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# Genetic diversity analyses reveal novel recombination events in *Grapevine leafroll-associated virus* 3 in China☆

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ABSTRACT

*Grapevine leafroll-associated virus* 3 (GLRaV-3) is the most prevalent causal agent of grapevine leafroll disease (GLD). Of the 75 grapevine samples collected from three regions in China, 46.7% and 94.7% of samples tested positive for GLRaV-3 in reverse transcription-PCR (RT-PCR) and reverse transcription nested PCR (RT-nPCR), respectively. The SSCP analysis for the clones of complete CP gene from 16 GLRaV-3 isolates showed that 15 isolates contained one predominant haplotype and one isolate had no predominant haplotype. The sequences of the CP genes showed 89.9–100% identities at the nucleotide level. Phylogenetic analysis of the CP gene sequences revealed the existence of four well defined variant groups, which corresponded to previously reported phylogenetic groups (1, 2, 3, and 5). Two new sub-groups designated as sub-group 1B and sub-group 3B in groups 1 and 3, respectively, were identified in the Chinese GLRaV-3 population. Recombination analyses illustrated that those two new sub-groups (1B and 3B) were emerged as a result of recombination events between variants in groups 1 and 2, and variants in groups 1 and 3, respectively. These results further indicated that the variants in those new sub-groups are viable and evolutionary successful. Recombinants with highly similar coat protein structure to variants of group 1 were abundantly found in the viral population. In addition, these analyses provided evidence about CP gene as one of the recombination hotspots in GLRaV-3 genome. The population genetic parameters of all available CP sequences suggested that the recombinants might have emerged due to population bottlenecks during transmission. The results provide new insights into the variability and evolution of GLRaV-3.

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## 1. Introduction

Grapevine leafroll disease (GLD) is one of the most important and widespread viral diseases of cultivated grapevines worldwide. The disease can cause significant yield losses (up to 62%), delays fruit ripening and severely affects fruit quality (Walter and Martelli, 1997). In the recent taxonomic revision of the family *Closteroviridae*, 5 genetically distinct phloem-limited filamentous grapevine leafroll-associated viruses (GLRaVs) have been recognized (Martelli et al., 2012). *Grapevine leafroll-associated virus* 3 (GLRaV-3) is the most prevalent causal agent of GLD. GLRaV-3 is the type species of the genus *Ampelovirus* in the family *Closteroviridae* (Martelli et al., 2002). The virus can be transmitted in a semi-persistent manner by numerous species of mealybugs and soft-scale insects. Its flexuous and filamentous virion is about 1800 nm long and contains a linear, monopartite, positive sense single strand genomic RNA, which is organized into 13 open reading frames (ORFs), encoding a proteinase, replicase, movement protein, coat protein, and replication associated proteins (Ling et al., 2004).

RNA viruses have high mutation rates and large population sizes, which result in accretion of abundant genetic variations in viral populations (Holmes, 2009). It is presumed that viral coat proteins evolved more rapidly than proteins involved in replication and expression of virus genomes (Callaway et al., 2001), thus providing a strong incentive to study the diversity of viruses based on CP genes. However, information on the genetic diversity within GLRaV-3 populations is limited compared to other plant viruses. To date, nine complete and near complete genomes for isolates of GLRaV-3 are available (Ling et al., 2004; Engel et al., 2008; Maree et al., 2008; Jarugula et al., 2010; Jooste et al., 2010; Bester et al., 2012). Since GLRaV-3 has a large genome of ca. 18 kb, the diversity studies by generating whole genome sequences are technically

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difficult. Wang et al. (2011) analyzed the GLRaV-3 genetic variability by sequencing a 4.7-kb fragment in the 3′ terminal region, and found that the phylogenetic tree obtained with the 3′ terminal region showed topology similar to the tree with its CP gene. Some studies suggested that GLRaV-3 is composed of three to seven groups of sequence variants based on the analysis of sequences derived from the partial or complete heat shock protein 70 (HSP70) and/or coat protein (CP) gene sequences of the virus isolates from Brazil, Portugal, South Africa, and USA (Fajardo et al., 2007; Fuchs et al., 2009; Jooste et al., 2010; Gouveia et al., 2011; Sharma et al., 2011; Bester et al., 2012). Turturo et al. (2005) analyzed the partial sequences of RdRp, HSP70 and CP genes of GLRaV-3 isolates from various geographical areas, and found mixed infections of different molecular variants in some plants, and observed an estimated mean genomic diversity of 4.9%. The existence of five well defined variant groups in the Portuguese GLRaV-3 population was revealed by examining the diversity within the complete CP gene (Gouveia et al., 2011). Significant genetic variability of GLRaV-3 isolates was also reported from New Zealand (Chooi et al., 2009). Previously, two recombination events, one each in partial CP and HSP sequences were identified in GLRaV-3 (Turturo et al., 2005).

China has a long history of grapevine cultivation. According to data compiled by food and agricultural organization of the United Nations (FAO) in 2010, China is leading grape producing country in the world accounting for 12.67% of total yield of the world. It is imperative to study the diversity of GLRaV-3 in Chinese vineyards to successfully curb the disease caused by the virus. Previous surveys revealed the presence of GLRaV-3 in cultivated grapevine in China (Hong et al., 2005; Xu et al., 2006). However, little is known about the incidence and molecular characteristics of GLRaV-3 in Chinese vineyards. The knowledge of genetic variability of GLRaV-3 is essential for the development of reliable diagnostic assays in clean plant programs and the management of GLD in vineyards. In this study, the prevalence of GLRaV-3 in china was assessed by reverse transcription-PCR (RT-PCR) and reverse transcription nested PCR (RT-nPCR). The population structure and genetic diversity of GLRaV-3 isolates from China was evaluated by single stranded conformation polymorphism (SSCP) and analysis of the complete CP gene of 35 isolates. The novel recombination events and recombination hotspots were identified that may significantly contribute to our understanding of molecular and biological evolution of the virus.

## 2. Materials and methods

### 2.1. Virus sources

Grapevine canes of 75 non-grafted varieties showing typical leafroll disease symptoms were randomly collected from grapevine germplasm collection centers in three regions in China. Fifty five and 17 samples were collected from varietal collection centers of *Vitis vinifera* in Henan and Hubei provinces, respectively. Three samples were obtained from a vineyard in Hubei province. Samples are listed in Supplementary Table S1. Grapevine canes from the same variety in the same field were considered as one sample, and viral population derived from a single variety was considered as one isolate.

### 2.2. dsRNA extraction

Approximately 1 g GLRaV-3 infected cortical scrapings of grapevine canes was pulverized in liquid nitrogen, and viral dsRNA was recovered by chloroform extraction and purification through cellulose CF-11 (Whatman) as described by Rezaian et al. (1991).

### 2.3. RT-PCR, RT–nPCR

Approximately 600 ng dsRNA was denatured with 400 ng 6-mer random primers (TaKaRa, Dalian, China) at 99 °C for 5 min, and chilled on ice. The cDNA synthesis was performed with M-MLV reverse transcriptase in a 40 μL final reaction volume according to the instructions of the manufacturer (Promega, USA). For the nested PCR, the outer primers GLR-3-L (5′-ATGGCG/ATTC/TGAACTGAAATTAGGGC-3′) and GLR-3-R (5′-CTAC/G/TTTCTTC/TTGCAATAGTTGGAA-3′) were designed from multiple alignments of nucleotide sequences available in GenBank. The expected size of the PCR amplicon encompassing the complete CP gene of GLRaV-3 was 942 bp. Inner primers GLR-3-LN (5′-CAGGAAACCGATATAGGGGTAG-3′) and GLRaV-3-RN (5′-TCGAACTCTTTGAACTCTGTCG-3′) were designed by aligning sequences obtained after first amplification. The expected size of RT-nPCR product was 315 bp. The first round of PCR was performed in a 25 μL reaction volume containing 2.5 μL of 10× PCR buffer, 0.5 mM dNTPs, 0.5 mM of each primer, one unit of Taq DNA polymerase (TaKaRa, Dalian, China), and 3 μL cDNA. Reactions were conducted in a PCR Thermal Cycler (Model PTC-200, MJ Research, USA). The PCR cycling conditions comprised of an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of denaturation for 30 s at 94 °C, annealing at 48 °C for 45 s, elongation for 1 min at 72 °C, and final extension for 10 min at 72 °C. The PCR products were separated by electrophoresis in a 1.2% agarose gel, stained with ethidium bromide and visualized under UV light. Initial RT-PCR products were diluted to 1:10 before using as template for RT-nPCR. For RT-nPCR, all the reaction conditions were kept same as those for RT-PCR.

### 2.4. Cloning, SSCP analysis and sequencing

Amplicons of complete GLRaV-3 CP genes were gel purified and ligated into the PMD18-T vector (Takara, Dalian, China) following the manufacturer's instructions. The recombinant plasmids were identified after transformation into *Escherichia coli* DH5α (Sambrook et al., 1989). Out of 35 samples tested positive for GLRaV-3 by RT-PCR, 16 samples were selected randomly for SSCP analysis which was performed according to the protocol of Palacio and Duran-Vila (1999) with minor modifications. At least 10 positive clones from each of the 16 isolates were analyzed by SSCP. An aliquot of 1 μL PCR product was mixed with 2 μL denaturing solution (95% deionized formamide, 10 mM NaOH, 0.05% bromophenol blue), boiled for 10 min, and chilled on ice immediately, where after it was separated by non-denaturing polyacrylamide gel electrophoresis (8%) at 200 V and 4 °C for 18 h. SSCP profiles were visualized by silver staining. The clones showing unique SSCP patterns and at least one positive clone from other 19 isolates were sequenced by Genscript Corporation (Nanjing, China).

### 2.5. Phylogenetic and nucleotide sequences analyses

Multiple alignments of nucleotide sequence were conducted using CLUSTALX 1.8 (Thompson et al., 1997) with default settings. Genetic distances with standard error were evaluated according to the method of Jukes and Cantor (1969) for correction of superimposed substitutions with MEGA 5. The phylogenetic tree was inferred by importing the aligned sequences (produced with CLUSTALX 1.8) into the MEGA5 and constructed using the neighbor-joining method with 1000 bootstrap replicates to assess the robustness of nodes (Tamura et al., 2011). Only the complete CP gene sequences of GLRaV-3 available in GenBank were used in analyses. Numbers of synonymous and non-synonymous nucleotide substitutions and the population genetic parameters ($\pi$; estimated by the average number of nucleotide differences between two

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