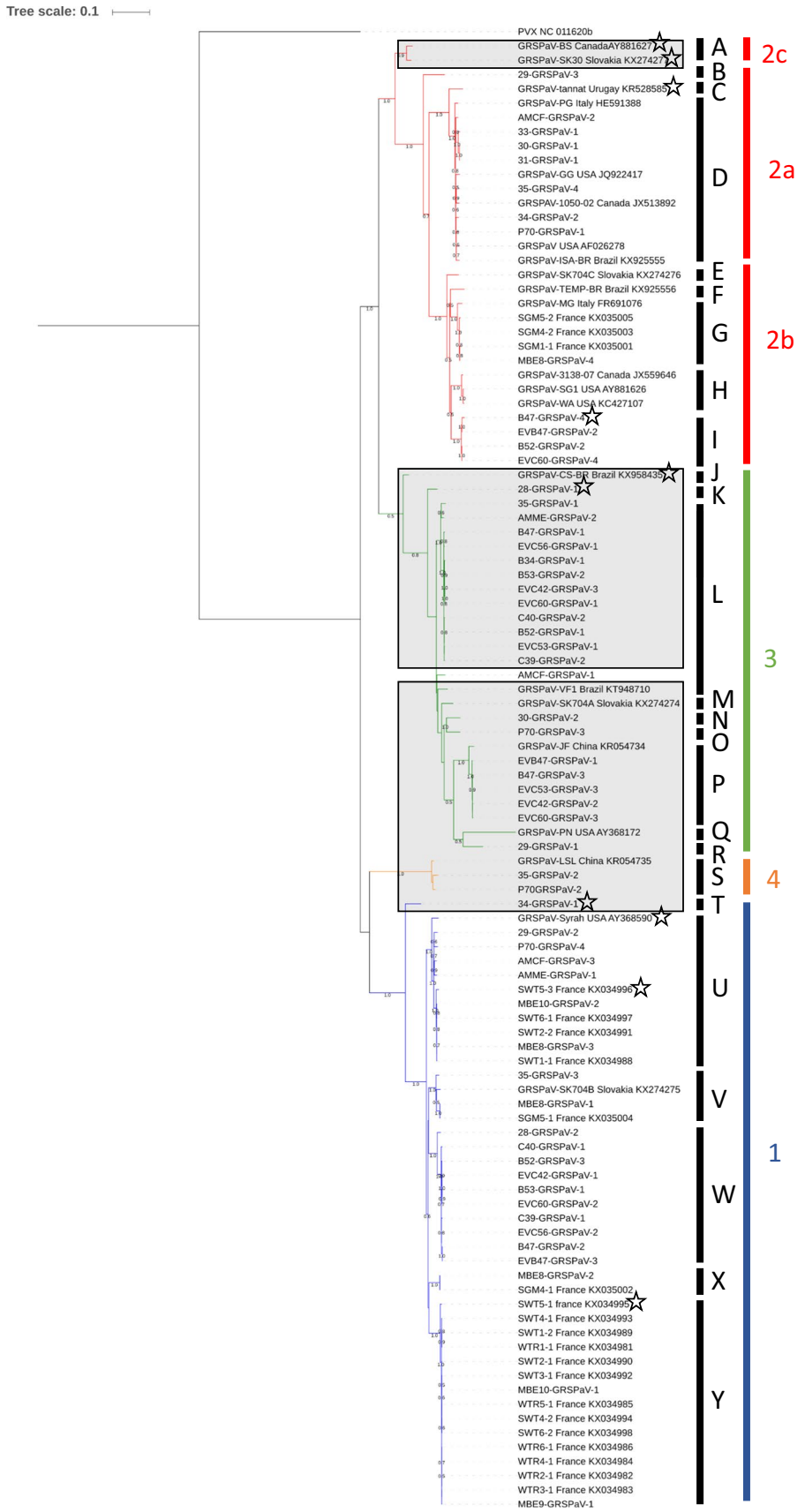
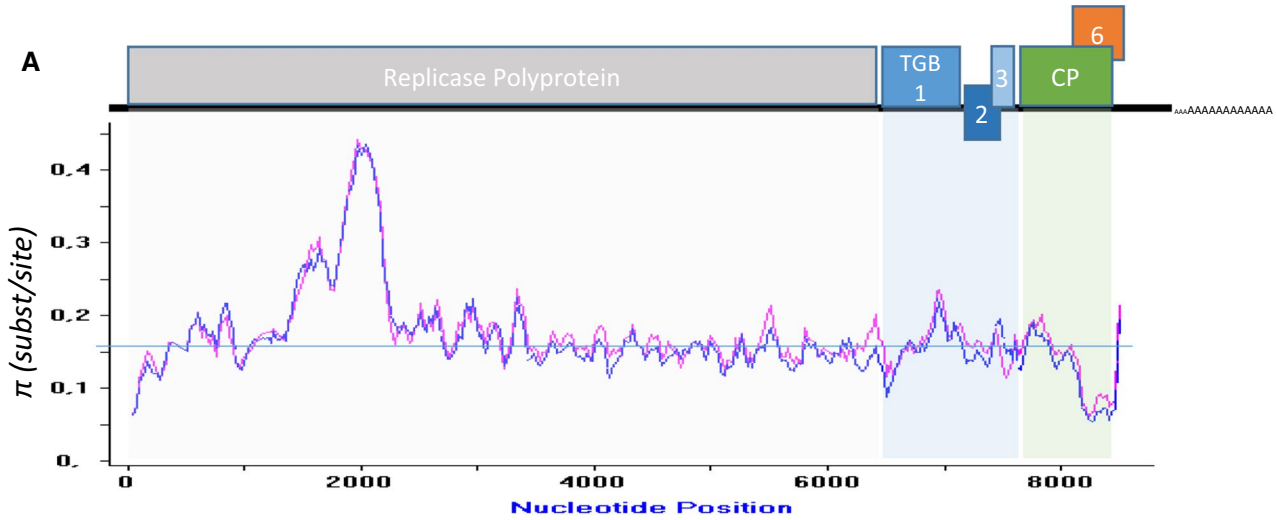


Fig. 1 Phylogenetic relationships of 103 genome sequences of grapevine rupestris stem pitting-associated virus (GRSPaV). Twenty-two genomes of GRSPaV are available at NCBI (Nov. 2017). The other 81 genomes were obtained from different RNAseq runs (see Table 1) which were assembled with CLC Workbench 8.5.1 software. Nucleotides alignment analysis and tentative Maximum Likelihood (ML)-based phylogenetic trees were performed using MUSCLE [8] and MEGA7 [18] software. The best ML-fitted model was used and bootstrapping analyses of 100 replicates was achieved. A potato virus X (PVX) genome sequence was used as an outgroup. Stars indicate recombinant genomes (see Fig. 2B). Grey boxes indicate genomes were ORF6 was not predicted in CLC Workbench analyses. Numbers correspond to previously described clades [14] and letters identify groups of sequences sharing at least 95% identical nucleotides



B

number	recombinant	Breakpoint location begining	end	major	% sim	minor	% sim	RDP MC corrected	Softwares
1	34-GRSPaV-1	6537-6613	end	GRSPaV-Syrah-USA	93,4%	GRSPaV-PN	93,9%	6,14 E-112	R, G, M, C, S, 3
2	SWT5-1	1	1220-1344	WTR5-1	99,9%	SWT6-1	99,9%	2,2 E-45	R, G, M, C, S, 3
3	GRSPaV-Syrah-USA	4498-4724	5258-5393	AMCF-GRSPaV-3	98,0%	34-GRSPaV-1	98,5%	2,7 E-23	R, G, M, C, S, 3
4	GRSPaV-Syrah-USA	5506-5657	6564-6700	AMCF-GRSPaV-3	97,2%	34-GRSPaV-1	99,0%	8,5 E-17	R, G, S, 3
5	SWT5-3	1-144	1220-1339	MBE8-GRSPaV-3	99,8%	WTR3-1	99,9%	2,0 E-46	R, G, M, C, S, 3
6	GRSPaV-BS-Canada	6292-6320	end	29-GRSPaV-3	85,2%	28-GRSPaV-1	97,9%	4,0 E-99	R, G, M, C, 3
7	B47-GRSPaV-4	8192-8237	end	EVB47-GRSPaV-2	100,0%	C39-GRSPaV-1	99,2%	7,5 E-81	R, G, M, C, S
8	GRSPaV-SK30	6274-6311	9200	29-GRSPaV-3	84,20%	28-GRSPaV-1	97,70%	1,3 E-97	R, G, M, C, 3
9	GRSPaV-tannat-Uruguay	1	238-278	GRSPaV-PG-Italy	96,4%	34-GRSPaV-1	99,6%	1,2 E-30	R, G, M, C
10	GRSPaV-tannat-Uruguay	3815-3853	4787-4851	35-GRSPaV-4	95,9%	GRSPaV-Syrah-USA	96,2%	1,9 E-108	R, G, M, C, S, 3
11	GRSPaV-tannat-Uruguay	6856-6914	7656-7786	GRSPaV-PG-Italy	97,3%	29-GRSPaV-3	95,6%	5,6 E-22	R, M, C, S, 3
12	GRSPaV-CS-Brazil	1	1082-1200	GRSPaV-SK30	96,00%	GRSPaV-SK704C	95,70%	3,2 E-20	R, G, S, 3
13	GRSPaV-CS-Brazil	2131-2337	end	MBE8-GRSPaV4	87,10%	AMME-GRSPaV-2	98,70%	8,0 E-139	R, G, M, C, S, 3
14	28-GRSPaV-1	1	669-727	B47-GRSPaV-1	94,70%	P70-GRSPaV-1	99,40%	1,2 E-93	R, G, M, C, S, 3
15	28-GRSPaV-1	745-799	1002-1087	AMME-GRSPaV-2	95,40%	AMCF-GRSPaV-2	95,40%	9,2 E-28	R, G, M, C

Fig. 2 Nucleotide diversity index (π) study and location of recombination events within all grapevine rupestris stem pitting-associated virus (GRSPaV) genomes. The variation of π along the GRSPaV genome (A) was evaluated by sliding window analyses using DnaSP v. 5.10 [19], with parameters set at 100 nucleotides for windows and 25 nucleotides for step size. Comparison was performed on the alignment of genome obtained from French (blue) or around the world samples (pink). Recombination event detection (B) was performed using Recombination Detection Program package (RDP v.4.46) [23], with GeneConv (G), MaxChi (M), Chimaera (C), SiScan (S) and 3Seq (3) software being included. Numbers correspond to recombination location along the genome with major and minor parents as well as confidence level being detailed in the table below. To confirm the biological occurrence of recombination events, one site (highlighted in grey in the table) was tested by RT-PCR on RNA samples that had been used for RNAseq library construction. Resulting PCR amplicons were Sanger-sequenced confirming the existence of recombinant isolate in tested plants. TGB: Triple Gene Block, CP: Coat Protein

acquired from host plants through horizontal gene transfer. This hypervariable region has also been described in the genomes of viti- and ampeloviruses belonging to two other genera of grapevine-infecting viruses, potentially infecting a plant at the same time [4]. However, it is worth mentioning that a long stretch of 300 nt located at the 3'end of ORF5 (8250-8550) presents lower genetic diversity as compared to the rest of the genome (Fig. 2A). This could be potentially due to (1) the many functional constraints of the coat protein [5], or (2) the presence of the overlapping ORF6, adding selective constraints. Using SNAP [17] (from the suite of tools from Datamonkey [http://www.datamonkey.org/, last visited January 2018]), we performed an analysis of the behavior of each codon along the different ORFs. Only a few non-synonymous mutations were detected within the 3'-end region (corresponding to the overlap with ORF6) of the *CP* gene, compared to the rest of the genome (supplemental Fig 1E). Such a low level of non-synonymous mutations was not observed in other parts of the GRSPaV genome that also display gene overlap (*i.e.* TGBp2 and TGBp3, supplemental Fig 1C and 1D). In addition, the genetic diversity of genomes for which the ORF6 is predicted compared to genomes that do not harbor it were similar (supplemental Fig 1F), underlying the fact that *CP* gene is in fact under

high negative selective pressure, supporting previous reports for GRSPaV [29] and for other plant viruses [15, 33].

In addition to mutations introduced during viral replication by the viral polymerases, which lack proofreading capability, recombination events constitute another major evolutionary driving force shaping the diversity of RNA viruses. To pinpoint such potential recombination events, the 103 aligned sequences were analyzed using RDP4 [23]. Twelve recombination events were identified within nine GRSPaV genomes, with an associated Bonferoni-corrected p-value lower than 1.0×10^{-10} (Fig. 2B). The majority of the recombination events involved two parental variants belonging to different clades. One of the detected recombination event has been tested and confirmed to be biologically genuine in the RNA sample used for RNAseq library construction (Fig. 2B, highlighted in grey in table).

Our molecular diversity data on complete to near-complete GRSPaV genome sequences has confirmed previous studies based on fragments of the genome. GRSPaV is genetically very diverse and is composed of at least four distant variant clusters. While GRSPaV has been detected in most grapevine growing areas, no structuration based on geographical regions can be observed, which might reflect human activity with the exchange and trade of infected grapevine material. Modern molecular techniques, and HTS in particular, have allowed the deciphering of the virome which is more complex than anticipated, with the detection of many viral species as well as many different variants of the same virus inhabiting a plant [4]. This complex and dynamic viral infection might and can affect plant functions and responses to pathogen invasion [9]. Recent results on the virome (comprising commensal and pathogenic viruses) are ultimately shaping old postulates into new ones: from Koch's original postulates to Koch's adapted postulate, taking into consideration the corpus of viruses and their dynamic adaptation [7].

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Compliance with ethical standards

Conflict of interest The authors declare there are no conflicts of interest.

Research involving human participants and/or animals The research did not involve human participants or animals.

Informed consent The research did not involve human participants or animals.

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