Occurrence of Grapevine Leafroll-Associated Viruses in China

M.-H. Liu and M.-J. Li, Department of Pomology/Lab of Stress Physiology and Molecular Biology for Tree Fruits, A Key Lab of Beijing Municipality, College of Agronomy and Biotechnology, China Agricultural University, Beijing 100193; H.-H. Qi, Plant Science and Technology College, Beijing University of Agriculture, Beijing 102206; R. Guo and X.-M. Liu, Department of Pomology/Lab of Stress Physiology and Molecular Biology for Tree Fruits, A Key Lab of Beijing Municipality, College of Agronomy and Biotechnology, China Agricultural University; Q. Wang, Department of Plant Pathology, College of Agronomy and Biotechnology, China Agricultural University; and Y.-Q. Cheng, Department of Pomology/Lab of Stress Physiology and Molecular Biology for Tree Fruits, A Key Lab of Beijing Municipality, College of Agronomy and Biotechnology, China Agricultural University

Abstract

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To characterize the prevalence of viruses associated with grapevine leafroll disease in China, 249 grapevine (*Vitis* spp.) samples (86 popular cultivars and a rootstock) from 19 provinces and regions were collected and tested for *Grapevine leafroll-associated virus 1* (GLRaV-1), GLRaV-2, GLRaV-3, GLRaV-4, and GLRaV-4 strain 5 by SYBR Green real-time reverse-transcription polymerase chain reaction (RT-PCR), and RT-PCR and sequencing. GLRaV-3 was found in 100% of the samples while GLRaV-1, GLRaV-2, and GLRaV-4 were detected in 24.9% (62/249), 15.3% (38/249), and 0.80% (2/249) of the samples, respectively. Single infections with GLRaV-3 were found in 66.3% (165/249) of the samples, and the remaining samples were mixed infections of GLRaV-3 with one or two other GLRaVs, those with GLRaV-1 being the most common (18.5%, 46/249). The genetic varia-

bility of Chinese GLRaV-3 isolates was characterized based on the coat protein (CP) gene. In total, 153 full-length CP gene sequences (94 sequences newly generated) of Chinese GLRaV-3 isolates from different grapevine-growing regions showed 89.3 to 100.0% and 92.7 to 100.0% identity at the nucleotide and amino acid levels, respectively. The average nucleotide diversity for the population of Chinese GLRaV-3 isolates was estimated at 0.037 (standard error = 0.0032). GLRaV-3 isolates from China segregated into five distinct phylogenetic groups and two novel recombination events were found in the viral population. This is the first and most extensive report of the prevalent species of GLRaV in China, which also provides an assessment of genetic variability of GLRaV-3 Chinese isolates.

Grapevine leafroll disease (GLD) is considered one of the most economically important viral diseases in all grapevine-growing countries, causing decreases in berry weight and total soluble solids and increases in titratable acidity (27). Graft incompatibility has also been associated with GLD (3). Several positive single-stranded RNA virus species, referred to as grapevine leafroll-associated viruses (GLRaVs), from the family *Closteroviridae* have been reported to be associated with GLD (16). The genus *Ampelovirus* comprises mealybug-transmitted GLRaV-1 and -3 in subgroup I and GLRaV-4, including the genetically divergent variants GLRaV-4 strain 5, 6, 9, De, Pr, and Car, in subgroup II (22). GLRaV-2 is included in the genus *Closterovirus* comprising aphid-transmitted closteroviruses, whereas GLRaV-7 is in the provisional genus “*Velarivirus*” comprising viruses with unknown vectors (2,22).

GLRaV-1, GLRaV-2, and GLRaV-3 are often prevalent in leafroll-affected grapevines (11). GLRaV-1 in Turkey (1) and GLRaV-2 in Chile (10) and in Argentina (30) have been reported to be the main causative agents of GLD. However, GLRaV-3 has been found to be the most frequently encountered virus associated with the disease in many grapevine-growing countries (4,7,14,15,20,21,23,28). GLRaV-4 and GLRaV-4 strain 5 have only been found in extremely low frequency in grapevines (28).

Understanding the genetic diversity of a virus population can help epidemiological investigations and diagnosis. The genetic variability of GLRaV-3 isolates has been characterized by analyses of partial or complete heat-shock protein 70 homolog (HSP70h) or coat protein (CP) genes (5,11,13,15,28,29), and of a large fragment at the 3' end of GLRaV-3 isolates (31). Those authors recognized the existence of three to seven phylogenetic groups, with variants from group II, group III, and, most notably, group I being by far the most common. Groups I, II, and III of GLRaV-3 isolates are identical groups on the basis of the CP and HSP70h genes (15), except for the recently reported Indian isolate (17). Recombination events have been reported in the CP (8,29) and HSP70h (29) genes of the GLRaV-3 genome.

China is one of the world's leading grape-production areas, recently estimated to cover a total area of 438,000 ha (of which nearly 13% is for wine and 73% for table grape). Vineyards are mainly distributed in Xinjiang Uygur Autonomous Region, and Shandong, Hebei, Henan, and Liaoning Provinces, with their acreage accounting for nearly 64% of the total. A large number of cultivars are grown in China. The primary wine grape cultivars are ‘Cabernet Sauvignon’, ‘Carmenère’, and ‘Merlot’, whereas the prevailing table grape cultivars are ‘Kyoho’, ‘Red Globe’, ‘Thompson Seedless’, ‘Summer Black’, and ‘Manicule Finger’, among others. GLRaV-1, 2, 3, 4, and GLRaV-4 strain 5 have been identified in China (26,32,33); more recently, we reported the full genome sequence of Chinese GLRaV-3 isolate LN (9), and GLRaV-7 was found in two native Chinese grapevine cultivars (19). Due to the frequent exchange of virus-infected propagation material among growers, leafroll is now one of the most important viral diseases of grapevines in China. Farooq et al. (8) recently reported on the genetic diversity of GLRaV-3; however, their study was based on a relatively small number of isolates obtained from only three different grapevine-growing regions in China. Thus, a comprehensive overview of the incidence and distribution of GLRaVs as well as the genetic variability of Chinese GLRaV-3

Corresponding author: Y.-Q. Cheng, E-mail: chengyuqin@cau.edu.cn

M.-H. Liu, M.-J. Li, and H.-H. Qi contributed equally to this work.

*The e-Xtra logo stands for “electronic extra” and indicates that a supplementary table is available online.

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isolates is still lacking. Therefore, we conducted an extensive survey of grapevines for GLRaVs using SYBR Green real-time quantitative reverse-transcription polymerase chain reaction (SG qRT-PCR) and RT-PCR-based testing and sequencing. The specific objectives of this research were to (i) determine which GLRaV species are most commonly associated with GLD in China and (ii) characterize genetic variability of the GLRaV-3 isolates obtained from different grapevine-growing regions in China.

Materials and Methods

Surveys and sample collection. The vineyard surveys and sample collection were performed in late August through October during four growing seasons (2009 to 2012) in 19 different grapevine-growing regions in North China (Liaoning, Jilin, and Hebei Provinces, Beijing and Tianjin regions), East China (Shanghai region, Shandong, Jiangsu, Fujian, and Zhejiang Provinces), West China (Shanxi and Gansu Provinces, Xinjiang Uygur Autonomous Region, and Ningxia Hui Autonomous Region), Central China (Anhui, Hubei, and Henan Provinces), and South China (Yunnan and Sichuan Provinces). In total, 72 vineyards of different sizes and ages and one varietal collection of *Vitis vinifera* in Henan Province were surveyed. One to three samples (from one to three individual grapevines) were collected per cultivar and vineyard. Each sample consisted of 10 to 15 petioles from a single plant that showed mild (very slightly curled leaves) or typical (interveinal reddening or mild yellowing and curled leaves) leafroll symptoms. Samples were stored at -40°C until testing.

Virus detection. Primer pairs HSP70-149 F/HSP70-293 R, 198 F/290 R, 56 F/285 R, and HSPV F/HSPC R (24) and LR4F/LR4R (26) used in SG qRT-PCR were previously reported. Positive controls for all GLRaV species tested in this study were kept in our laboratory. RNA extraction, SG qRT-PCR, and RT-PCR were basically carried out as described previously (6,31). Total RNA was extracted from phloem scrapings (100 mg) with the RNeasy

plant mini kit (Qiagen). SG qRT-PCR was performed with SYBR PrimeScript RT-PCR Kit (TaKaRa) according to the manufacturer's instructions. Synthesis of cDNA was primed with a mix of random primers and oligo dT provided by the kit using 800 to 1,000 ng of total RNA. Each qPCR (20 μl) contained 10 μl of SYBR *Premix Ex Taq*, 1 μl of RT products (at 10-fold dilution), 0.2 μl of each primer (20 μM), 0.4 μl of ROX Reference DyeII (at 50-fold dilution), and 8.2 μl of dH₂O. An ABI 7500 Fast Real-Time PCR System (Applied Biosystems) was used for PCR amplification. Amplification conditions were as follows: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, 61°C for 30 s, and 72°C for 35 s. Data were analyzed by measuring the threshold cycles and the peak in the melting curve.

Randomly selected samples that were positive for GLRaV-1, GLRaV-2, or GLRaV-4 were further assayed by RT-PCR-based testing and sequencing. The sequences were deposited to GenBank, KC477189-KC477195, or provided in Supplementary Table S1.

Cloning and sequencing of the GLRaV-3 CP gene. The primer pair CPF (5'-ATGGCATTGAACTGAAAT-3') and CPR (5'-CTACTTCTTTTGCAATAGT-3') for amplification of the GLRaV-3 CP gene was designed based on sequences available in GenBank. GLRaV-3 derived from a single grapevine was considered one isolate. We named each isolate based on their origin, using letters to represent the province or region. First-strand cDNA was synthesized from total RNA (4 μl of RNA per 20- μl reaction) using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Promega) and the specific reverse primer CPR. The RT reaction (2 μl) was added to a final PCR volume of 25 μl . The PCR program consisted of 5 min at 95°C , followed by 35 cycles of 95°C for 30 s, 60.3°C for 30 s, and 72°C for 1 min. This was followed by a final extension for 10 min at 72°C . The amplicons were cloned individually into pMD18-T simple vector. At least three independent clones in both orientations were sequenced, and a

Fig. 1. Map of China showing the provinces and regions (marked with pentagram symbols) where grapevines were surveyed for the presence of grapevine leafroll-associated viruses.

consensus sequence was derived when the three independent clones showed $\geq 99\%$ identity, so as to exclude in vitro RT-PCR errors. This consensus was termed “unique sequence”. If the isolate showed $<99\%$ nucleotide sequence identity between the three initially sequenced clones, additional clones were sequenced to investigate the possible occurrence of mixtures of distinct sequence variants within individual grapevines.

Sequence analysis. Phylogenetic analyses included 153 sequences of Chinese isolates (94 new sequences generated in this study and 59 sequences available in GenBank), and 12 representative sequences belonging to different groups which have been described previously (5,13,15,28,29). A neighbor-joining phylogenetic tree was constructed with the Mega 4 software package with 1,000 bootstrap replicates to assess node robustness. The occurrence of putative recombination events was assessed by at least six programs in Recombination Detection Program version 3 (RDP3) with default parameters (highest acceptable probability value = 0.05). The ratios of the rates of nonsynonymous (dN) to synonymous (dS) mutations for the CP gene of the Chinese isolates were calculated using the PBL method (18,25) implemented in Mega 4. The nucleotide diversities were determined using the DnaSP5 computer program. Multiple sequence alignment was performed using DNAMAN version 6.

Results

Prevalence of GLRaV-1 to -5 in leafroll-affected grapevines in China. In total, 72 vineyards of a wide range of table and wine grape cultivars and one varietal collection of *V. vinifera* from different provinces or regions in China (Fig. 1) were visually surveyed for leafroll symptoms. Over half of the vineyards surveyed were more than 20 years old. Inspected vines were largely own-rooted plants, except plants of *V. amurensis* cultivars such as ‘Shuanghong’, ‘Shangfeng’, ‘Zuoshan No. 1’, and ‘Zuoshan No. 2’ planted in Jilin Province (North China), which were grown on rootstocks (‘Beida’ is the most common) due to their low rooting ability. During the period of survey (August to early October), GLD seemed to be widespread because 71 vineyards and the varietal collection presented 85 to 100% symptomatic plants regardless of vineyard age. Leaves of most cultivars exhibited distinct downward curling; only some cultivars such as ‘Cow’s Nipple’ and ‘Dragon’s Eye’ displayed very slight curling of the leaves. Intervinal reddening or mild yellowing of leaves was often observed in wine grape cultivars, and some table grape cultivars such as ‘Hongshuangwei’ and ‘Jingxiu’ (red-berried cultivars) or ‘Victoria’ and ‘Rizamat’ (white-berried cultivars) also exhibited slight red-

dening or yellowing, respectively, between major veins of leaves. The single vineyard which was established with certified, clean propagative material showed a very small number of symptomatic plants and was not sampled. Of 840 samples with leafroll symptoms that were collected, 249 samples were further selected for the detection of GLRaV-1, 2, 3, 4, and GLRaV-4 strain 5 by SG qRT-PCR. These samples were from 86 cultivars (more than two-thirds from table grape cultivars) and one rootstock (Beida) as well as from different geographical regions.

All samples positive for GLRaV-3; GLRaV-1, GLRaV-2, and GLRaV-4 were found in 24.9, 15.3, and 0.80% of the samples, respectively (Table 1). This result showed that GLRaV-3 is the predominant virus associated with GLD in China. In addition, 66.3% (165/249) of the samples were infected with only GLRaV-3, and GLRaV-1, -2, and -4 were only detected in mixed infections with GLRaV-3. Mixed infections with GLRaV-1 and GLRaV-3 (18.5%, 46/249) were the most common, followed by GLRaV-2 and GLRaV-3 (8.8%, 22/249). In addition, 6.4% (16/249) of the samples were mixed infected with GLRaV-1, -2, and -3, whereas only two samples showed mixed infections with GLRaV-3 and GLRaV-4. GLRaV-4 strain 5 was not detected in any sample.

GLRaV-3 was detected in all grapevine cultivars tested whether they were native to China, such as Dragon’s Eye, Cow’s Nipple, ‘Munake’, and ‘Amur’ grapevine cultivars (Shuanghong, Shangfeng, Zuoshan No. 1, and Zuoshan No. 2 of *V. amurensis*) or new introductions (e.g., Red Globe, Kyoho, Summer Black, and Manicure Finger). In addition, mixed infections of two or three GLRaVs were found in many grapevine cultivars such as Cabernet Sauvignon, Merlot, Carmenère, ‘Chardonnay’, Kyohou, Red Globe, Thompson Seedless, Summer Black, Manicure Finger, ‘Yatomi’, ‘Gold Finger’, ‘Muscat’, ‘Ardeleanca’, Victoria, ‘August’, and Amur grapevines (Zuoshan No. 1 and Shuanghong) and the rootstock Beida. For instance, Cabernet Sauvignon (the most popular grapevine cultivar in China, with a total cultivation area of approximately 23,000 ha) and the popular table grape cultivars Kyoho, Manicure Finger, and Summer Black often showed mixed infections of GLRaV-3 with GLRaV-1 or GLRaV-2. The other major table grape cultivar in China, Red Globe, frequently showed a mixed infection with GLRaV-3 and GLRaV-2. ‘Autumn Royal’ was the only cultivar that tested positive for GLRaV-4 in this survey.

Genetic diversity of GLRaV-3 isolates from China. In total, 94 GLRaV-3-positive samples were selected (based on different geographical origins and the economic importance of the grapevine cultivars) to amplify the CP gene with primer pair CPF/CPR. Initially, three independent clones were sequenced in both orienta-

Table 1. Distribution of *Grapevine leafroll-associated virus 1* (GLRaV-1), -2, -3, -4, and GLRaV-4 strain 5 in leafroll-affected grapevines in China

Province, region	N ^a	Number positive for GLRaVs				
		GLRaV-1	GLRaV-2	GLRaV-3	GLRaV-4	GLRaV-4 strain 5
Hebei	37	6	10	37	0	0
Ningxia	25	5	0	25	0	0
Xinjiang	23	3	2	23	0	0
Henan	20	4	1	20	0	0
Jiangsu	20	9	5	20	2	0
Jilin	18	6	1	18	0	0
Gansu	15	7	1	15	0	0
Beijing	14	0	2	14	0	0
Shandong	13	2	2	13	0	0
Yunnan	13	3	0	13	0	0
Liaoning	9	2	5	9	0	0
Zhejiang	8	5	0	8	0	0
Shanghai	8	0	0	8	0	0
Tianjin	6	0	1	6	0	0
Shanxi	6	2	4	6	0	0
Anhui	5	0	1	5	0	0
Hubei	4	4	0	4	0	0
Fujian	3	2	3	3	0	0
Sichuan	2	2	0	2	0	0
Total	249	62	38	249	2	0

^a Number of samples tested.

tions per isolate. If the three clones from one isolate showed sequence identity <99.0%, additional clones were sequenced to investigate the possible occurrence of mixtures of distinct sequence variants within individual grapevines. However, the initial three clones for each isolate in this study showed ≥99.0% identity and, thus, were considered to present a single sequence. The sequences of these isolates were deposited in GenBank (Table 2).

In pairwise comparisons, the 94 CP gene sequences of Chinese isolates (Hebei = 13, Ningxia = 13, Henan = 10, Beijing = 9, Jilin = 8, Shandong = 7, Xinjiang = 7, Liaoning = 6, Yunnan = 5, Tianjin = 5, Gansu = 4, Zhejiang = 3, Jianshu = 2, Anhui = 1, and Shanxi = 1) newly generated from 60 grapevine cultivars showed identities of 89.3 to 100.0 and 92.7 to 100.0% at the nucleotide and amino acid levels, respectively. Similar ranges of values were observed when comparisons were made with corresponding sequences of GLRaV-3 isolates ($n = 59$) from China available in GenBank. The overall mean value of nucleotide diversity of this set of 153 sequences was estimated to be 0.037 (standard error = 0.0032), indicating a low level of genetic variability.

To gain insight into the evolutionary forces and constraints acting on the GLRaV-3 CP gene, the dN/dS ratio among Chinese isolates was computed. The mean dN/dS ratio for the CP gene of

the Chinese isolates was 0.086, suggesting a strong tendency toward negative (or purifying) selection.

GLRaV-3 isolates from China belong to five genetic groups.

A phylogenetic analysis was done with a total of 153 CP gene sequences of GLRaV-3 isolates from China and 12 representative sequences from other grapevine-growing regions to maintain a standardized nomenclature of GLRaV-3 sequence variant groups by analogy with previous reports (5,13,15,28,29). The additional sequence (43-15) that clustered separately in the phylogenetic tree presented by Sharma et al. (28) is a partial sequence of the CP gene. Therefore, it was not included in our phylogenetic analysis. The results revealed the existence of six well-defined clusters, and all sequences of GLRaV-3 Chinese isolates segregated into five groups (Fig. 2) corresponding to previously defined variant groups I to V (5,15), regardless of cultivar or vineyard origin. Most of the Chinese GLRaV-3 isolates were placed in group I. Group III contained three isolates (HN-3, XJ-1, and XJ-4) obtained in this work and previously reported sequences of Chinese isolates. Four sequences of Chinese isolates (BJ-6, HB-1, HB-2, and HB-3) were placed in group IV. Group V contained three sequences of Chinese isolates previously reported by Farooq et al. (8).

The genetic distance within variant group was highest in group III (0.020), followed by group I (0.009), group II (0.008), group V

Table 2. Name, cultivar, GenBank accession number (GenBank), and other details of *Grapevine leafroll-associated virus 3* coat protein gene sequences generated in this study

Isolate	Source	Cultivar	GenBank
AH-1	Anhui	Red Fuji	KC417451
BJ-1	Beijing	Aranèle	KC477101
BJ-2	Beijing	Aranèle	KC477102
BJ-3	Beijing	Flame Seedless	KC477103
BJ-4	Beijing	Rizamat	KC477104
BJ-5	Beijing	Rizamat	KC477105
BJ-6	Beijing	Manicule Finger	KC477106
BJ-7	Beijing	Victoria	KC477107
BJ-8	Beijing	Yatomi Rosa	KC477108
BJ-9	Beijing	Yatomi Rosa	JX481786
GS-1	Gansu	Tamina	KC477109
GS-2	Gansu	Takatsuma	KC477110
GS-3	Gansu	Himrod Seedless	JX481787
GS-4	Gansu	Zinfandel	KC477111
HB-1	Hebei	Gold Finger	KC477112
HB-2	Hebei	Unknown	KC477113
HB-3	Hebei	Unknown	KC477114
HB-4	Hebei	Augusta	KC477115
HB-5	Hebei	Red Globe	KC477116
HB-6	Hebei	Red Globe	KC477117
HB-7	Hebei	Merlot	KC477118
HB-8	Hebei	Carmenère	KC477119
HB-9	Hebei	Carmenère	KC477120
HB-10	Hebei	60-1	KC477121
HB-11	Hebei	Red Globe	KC477122
HB-12	Hebei	Carmenère	KC477123
HB-13	Hebei	Exotic	KC477124
HN-1	Henan	Ardeleanca	KC477125
HN-2	Henan	Atebage	KC477126
HN-3	Henan	Daban	KC477127
HN-4	Henan	Aligote	KC477128
HN-5	Henan	Herbet	KC477129
HN-6	Henan	Hongjiu	KC477130
HN-7	Henan	Gold Finger	KC477131
HN-8	Henan	Kaifengwuhe	KC477132
HN-9	Henan	Shanxi No. 80	KC477133
HN-10	Henan	Summer Black	KC477134
JL-1	Jilin	Gongniang No. 1	KC477135
JL-2	Jilin	Gongniang No. 2	KC477136
JL-3	Jilin	Shangfeng	KC477137
JL-4	Jilin	Shuanghong	KC477138
JL-5	Jilin	Shuangyou	KC477139
JL-6	Jilin	Zuoshan No. 1	KC477140
JL-7	Jilin	Zuoshan No. 2	KC477141
JL-8	Jilin	Zuoyouhong	KC477142
JS-1	Jiangsu	Gold Finger	KC477143

(continued in next column)

Table 2. (continued from preceding column)

Isolate	Source	Cultivar	GenBank
JS-2	Jiangsu	Thompson Seedless	KC477144
LN-1	Liaoning	No. 27	KC477145
LN-2	Liaoning	No. 32	KC477146
LN-3	Liaoning	Unknown	KC477147
LN-4	Liaoning	Unknown	KC477148
LN-5	Liaoning	Unknown	KC477149
LN-6	Liaoning	Unknown	KC477150
NX-1	Ningxia	Muscat × Alicanfe Bouschet	KC477151
NX-2	Ningxia	Red Globe	JX481788
NX-3	Ningxia	Red Italia	KC477196
NX-4	Ningxia	Thompson seedless	KC477152
NX-5	Ningxia	Ribier	JX481789
NX-6	Ningxia	Carmenère	KC477153
NX-7	Ningxia	Cabernet Sauvignon	KC477154
NX-8	Ningxia	Italian Riesling	KC477155
NX-9	Ningxia	Carmenère	KC477156
NX-10	Ningxia	Garnacha Tinta	KC477157
NX-11	Ningxia	Pinot Gris	KC477158
NX-12	Ningxia	Carmenère	KC477159
NX-13	Ningxia	Sauvignon Blanc	KC477160
SD-1	Shandong	Cow's Napple	KC477161
SD-2	Shandong	Cabernet Sauvignon	KC477162
SD-3	Shandong	Blue French	KC477163
SD-4	Shandong	Kyoho	KC477164
SD-5	Shandong	Cabernet Franc	KC477165
SD-6	Shandong	Carmenère	KC477166
SD-7	Shandong	Carmenère	KC477167
SX-1	Shanxi	Red Globe	KC477168
TJ-1	Tianjin	Rosario Bianco	KC477169
TJ-2	Tianjin	Triumph	KC477170
TJ-3	Tianjin	Red Globe	KC477171
TJ-4	Tianjin	Thompson Seedless	KC477172
TJ-5	Tianjin	Summer Black	KC477173
XJ-1	Xinjiang	Munage	KC477174
XJ-2	Xinjiang	Fenyuan	KC477175
XJ-3	Xinjiang	Red Globe	KC477176
XJ-4	Xinjiang	Rizamat	KC477177
XJ-5	Xinjiang	Manicule Finger	KC477178
XJ-6	Xinjiang	Unknown	KC477179
XJ-7	Xinjiang	Unknown	KC477180
YN-1	Yunnan	8612	KC477181
YN-2	Yunnan	Jinxu	KC477182
YN-3	Yunnan	Venus Seedless	KC477183
YN-4	Yunnan	Rose Honey	KC477184
YN-5	Yunnan	Merlot	KC477185
ZJ-1	Zhejiang	Gold Finger	KC477186
ZJ-2	Zhejiang	Kyoho	KC477187
ZJ-3	Zhejiang	Thompson Seedless	KC477188

(0.007), and group IV (0.003). The genetic distances between variant groups were 0.056 to 0.105.

Novel recombination events in the CP gene. Two new recombination events were detected using RDP3 (Table 3). Based on

these analyses, isolate NX-10 and the previously reported sequence S3-33-2 were detected as recombinants with highly significant statistical values by at least six detection methods. The recombinant NX-10 had isolate ZJ-1 in group I as its major parent and the

Fig. 2. Phylogenetic tree of the complete coat protein gene from *Grapevine leafroll-associated virus 3* Chinese isolates. Reference isolates are marked with an asterisk (*). Sequences of previously reported Chinese isolates are indicated by their isolate name and GenBank accession numbers. Bootstrap analysis with 1,000 replicates was performed. Only bootstrap values of $\geq 75\%$ are shown, and branch lengths are proportional to the genetic distances.

sequence BZ15-4 in group II as its minor parent, while the recombinant S3-33-2 had the sequence BZ15-9 (in group I) and HN-3 (in group III) for its major and minor parents, respectively. The locations of identified recombination break points, 3 to 515 nucleotides (nt) for NX-10 and 201 to 318 nt for S3-33-2, were different from those previously reported by Turrero et al. (29) and Farooq et al. (8).

Discussion

Our survey shows that GLD is widespread in China. GLRaV-3 was the most frequently encountered virus in Chinese leafroll-affected grapevines associated with this disease, similar to other grapevine-growing areas of the world (4,11,20,23,28). GLRaV-1, -2 and -4 were only detected in mixed infections with GLRaV-3. Current Chinese regulations state that certified plant material cannot contain GLRaV-1 or GLRaV-3 but do not consider GLRaV-2 or the other leafroll viruses. However, we found 15.3% of the samples positive for GLRaV-2, suggesting that it should be included in Chinese regulations for grapevine certification. Mixed infections were observed in 33.7% (84/249) of the samples in this survey, significantly higher than the number found in the Finger Lakes region (11) and Napa Valley (28) in the United States, where the authors used RT-PCR/enzyme-linked immunosorbent assay and RT-PCR, respectively.

Our results indicated that GLRaVs were also present in Amur grapevine cultivars planted in Jilin Province. It might be due to the grafting onto an infected rootstock, because Beida, the most widely used rootstock in the area, showed mixed infection of GLRaV-3 and GLRaV-2. Alternatively, the Amur grapevine cultivars might have been already infected with GLRaV before being grafted onto a rootstock. Further studies are needed to test this hypothesis.

GLRaV-4 was present at a very low level (0.80%; Table 1); a similar trend was observed in Napa Valley (28). In addition, Autumn Royal was found to be the only cultivar planted in China that was positive for GLRaV-4 in our survey (samples obtained from vineyards in Jianxu Province) and in a previous report (26), where authors collected samples from the varietal collection of *V. vinifera* in Liaoning Province in China, suggesting the introduction of contaminated planting materials.

Although SG qRT-PCR and RT-PCR are sensitive methods for GLRaV detection (6), GLRaV-4 strain 5 was not detected in this survey. Similar results were reported in Napa Valley (28). This might be due to the low distribution frequency of GLRaV-4 strain 5 in leafroll-affected grapevines. In addition, the limited number of vineyards and samples in the present study might have reduced the chances of detecting the virus. We also cannot rule out the possibility that the primer set might not have been suitable for the detection of GLRaV-4 strain 5 in China.

In this study, the genetic variability of GLRaV-3 isolates from different grapevine-growing regions in China was established using the CP gene nucleotide sequence. Together with a recent report (5), our phylogenetic analysis supports segregation of GLRaV-3 isolates into six phylogenetic groups (Fig. 2), with GLRaV-3 Chinese isolates segregated into five groups. A previous report showed that isolates from China segregated into groups I, II, III, and V (8); however, one new group (group IV) was identified here for the Chinese isolates BJ-6, HB-1, HB-2, and HB-3. This result suggests that the GLRaV-3 population in China is more diverse than initially thought. No Chinese isolate was found to belong to group VI.

Similar to previous results (5,13,28,31), variants from groups I, II, and III were the most common; nevertheless, we also observed that the vast majority of Chinese isolates belonged to group I. These results most likely explain why the genomic diversity (0.037) for the entire population of Chinese isolates was lower than previous estimates of 0.063 by Gouveia et al. (13) and 0.049 by Turrero et al. (29). Our phylogenetic analysis showed a lack of clustering by geographical origin, similar to other studies (11,29,31), further emphasizing the importance of the exchange and use of infected plant material in the dissemination of this virus.

Previous reports have shown mixed infections of two or more GLRaV-3 variants in a single plant (28,29). In this study, no multiple infections of GLRaV-3 variants were detected but this was expected, given the number of clones sequenced per isolate; it is possible that mixed infections of different GLRaV-3 variants within a single plant were present in the samples but not detected.

Recombination results in genetic exchange, and may be a major source of evolutionary variation (12). Turrero et al. (29) suggested the CP gene as one of the recombination hotspots in the GLRaV-3 genome, and Farooq et al. (8) reported a high recombination frequency in the CP gene of Chinese isolates, of which most were from the varietal collection of *V. vinifera* in Henan Province. In this study, we observed two novel recombination events in the CP gene of the Chinese GLRaV-3 population. The results of our recombination analysis agreed with those of the phylogenetic analysis. The recombinant NX-10, together with previously reported recombinants (HDQ-7, ADLG-3, and BZ-15) included in subgroup I B (8), and the other recombinant S3-33-2, did not cluster clearly into any variant group. Furthermore, we noted that two sequences, BZ15-9 and HN-3, are the major and minor parents for recombinant S3-33-2 identified in this study and those previously reported by Farooq et al. (8), where one or both parents of these recombinants were not detected (*data not shown*). These two sequences both originated from the same geographic region, the varietal collection of *V. vinifera* in Henan Province. A possible explanation for these recombination events might be that numerous grapevine cultivars carrying different GLRaV-3 variants were planted in one garden at the collection, and mealybug-mediated transmission might have provided more chances for mixed infection of different variants in the same grapevine, resulting in recombination. However, grapevines at the varietal collection might come originally from grafted vines collected from different sources worldwide prior to being planted as own-rooted vines at the collection. Therefore, mixed infections of distinct GLRaV-3 variants in the same grapevine could also be obtained by grafting onto an infected rootstock.

Similar to previous observations (13,28,29,31), there was no evidence for positive selection in the CP gene, indicating that the five identified groups of GLRaV-3 isolates have been present in China for some time and that they are not increasing in prevalence in the region.

This is the first and the most extensive report of the GLRaV species prevalent in China as well as on the genetic variability of GLRaV-3. Our results could help improve regulatory measures, including quarantine, to improve the sanitary status of the planting material.

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Table 3. Characteristics of two novel recombination events detected in the Chinese *Grapevine leafroll-associated virus 3* population^a

Recombinant	Parental (major × minor) sequences	Breakpoints (begin–end)	Average <i>P</i> value
NX-10	ZJ-1 × BZ15-4	3–515	G (1.780×10^{-2}), B (1.380×10^{-3}), M (9.903×10^{-5}), C (1.403×10^{-3}), S (9.251×10^{-7}), L (5.277×10^{-11}), 3s (2.839×10^{-5})
S3-33-2	BZ15-9 × HN-3	201–318	B (4.789×10^{-3}), M (7.014×10^{-7}), C (5.378×10^{-7}), S (1.073×10^{-11}), L (1.620×10^{-14}), 3s (3.171×10^{-15})

^a The suite of recombination detection programs used for the detection of recombination events and the corresponding average *P* values were: G, GeneConv; B, Bootscan; M, MaxChi; C, CHIMAERA; S, SiScan; 3s, 3SEQ; *P* > 0.05.

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