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Genetic diversity and phylogenetic analysis of *Australian Grapevine Viroid* (AGVd) isolated from different grapevines in China

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Abstract *Australian grapevine viroid* (AGVd) is found in only three countries in the world. Here, the genetic diversity and phylogenetic relationships of AGVd isolates from three different grape varieties (Thomson Seedless, Jingchuan and Zaoyu) in China were studied. A hundred of independent cDNA clones from each of the three isolates, in total of 300, were sequenced. We identified 29 sequence variants including two predominant ones in Thomson Seedless, and 48 each including a unique predominant one in Jingchuan and Zaoyu. In silico structure analysis revealed that base changes were clustered in the left terminal domain of the predicted secondary structure in all three isolates. Further, these changes were shown to affect their secondary structures to varying degrees. Genetic diversity and phylogenetic analysis of four predominant sequence variants from this study, plus four others from Australia and Tunisia, revealed obvious regional disparity and variety-specificity in AGVd.

Keywords *Australian grapevine viroid* · Phylogenetic analysis · Sequencing analysis · Secondary structure

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Introduction

Viroids are small (246–475 nucleotides) covalently closed single-stranded RNAs. Like viruses, viroids replicate in host plants and act as phytopathogenic agents; however, unlike viruses they do not code for proteins. Viroids are classified into two families: *Pospiviroidae,* composed of species with a central conserved region (CCR) and no hammerhead ribozymes, and *Avsunviroidae,* composed of species lacking CCR but able to self-cleave in both polarity strands through hammerhead ribozymes [1].

So far, *Australian grapevine viroid* (AGVd), *Citrus exocortis viroid* (CEVd), *Hop stunt viroid* (HSVd), *Grapevine yellow speckle viroid* 1(GYSVd1), and *Grapevine yellow speckle viroid* 2 (GYSVd2) have been isolated from grapevines [2–4]. AGVd is 369 nt in length and was first described in Australia [2]. It contains the entire central conserved region (CCR) of the apple scar skin viroid group and is a member of the genus *Apscaviroid*, family *Pospiviroidae*. AGVd has only been isolated from grapevines and its entire sequence can be divided into regions, each with a high sequence similarity with segments from *Potato spindle tuber viroid* (PSTVd), *Apple scar skin viroid* (ASSVd), CEVd and GYSVd [5]. So far, AGVd has only been reported in Australia, Tunisia and China [2, 6, 7], and could be distinguished from other viroids by a combination of its electrophoretic properties, its ability to replicate in cucumber and in tomato, and its lack of hybridization to other viroid probes [5]. Considering present research, we know that grapevines are the only natural host of AGVd, and the relationship of AGVd to grapevine diseases is unclear [2].

The genetic diversity and variability of AGVd has not been documented and few reports on AGVd have been published. The present work describes the molecular

characterization of AGVd isolates from three grape varieties in China and provides information regarding the variability found within each isolate. The variability among isolates from China, Australia and Tunisia was also studied.

Materials and methods

Viroid sources

From 2006 to 2007, young leaves of more than 130 samples from different grape varieties were collected from Xinjiang autonomous region and Beijing, China.

Isolation and extraction of AGVd

Low molecular weight RNAs were extracted according to Li et al. [8]. In brief, 5 g of tissue were powdered in liquid nitrogen, extracted with 10 ml of 1 M K_2HPO_4 containing 0.1% β-mercaptoethanol and homogenized with 10 ml phenol: chloroform (1:1, v/v). After eliminating polysaccharides by 2-methoxyethanol extraction and CTAB precipitation, 2 M LiCl was used to precipitate low molecular weight RNAs. The resulting preparation was dissolved in 30 µl of distilled water.

Reverse transcription-polymerase chain reaction (RT-PCR)

cDNA was generated from viroid RNA by RT-PCR. Template liquid (1 µl) was mixed with 0.5 µl (20 pmol) of primer AGVd P8 (5′-CCCTGCAGGTTTCGCCAGCAAGCGC-3′, complementary to nucleotides 222–224.) and distilled water, heated at 98°C for 5 min, and quenched in ice water for more than 2 min. One microliter of 2.5 mM (each) dNTPs, 1 µl (200 U) MMuLV reverse transcriptase (Promega), 2 µl M-MLV 5X Reaction Buffer, 0.25 µl (40 U) Recombinant RNasin ribonuclease inhibitor and distilled water were added to the RT mixture to yield a final volume of 10 µl. The resulting mixture was incubated at 42°C for 60 min, and at 98°C for 5 min. After the RT reaction, 5 µl of the reverse transcription solution was mixed with 25 µl 2X PCR Ex-TaqMix, 18 µl distilled water and 1 µl (20 pmol) each of primers AGVd P7 (5′-ACCTGCAGGGAAGCTAGCTGGGTC-3′, homologous to nucleotides 239–260.) and AGVd P8 (5′-CCCTGCAGG TTTCGCCAGCAAGCGC-3′) to yield a final reaction volume of 50 µl. The cycling parameters for the PCR amplification consisted of one cycle of heat denaturation at 94°C for 5 min, 30 amplification cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s. The final elongation step was conducted for 5 min at 72°C.

Cloning and sequencing

After RT-PCR, electrophoresis confirmed the presence of a PCR product of the expected size. The products were purified with the PCR purification kit (Tiangen). The resulting fragments were cloned into a pGEM-T vector (Promega) and were transformed into *E. coli* DH5α. Recombinant DNA clones containing a 369 bp insert were identified by restriction analysis. The selected clones were sequenced using an automated DNA sequencer (ABI PRISMTM 3730XL DNA Analyzer) and analyzed by DNAMAN Version 5.2.2.

Phylogenetic analysis and determination of secondary structures

The sequences were aligned with those of the other AGVd sequences deposited in the GenBank databases using the Clustal W (Ver.1.83) program, and phylogenetic analysis were performed using neighbor joining (NJ) and maximum parsimony (MP) methods (the Molecular Evolutionary Genetics Analysis [MEGA] software; [9]). Phylogenetic tree was drawn with TreeView 68 K (Ver.1.5.1). Possible secondary structures were calculated with the CLC RNA Workbench package downloaded from the World Wide Web (version 3.0.1, http://www.clcrnaworkbench.com/).

Results

Genetic diversity of AGVd within each isolate of Thomson seedless, Jingchuan and Zaoyu

Of the more than 130 samples examined, AGVd was dot-blot or northern hybridization positive in three samples: i.e., Thomson Seedless was collected from Xinjiang autonomous region, and Jingchuan and Zaoyu were collected from a grapevine nursery in Beijing. Complementary DNA of AGVd was reverse transcribed and amplified by PCR for cloning. A hundred cDNA clones were chosen randomly from each isolate, and a total 300 independent cDNA clones were sequenced. From them, a total of 125 sequence variants were detected: i.e., 29 from Thomson Seedless, 48 from Jingchuan and 48 from Zaoyu. The 29 sequence variants of Thomson Seedless consisted of eight types of major sequence variants (Tv1–Tv8) and 21 singletons. The major sequence variants in this text mean the sequence variants being comprised of at least two cDNA clones. Two types of variants (Tv1 = 33% and Tv2 = 32%) were predominant in this population (Fig. 1, left). The 48 sequence variants of Jingchuan consisted of five types of major sequence variants (Jv1–Jv5) and 43 singletons, and only one (Jv1 = 43%) was predominant

(Fig. 1, middle). The 48 sequence variants of Zaoyu consisted of seven types of major sequence variants (Zv1–Zv7) and 40 singletons, and only one (Zv1 = 46%) was predominant (Fig. 1, right). These results suggested that the genetic diversity was lower, that means only one was predominant, in the latter two isolates. For example, the overall sequence homology among the 48 sequence variants of Jingchuan isolate was 98.64–99.73%. The predominant sequences from each isolate (Tv1, Tv2, Jv1, and Zv1) were therefore presumed the representative of each population, suggesting that each isolate was a mixture of RNA species, in agreement with the quasispecies concept [10, 11], described for many RNA viruses.

Genetic diversity among isolates from different grapevine varieties and different countries

When aligned, the predominant sequences from the Thomson Seedless, Jingchuan and Zaoyu isolates showed nucleotide differences of 1–6 bases and homologies ranging from 98.38 to 99.73%. The predominant sequence variants from each isolate showed specific variations. For example, the predominant variants of Thomson Seedless, Jingchuan and Zaoyu could be discriminated by the combination of the nucleotides at the positions 11 and 15, in which (− and A) in Thomson Seedless, (− and U) in Jingchuan, and (A and U) in Zaoyu (Fig. 2). Comparison of the predominant sequences of the three Chinese isolates with those from Australia and Tunisia also revealed seven characteristic variations in the Chinese isolates: $A_{28} \rightarrow T$; $T_{30} \rightarrow A$; $C_{47} \rightarrow G$, $G_{48} \rightarrow C$; $A_{55} \rightarrow -$; $-_{322} \rightarrow C$ and $G_{351} \rightarrow A$. In addition to these, Jingchuan isolate showed additional A to T substitution at the position 15 ($A_{15} \rightarrow T$), and Zaoyu isolate also showed two mutations at the positions 11 and 15 ($-_{11} \rightarrow A$ and $A_{15} \rightarrow T$) (Fig. 2, boxed sequences). The isolate from Thomson Seedless was more similar to those from Tunisia and Australia.

Phylogenetic analysis

A phylogenetic analysis was carried out on the four predominant sequence variants isolated from Thomson Seedless, Jingchuan and Zaoyu, (Tv1, Tv2, Jv1 and Zv1) and four others previously reported in Australia and Tunisia [2, 6]. As shown in Fig. 3, AGVd variants from China can be clearly distinguished from the two others from Australia and Tunisia. Further, Jingchuan and Zaoyu variants, both collected from Beijing, showed a closer relationship with each other than with variants of Thomson Seedless collected from Xinjiang province. It was again confirmed that Thomson Seedless isolate is more closely related to Tunisian and Australian isolates.

Genetic diversity on proposed secondary structure

Most of the variations found in the three AGVd isolates in China were clustered in the left terminal domain including the terminal conserved sequence (TCR) and the pathogenicity domain (P) of the predicted secondary structure (Fig. 2). Variations were not found in the CCR [5], at the positions 82–123, of the molecule (Fig. 2). In silico analysis on the predicted secondary structures of the variants suggested that some of the sequence variations could have some influence on the structure. For example, two mutations found at the positions 11 and 15 of Zaoyu (Zv1) variant, as well as the other two mutations found at the positions 28 and 30 of Thomson Seedless (Tv1, Tv2), Jingchuan (Jv1) and Zaoyu (Zv1) variants, could have changed their predicted secondary structures (Fig. 4).

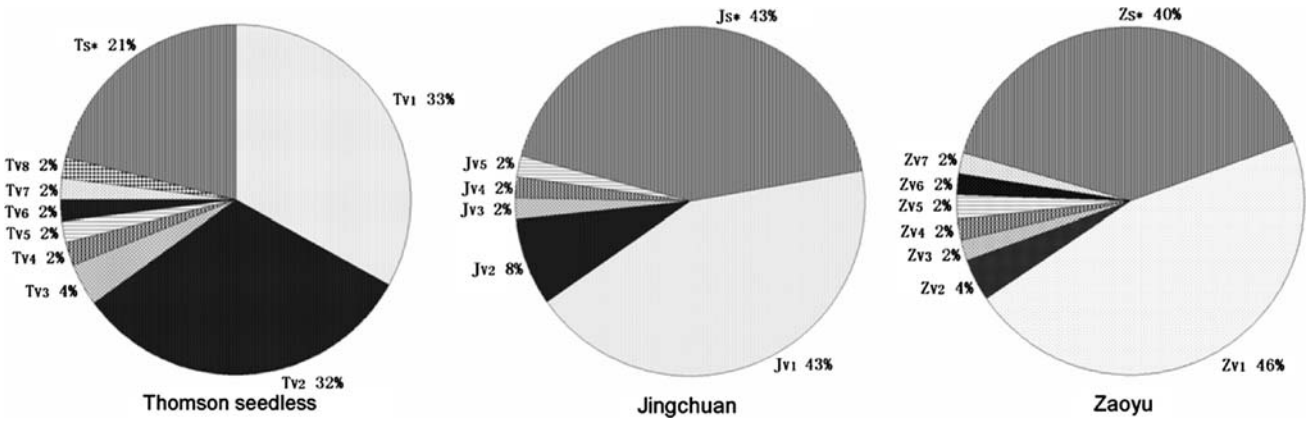

Fig. 1 AGVd variants from the three different grape varieties (Thomson seedless, Jingchuan and Zaoyu) in China. A hundred of independent cDNA clones from each of the three isolates, in total of 300, were sequenced. We identified 29 sequence variants including two predominant ones (Tv1 and Tv2) in Thomson Seedless, and 48 each including a unique predominant one in Jingchuan (Jv1) and Zaoyu (Zv1). (Tvn: variants from Thomson seedless. Jvn: variants from Jingchuan. Zvn: variants from Zaoyu. S*: Singleton sequences.)

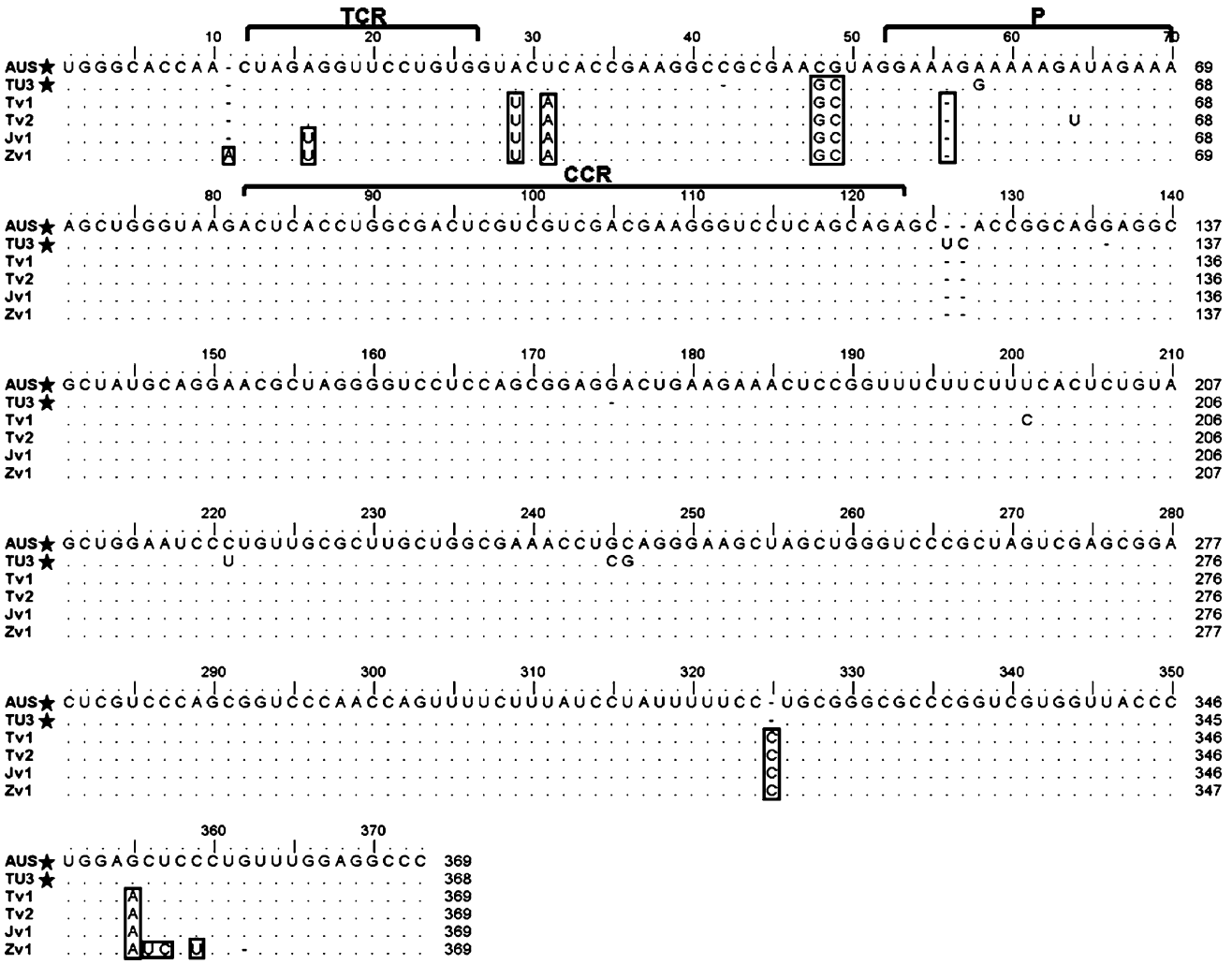

Fig. 2 Sequence alignment of AGVd variants isolated from grapevines in China, Australia and Tunisia. Isolates from different grape varieties and different countries displayed specific sequence variants and the CCR domain and the consensus sequence of TCR of all the variants presented conserved sequences. Abbreviation of the sample names such as "Tv1" were described in the text. Samples with asterisk (★) indicate those obtained from GenBank. (AUS Australia; TU3 Tunisia; Tv1, Tv2: China (Thomson Seedless); Jv1: China (Jingchuan) and Zv1: China (Zaoyu))

Discussion

The genetic structure of viroid populations must be characterized to understand their evolution. Many reports describe the characterization of viroid populations such as HSVd, CEVd, GYSVd1 and PLMVd [12–20], but this is the first report on the genetic diversity and phylogenetic analysis of AGVd. Here, a hundred of independent cDNA clones from each of the three isolates, in total of 300, were sequenced. There were 125 sequence variants different from each other and we identified 29 sequence variants including two predominant ones in Thomson Seedless, and 48 each including a unique predominant one in Jingchuan and Zaoyu. Although we cannot rule out the possibility that the singleton sequences were the result of PCR artifacts, it is likely that they are naturally occurring mutations for the following reasons: First, the frequency of error resulting from Ex-*Taq* polymerase is only about 10^{-4}–10^{-5} [21]. Second, most of the changed bases emerged multiple times in different cDNA clones. For example, at least five sequence variants changed from A to T at the position of the 63rd nt in the Jingchuan isolate. Additionally, most changes were located in the TL and P domains and they never occurred in strictly conserved regions, including the CCR and the consensus sequence of TCR, indicating that they were naturally occurring mutations. To be conservative, however, we submitted only the 20 predominant sequences to GenBank. In addition, the TCR domain of all the variants presented a conserved sequence in accord with previous reports [17, 22] [22, 23], and the observations support the low genetic diversity found in the Terminal Left domain (Fig. 5).

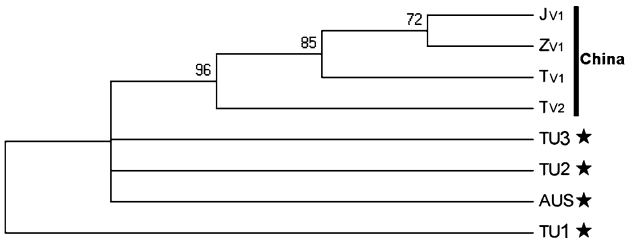

Fig. 3 A phylogenetic tree of 8 sequence variants of AGVd from Australia, Tunisia and China using MEGA 4 program. Branches with bootstrap support less than 70% were collapsed. Numerical numbers in the branches indicate bootstrap support from NJ (100 replicates, 1,000 seeds). AGVd variants from China could be clearly distinguished from the variants from Australia and Tunisia, and Jingchuan and Zaoyu variants, both collected from Beijing, showed a closer relationship with each other than with variants of Thomson Seedless collected from Xinjiang province. Abbreviation of the sample names such as "Tv1" were described in the text. Samples with asterisk (★) indicate those obtained from GenBank. (AUS Australia, TU1–TU3 Tunisia, Jv1 China (Jingchuan) Zv1 China (Zaoyu), Tv1 and Tv2 China (Thomson Seedless)

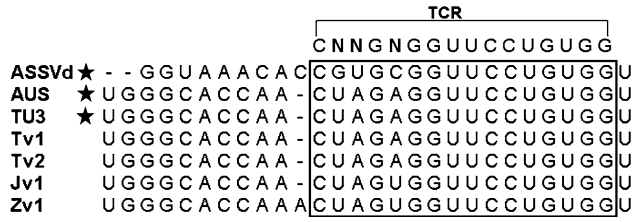

Fig. 5 Analysis of the TCR (Terminal Conserved Region) domain of AGVd isolates in the *Apscaviroid* group. The sequence proximal to the 3' end of the motif is highly conserved while considerable variability is found in the nucleotides near the 5' terminus as evidenced in the three "N" nts in the five terminal positions [22]. Abbreviation of the sample names such as "Tv1" were described in the text. Samples with asterisk (★) indicate those obtained from GenBank. (AUS: Australia TU3: Tunisia Tv1, Tv2: China (Thomson Seedless). Jv1: China (Jingchuan) and Zv1: China (Zaoyu)

The V and P domains of most variants were highly variable, supporting the idea that the variability of viroids of the family Pospiviroidae is mainly found in these two domains

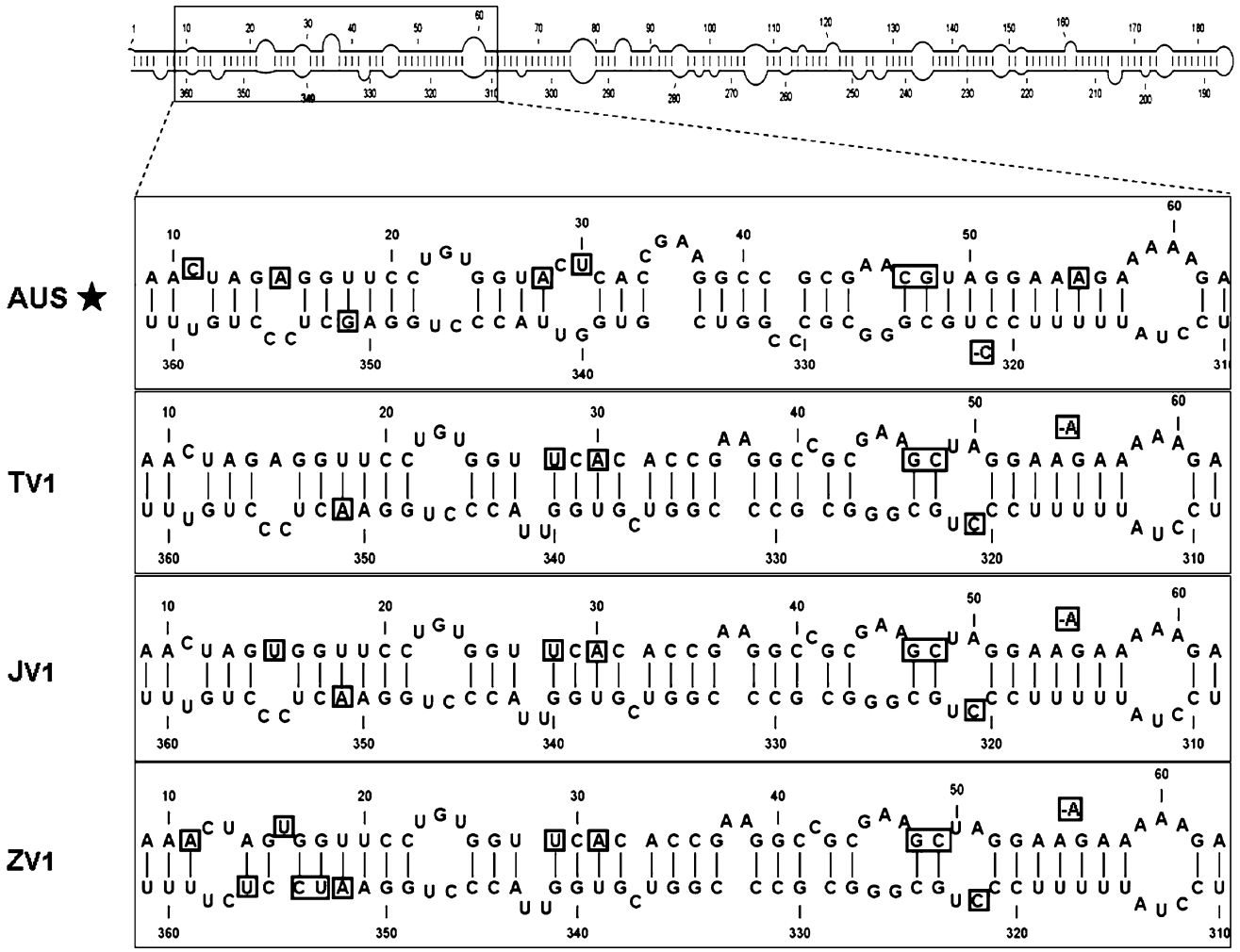

Fig. 4 Predicted secondary structure of AGVd isolates. Compared to AUS, the first reported sequence of AGVd, base changes in Thomson Seedless (Tv1), Jingchuan (Jv1) and Zaoyu (Zv1) isolates affected the secondary structure, as shown in the boxes above. Abbreviation of the sample names such as "Tv1" were described in the text. Samples with asterisk (★) indicate those obtained from GenBank

[24]. Sequence alignment among the variants from Tunisia and Australia also showed that isolates from different countries displayed specific sequence variants. For example, all the sequence variants from the three Chinese isolates showed the seven variations: $A_{28} \rightarrow T$; $T_{30} \rightarrow A$; $CG_{47,\ 48} \rightarrow GC$; $A_{55} \rightarrow -$; $-_{322} \rightarrow C$ and $G_{351} \rightarrow A$. These differences suggest sequence variants from different countries may be shaped from different ancestors. In addition, isolates from different grape varieties also displayed specific sequence variants. An additional variation from A to T ($A_{15} \rightarrow T$) was found in the Jingchuan and Zaoyu isolates at the position 15, and another one isolate was also found in the Zaoyu variant at the position 11 ($-_{\mathbf{11}} \rightarrow A$). Thomson Seedless isolate was from a grapevine that has never been grafted and is more than 100 years old. Jingchuan and Zaoyu isolates were collected from the same grapevine nursery in Beijing. From this, we believe that Jingchuan and Zaoyu isolates have close affinity and may have evolved from Thomson Seedless isolate.

The results of phylogenetic analysis showed that AGVd variants from China could be clearly distinguished from the variants of Australia and Tunisia, and the result also showed that Jiangchuan and Zaoyu variants, both collected from Beijing, showed a closer relationship with each other than with variants of Thomson Seedless collected from Xinjiang province. The result revealed obvious regional disparity and variety-specificity in AGVd.

The most stable secondary structures, in terms of energy, were predicted for the three predominant variants from China and the AGVd variant from Australia, which is the first reported variant. Comparing to the variant from Australia, most of the variations in the three Chinese isolates were located in the terminal left and the pathogenicity domains of the secondary structure, which may have some influence on their secondary structures. Possibly, these changes to the secondary structure affected the pathogenicity of the viroid, and this possible connection needs further biological testing. Among the changed bases, $A_{15} \rightarrow T$ in Jv1 did not influence the secondary structure, however, when $-_{11} \rightarrow A$ and $A_{15} \rightarrow T$ simultaneously happened in Zv1, the structure was changed (Fig. 4). This might be due to reciprocity among the bases.

Our results confirmed that AGVd follows the quasispecies model and we verified existence of regional disparity and variety-specificity in AGVd. We obtained many AGVd cDNA clones, however, their infectivity remains to be determined by further biological testing and study.

GenBank accession numbers

GenBank accession numbers for the predominant sequence variants within AGVd isolate of Thomson Seedless Jingchuan and Zaoyu are DQ362908-DQ362915; EU74 3601-EU743605 and EU743606-EU743612, respectively.

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