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Genetic diversity analysis of grapevine rupestris stem pitting-associated virus from grapevine by colony PCR-SSCP and -RFLP

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Summary. – We have developed methods for detecting the genetic diversity of grapevine rupestris stem pitting-associated virus (GRSPaV) based on restriction fragment length polymorphism (RFLP) and single stranded conformational polymorphism (SSCP) in the 905 nt 3' sequence. The amplicons were cloned from six grapevine cultivars, and colony polymerase chain reaction (colony PCR) using recombination bacteria was subsequently analyzed by RFLP and SSCP. Four haplotypes of SSCP and six haplotypes of Sac I RFLPs were defined. The two methods had a 40% discrepancy rate in showing the degree of diversity. All clones were sequenced and were used to construct a phylogenetic tree with seven previously reported GRSPaV sequences. In the tree, all the newly acquired sequences were divided into three clusters, I, II, and III, which corresponded to haplotypes I, II, and III of SSCP, respectively. Haplotype IV of SSCP was grouped into cluster II. A recombination analysis showed that haplotype IV has undergone a recombination event. Together, these results indicate that the SSCP assay is useful for the rapid identification of genetic diversity of GRSPaV. This is the first report of an analysis of the large fragment of GRSPaV by colony PCR-SSCP.

Keywords: grapevine; grapevine rupestris stem pitting-associated virus (GRSPaV); RFLP; SSCP; genetic diversity analysis

Introduction

Grapevine rupestris stem pitting-associated virus (GRSPaV, the genus *Foveavirus*, the family *Betaflexiviridae*) is a common virus in cultivated grapevines worldwide (Meng and Rowhani, 2017). It usually exists in its latent state in *Vitis vinifera* cultivars, but it is considered to be associated with rupestris stem pitting, vein necrosis disease, Syrah decline, and several other symptoms and disorders (Nakaune *et al*., 2008; Lunden *et al*., 2010).

The genome of GRSPaV comprises five open reading frames (ORFs) that encode, in order, a replication-related protein, triple gene block proteins (TGB, TGBp1, TGBp2, and TGBp3), and the coat protein (Lima *et al*., 2006). GRSPaV exhibits extensive genetic diversity and has numerous sequence variants. The virus can be divided into four main clusters depending on the full genomic sequence: cluster I, cluster II comprising sub-clusters IIa and IIb, cluster III, and cluster IV (Beuve *et al*., 2013; Hu *et al*., 2015). Another two sub-clusters, IIc and IId, are defined when the replicase polyprotein and coat protein domains are used to classify GRSPaV isolates or variants (Mostert *et al*., 2018). The types of GRSPaV can differ among different locations, and there can also be multiple variants at a single location. For example, a previous study detected two to four distinct viral variants in the scion and one in the rootstock of a single plant (Meng *et al*., 2006). The existence of sequence variants can severely affect the analyses of complete viral genes and genomes in some samples.

Although cloning and sequencing of a sufficient number of clones is the best way to detect viral heterogeneity, however these techniques are laborious and expensive (Gulija *et al*., 2011). Other mutation-scanning approaches,

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Abbreviations: GRSPaV = grapevine rupestris stem pitting-associated virus; RFLP = restriction fragment length polymorphism; SSCP = single stranded conformational polymorphism

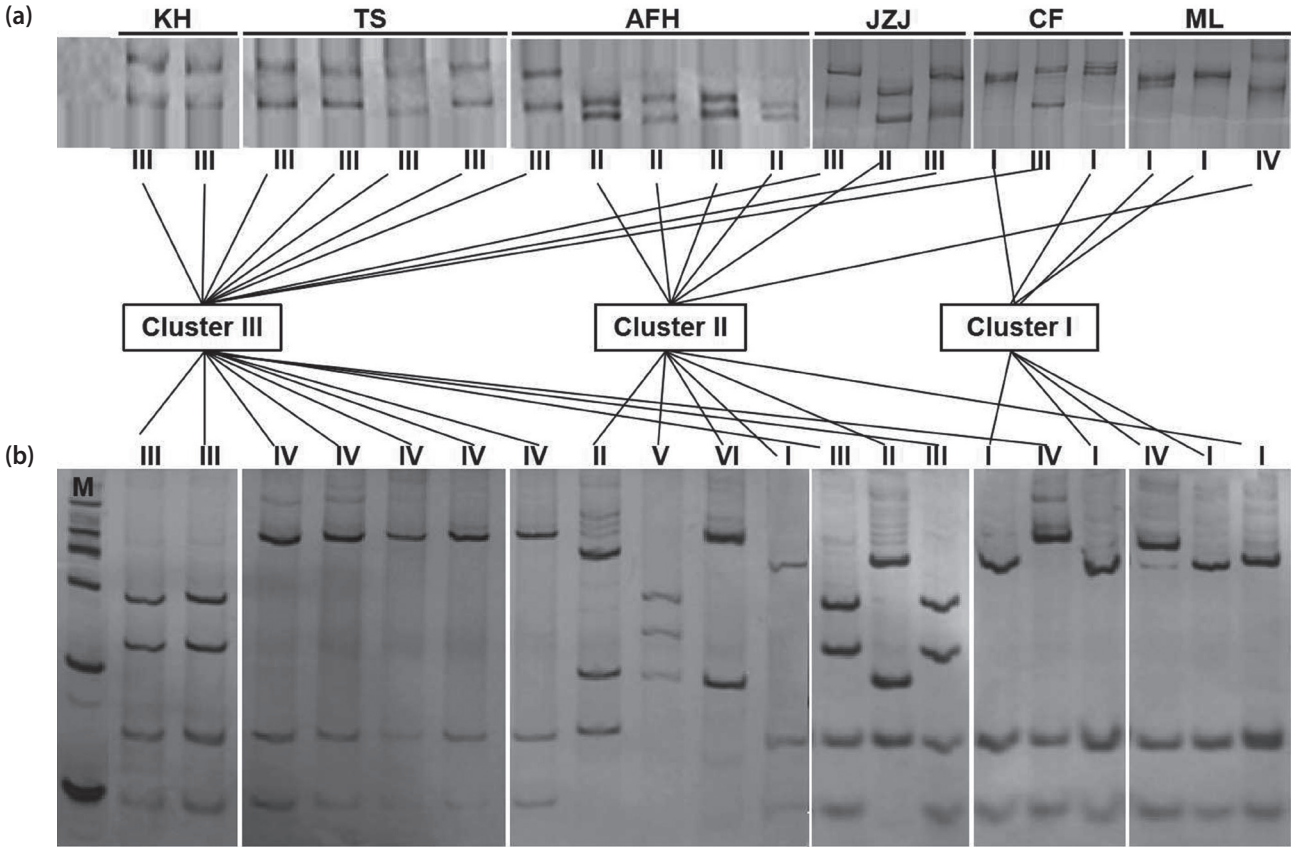

Fig. 1

SSCP and RFLP analysis of GRSPaV

(a) Single-strand conformation polymorphism (SSCP) and **(b)** restriction fragment length polymorphism (RFLP) analysis of variant structure of grapevine rupestris stem pitting-associated virus (GRSPaV) in grapevine. Cultivars: KH: Kyoho, TS: Thompson Seedless, AFH: A'fuhan; JZJ: Jingzijing; CF: Cabernet franc; ML: Merlot. Clusters I, II, and III: GRSPaV groups in phylogenetic tree.

such as restriction fragment length polymorphism (RFLP) and single stranded conformational polymorphism (SSCP) analyses, are often used to screen the structure of viral variants (Kuta *et al*., 2015; Youssef and Shalaby, 2016). However, these methods have scarcely been used for analyses of GRSPaV. In this study, RFLP- and SSCP-based methods based on the GRSPaV 3' sequence amplified by colony polymerase chain reaction (colony PCR) were developed. These methods were then applied to detect the genetic diversity of GRSPaV isolates from grapevines in China, and the results were verified by sequencing.

Materials and Methods

Samples. Branches were randomly collected from six grapevine cultivars (grafted plants with rootstock Beta): Kyoho (KH), Thompson Seedless (TS), A'fuhan (AF), and Jingzijing (JZJ) from Liaoning province, and Cabernet franc (CF) and Merlot (ML) from Ningxia province, China. One plant of each cultivar was used as the original material. These plants did not show any obvious symptoms associated with GRSPaV, however the status of GRSPaV-infection had been verified by RT-PCR before collection.

RNA isolation and RT-PCR. Total RNA was extracted from the dormant canes as described by Hu *et al.* (2015). First-strand cDNAs were generated by reverse transcription with random hexamer primers and M-MLV reverse transcriptase (Promega, USA). The primer pair RSP52F (5'-TGAAGGCTT TAGGGGT TAG-3')/RSP53R (5'-CTTAACCCAGCCTTGAAAT-3'), encompassing the entire CP gene and flanking sequences upstream in the TGBp3 and downstream in the 3' untranslated region (Alabi *et al*., 2010), was used to re-test GRSPaV.

Cloning. The PCR products were purified using a PCR purification kit (Takara, China), and the amplicons were inserted into the pMD18-T vector (Takara). After *E. coli* DH5' were transformed with recombinant DNA, positive clones were identified by colony PCR with the primer pair RSP52F/RSP53R. Three to

Table 1. Haplotypes of RFLP and SSCP for grapevine rupestris stem pitting-associated virus (GRSPaV)

Cultivars	Variants	SSCP	RFLP	
			Fragments (bp)	Groups
Kyoho	KH-3	III	85, 140, 269, 411	III
	KH-4			
Thompson Seedless	TS-1	III	85, 140, 680	IV
	TS-2			
	TS-3			
	TS-4			
A'fuhan	AFH-1	III	85, 140, 680	IV
	AFH-2	II	146, 225, 534	II
	AFH-3	II	225, 269, 411	V
	AFH-4	II	225, 680	VI
	AFH-5	II	85, 140, 146, 534	I
Jingzijing	JZJ-1	III	85, 140, 269, 411	III
	JZJ-2	II	146, 225, 534	II
	JZJ-3	III	85, 140, 269, 411	III
Cabernet france	CF-1	I	85, 140, 146, 534	I
	CF-2	III	85, 140, 680	IV
	CF-3	I	85, 140, 146, 534	I
Merlot	ML-1	I	85, 140, 680	IV
	ML-2	I	85, 140, 146, 534	I
	ML-3	IV	85, 140, 146, 534	I

five positive clones of one sample were used for RFLP and SSCP analysis (Supplementary Table S1).

RFLP and SSCP analysis. Colony PCR products (3 µl) were directly digested with 5 U Sac I and 10× L buffer according to the instruction manual (Takara). After incubation at 37°C for 1 h, RFLP fragments were separated by electrophoresis on a 6% polyacrylamide gel (Kong *et al*., 2003) for 1.5 h at room temperature (25°C). The gels were silver-stained as described by Beidler *et al*. (1982). The same PCR products (1 µl) and 2 µl of denaturing buffer (95% formamide, 50 mM EDTA and 0.05% bromophenol blue) were mixed and heated at 100°C for 10 min, and then cooled on ice for 3 min. The denatured PCR products were loaded onto a 6% polyacrylamide gel. After 14 h of electrophoresis at 4°C, the gels were stained with 10% silver nitrate. Clones of the different isolates were sent for sequencing and the results were analyzed by the program DNAMAN 5. 2. 2 (Lynnon Bio Soft, Canada) and ClustalX1.81 software.

Phylogentic analysis. The phylogenetic tree was derived using the neighbor-joining method with 1,000 bootstrap replicates in the Molecular Evolutionary Genetics Analysis (MEGA) 5.0 software package (Tamura *et al*., 2011). The occurrence of putative recombination events, putative recombination junctions, and statistical scores were assessed using at least five of the following seven programs-RDP, GENECONV, BootScan, MaxChi, Chimaera, SiScan, and 3Seq-implemented in the software package RDP4 (Martin *et al*., 2015) with default parameters (highest acceptable probability value = 0.05).

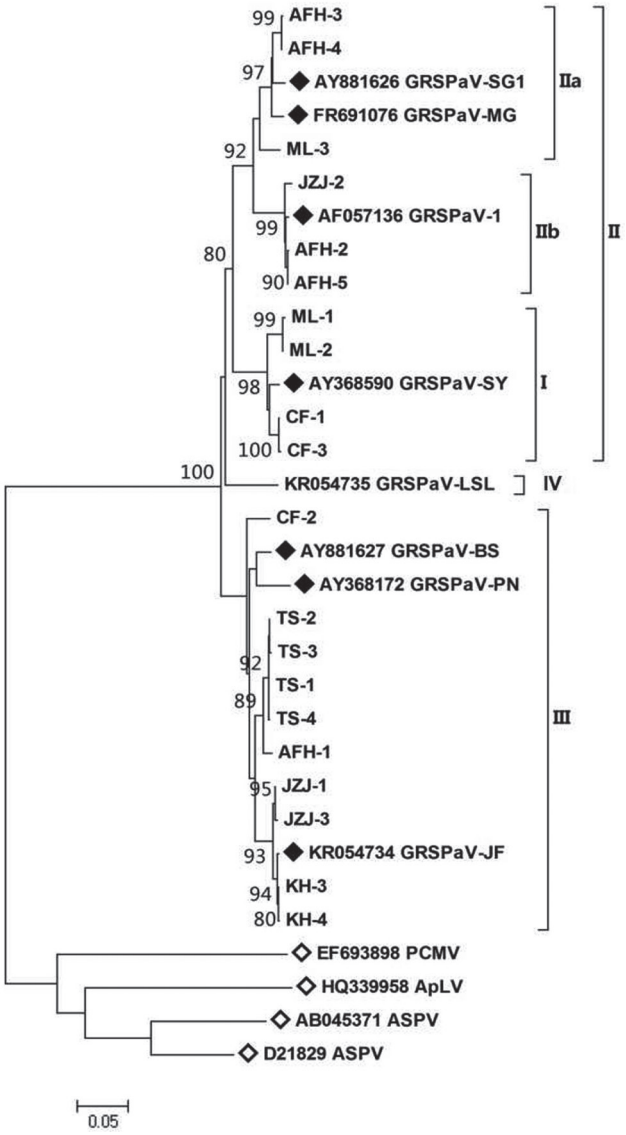

Fig. 2

Phylogenetic tree based on nucleotide sequences of the GRSPaV
Phylogenetic tree based on nucleotide sequences of the GRSPaV amplified by primer pair RSP52/53 and nucleotide sequences of seven GRSPaV isolates reported previously. For sources and GenBank Acc. Nos of reference isolates, see Supplementary Table S1. Nodes with bootstrap values below 70% are collapsed. Branch lengths correspond to genetic distances as indicated on the scale bar. The phylogenetic tree was constructed using the neighbor-joining method with 1,000 bootstrap replicates. Three other members of the s *Foveavirus*, apple stem pitting virus (ASPV, D21829 and AB045371), apricot latent virus (ApLV, HQ339958) and peach chlorotic mottle virus (PCMV, EF693898), were used as out-groups (marked by hollow diamonds).

Table 2. GRSPaV sequence showing putative recombination events

Isolates	Parental isolates		Break point[a]		RDP-implemented method (P-value)						
	Major	Minor	Start	End	R	G	B	M	C	S	3S
ML-3	AFH-4	ML-1	680	894	3.593×10^{-15}	7.747×10^{-13}	–	8.649×10^{-10}	3.106×10^{-09}	8.698×10^{-16}	1.686×10^{-2}
AFH-2	TS-4	MG050195	154	909	2.017×10^{-05}	4.300×10^{-17}	–	4.569×10^{-07}	2.013×10^{-07}	4.009×10^{-06}	1.494×10^{-12}
TS-4	KH-4	TS-3	713	907	–	2.616×10^{-08}	–	1.377×10^{-05}	9.614×10^{-11}	3.173×10^{-12}	5.990×10^{-20}

The following suite of recombination detection programs was used to detect recombination events and calculate average *P*-values: R: RDP; G: GENECONV; B: Bootscan; M: MaxChi; C: Chimaera; S: SiScan; 3S: 3Sep. [a]Position in alignment.

Results and Discussion

The silver staining results for colony PCR products of six grapevine samples revealed four and six haplotypes in the SSCP and RFLP analyses, respectively, and the two methods had a 40% discrepancy rate in showing the degree of diversity (Table 1 and Fig. 1). Only one haplotype was found in KH and TS, whilst the variant structures of the other four samples were complex, and one sample had at least two haplotypes of the virus. The sequence alignment results showed that three KH clones and one TS clone had nucleotide deficiencies and therefore they were thought to be invalid clones. There were large ranges of sequence identities among the clones of AF, JZJ, CF, and ML (80.0–99.8%, 80.2–99.7%, 81.7–99.7%, and 86.7–99.7%, respectively). The average identities of KH and TS were higher, both 99.8%, and in agreement with the results of the SSCP and RFLP analyses (Supplementary Table S1 and Fig. 1). Phylogenetic analyses were carried out by conducting multiple alignments of all sequence variants detected in this study (GenBank Acc. Nos MK867355–MK867374) and seven typical isolates of each cluster reported in the literature (Supplementary Table S1). In the tree, the GRSPaV sequence variants from the six grapevine cultivars separated into three groups (Fig. 2). These groups did not show clear relationships with the RFLP haplotypes. However, three haplotypes detected in the SSCP analysis corresponded to the clusters I, II, and III from the phylogenetic tree (Fig. 1), and each GRSPaV cluster had a representative SSCP haplotype (Supplementary Fig. S1). This phenomenon could have a relationship with the mutation of nucleotides in the clones. Moreover, it is most likely due to the effects observed on conformational mobility, which is caused by a few polymorphisms rather than a mutation in a single restriction site. Notably, clone ML-3 belonged to SSCP haplotype IV, but its sequence was in group IIa in the phylogenetic analysis. Three recombinants of the clone sequence (AFH-2, ML-3, and TS-4) were identified among all available sequences from the Genbank and the isolates from Chinese grapevine isolates obtained in this study. ML-3 was found to be a recombinant with strongly supported values of the isolate AFH-4 as a major parent and ML-1 as a minor parent (Table 2).

The present study describes RFLP and SSCP methods to analyze the genetic diversity of GRSPaV. Both methods could clearly reflect the molecular structure of GRSPaV in grapevine samples through polyacrylamide gel electrophoresis, which can be helpful when selecting samples for sequencing depending on the experimental purpose. The SSCP haplotypes were closely related to the phylogenetic groups of GRSPaVs. Shorter fragments, less than 400 bp, have been reported to be suitable for the detection of mutations in SSCP gels (Hongyo *et al*., 1993). The size of the RSP52/53 amplicon was 905 bp, more than double the recommended size of a suitable fragment, but the trends in sequence variations among different samples were still visible in the stained polyacrylamide gels. Furthermore, the SSCP analysis was able to distinguish most conformational changes caused by subtle sequence differences, such as a one-base substitution in a fragment several hundreds of bases long (Hayashi, 1991). Our results showed that the identities of clones with the same haplotype were not 100.0%, e.g., KH and TS, but the haplotypes of these clones were almost the same. Finally, the results demonstrated that longer fragments (more than 400 bp) could be used to analyze the genetic diversity of this virus by SSCP analysis. Studies on the molecular variability and genetic structure of viruses can provide information about viral evolutionary history (García-Arenal *et al*., 2001). As an RNA virus, GRSPaV represents a population of sequence variants resulting from the error-prone nature of RNA polymerases and from repeated infections in the field (Glasa *et al*., 2017; Hily *et al*., 2018). The SSCP, RFLP, and sequencing results revealed varying degrees of diversity among GRSPaV samples.

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Supplementary information is available in the online version of paper.

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