High-throughput sequence analysis of small RNAs in grapevine (*Vitis vinifera* L.) affected by grapevine leafroll disease

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SUMMARY

Grapevine leafroll disease (GLRD) is one of the most economically important virus diseases of grapevine (*Vitis* spp.) worldwide. In this study, we used high-throughput sequencing of cDNA libraries made from small RNAs (sRNAs) to compare profiles of sRNA populations recovered from own-rooted Merlot grapevines with and without GLRD symptoms. The data revealed the presence of sRNAs specific to *Grapevine leafroll-associated virus 3*, *Hop stunt viroid* (HpSVd), *Grapevine yellow speckle viroid 1* (GYSVd-1) and *Grapevine yellow speckle viroid 2* (GYSVd-2) in symptomatic grapevines and sRNAs specific only to HpSVd, GYSVd-1 and GYSVd-2 in nonsymptomatic grapevines. In addition to 135 previously identified conserved microRNAs in grapevine (Vvi-miRs), we identified 10 novel and several candidate Vvi-miRs in both symptomatic and nonsymptomatic grapevine leaves based on the cloning of miRNA star sequences. Quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) of selected conserved Vvi-miRs indicated that individual members of a miRNA family are differentially expressed in symptomatic and nonsymptomatic leaves. The high-resolution mapping of sRNAs specific to an ampelovirus and three viroids in mixed infections, the identification of novel Vvi-miRs and the modulation of certain conserved Vvi-miRs offers resources for the further elucidation of compatible host–pathogen interactions and for the provision of ecologically relevant information to better understand host–pathogen–environment interactions in a perennial fruit crop.

INTRODUCTION

Plants have evolved RNA silencing as an efficient defensive mechanism to ward off virus infections (Dunoyer and Voinnet, 2005). This defensive pathway is triggered in response to virus

invasion and generates small-interfering RNAs (siRNAs) to specifically target and cleave the viral genome into smaller non-functional fragments in a homology-dependent manner (Ding and Voinnet, 2007; Molnár *et al.*, 2005). Virus-induced RNA silencing involves the processing of viral double-stranded RNA (dsRNA) by one or more of the DICER-LIKE (DCL) homologues into 21–24-nucleotide-long, double-stranded, virus-derived siRNAs (vsRNAs). The antisense strand of the vsRNA duplex is recruited into the RNA-induced silencing complex (RISC) to target and destroy complementary RNA sequences, leading to the silencing of cognate viral RNAs. (For details, the reader is referred to recent reviews on the subject: Burgyán and Havelda, 2011; Katiyar-Agarwal and Jin, 2010; citations in these reviews.) Apart from viruses, viroid infections can also trigger host RNA silencing, and the mechanism of the biogenesis of viroid-derived small RNAs (vd-sRNAs) appears to show some similarities to, as well as differences from, that of vsRNAs (Navarro *et al.*, 2009). As a result of the lack of protein-coding capacity in viroid genomes, viroid dsRNAs are exclusively produced by host-encoded polymerases, in contrast with virus dsRNAs, some of which are produced by virus-encoded polymerases (Mlotshwa *et al.*, 2008).

Apart from siRNA-mediated gene silencing, recent studies have indicated that microRNAs (miRNAs), another class of sRNAs that play a regulatory role in diverse aspects of plant development and plant responses to biotic and abiotic stresses (Mallory and Vaucheret, 2006; Ruiz-Ferrer and Voinnet, 2009; Sunkar *et al.*, 2012), are also probably involved in the modulation of plant–virus interactions and the expression of disease symptoms (Carmen and Juan, 2006; Dunoyer *et al.*, 2004; Katiyar-Agarwal and Jin, 2010; Lu *et al.*, 2008). The biogenesis and mode of action of miRNAs in plants and animals have been extensively reviewed (Bartel, 2004; Chen, 2009; Czech and Hannon, 2011; Mallory and Vaucheret, 2006; Zhang *et al.*, 2006). Although virus-encoded miRNAs have been identified from a number of human oncogenic viruses in the family *Herpesviridae* (Grundhoff and Sullivan, 2011), there are no reports yet of miRNAs encoded by plant viruses. It is likely that plant viruses exploit the host cellular miRNA pathway to regulate networks of host genes for their advantage. So far, there is no evidence for the direct role of plant miRNAs in antiviral activity,

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although artificial miRNAs have recently been used as a promising approach to confer virus resistance in plants (Simón-Mateo and García, 2011; and references cited therein).

Studies involving pathogen–host systems have revealed that, in addition to the production of vsRNAs, changes in the levels of miRNAs occur in several vertebrate and invertebrate hosts infected with different viruses (Parameswaran *et al.*, 2010). Likewise, a few studies with plant viruses have analysed changes in host-specific miRNA populations accompanied by virus infections and discussed the role of virus-induced miRNA changes in several aspects of host–virus interactions, including the modulation of symptom production (Bazzini *et al.*, 2007; Cillo *et al.*, 2009; He *et al.*, 2008; Wang *et al.*, 2010). Most of these studies have been carried out with annual plant species under controlled environmental conditions, and only a few studies have been conducted with perennial plant species in controlled or natural environments. As a woody perennial adapted to the temperate-zone climate, grapevine (*Vitis* spp.) undergoes alternating periods of active growth from spring to autumn and dormancy during the winter. Therefore, unlike annual plant species, the dynamics of host–virus interactions may be much more complex in grapevine because of chronic infections and the fact that infected plants have to thrive over many years under the influence of seasonal changes and environmental variations. Consequently, the response of grapevine to viral infections might provide ecologically relevant information to better understand host–pathogen–environment interactions.

Grapevine leafroll disease (GLRD) is one of the most complex virus diseases producing distinct symptoms in red- and white-fruited wine grape cultivars of *V. vinifera* L. (Rayapati *et al.*, 2008). Several genetically distinct viruses, termed grapevine leafroll-associated viruses (GLRaVs, family *Closteroviridae*; Martelli *et al.*, 2002) and numbered sequentially GLRaV-1, GLRaV-2, GLRaV-3, etc., in the order of their discovery (Alabi *et al.*, 2011), have been documented in grapevines showing GLRD symptoms. Among them, GLRaV-3 (genus *Ampelovirus*) is the most widespread in different grape-growing regions around the world (Jarugula *et al.*, 2010).

In addition to several viruses (Oliver and Fuchs, 2011), viroids are ubiquitous in cultivated grapevines, causing symptomless infections (Jiang *et al.*, 2009; Kawaguchi-Ito *et al.*, 2009). *Hop stunt viroid* (HpSVd, genus *Hostuviroid*), *Grapevine yellow speckle viroid 1* (GYSVd-1, genus *Apscaviroid*) and 2 (GYSVd-2, genus *Apscaviroid*), *Citrus exocortis viroid* (CEVd, genus *Pospiviroid*) and *Australian grapevine viroid* (AGVd, genus *Apscaviroid*), all belonging to the family *Pospiviroidae* (Tsagris *et al.*, 2008), are five viroid species thus far documented in grapevines (Little and Rezaian, 2003). Some, like GYSVd-1, have been reported to be associated with vein-banding and yellow speckle symptoms that are largely a result of a synergistic interaction with *Grapevine fanleaf virus* (Szychowski *et al.*, 1995).

In a recent study, vsRNAs specific to viruses belonging to the genera *Foveavirus*, *Maculavirus*, *Marafivirus* and *Nepovirus* were characterized from grapevine cv. Pinot Noir by high-throughput sequencing (Pantaleo *et al.*, 2010a). To our knowledge, little information is available on the composition of vsRNAs specific to grapevine-infecting closteroviruses and the dynamics of grapevine miRNAs (Vvi-miRs) in virus-infected grapevines. In this study, we used high-throughput sequencing to compare the profiles of sRNA populations recovered from own-rooted Merlot grapevines with and without GLRD symptoms. The data revealed the presence of sRNAs specific to GLRaV-3 and three viroid species (HpSVd, GYSVd-1 and GYSVd-2) in symptomatic grapevines, and sRNAs specific only to the three viroid species in nonsymptomatic grapevines. Further, the results indicated that abundances of sRNA populations specific to certain viroid species are affected in virus-infected vines, probably as a result of antagonistic interactions. In addition to several known Vvi-miRs reported previously in grapevines (Carra *et al.*, 2009; Pantaleo *et al.*, 2010b; Wang *et al.*, 2011a), this study identified 10 novel and several candidate Vvi-miRs in a perennial fruit crop.

RESULTS

Detection of viruses and viroids in grapevine samples

Petioles from mature leaves of grapevines showing GLRD symptoms (GLRD+ve) and adjacent grapevines with no symptoms (GLRD–ve) were tested by reverse transcriptase-polymerase chain reaction (RT-PCR) for a panel of grapevine-infecting viruses and viroids included in standard virus indexing programs (Naidu *et al.*, 2006). Samples from GLRD+ve vines tested positive for GLRaV-3, HpSVd and GYSVd-1 and those from GLRD–ve vines tested positive only for HpSVd and GYSVd-1 (data not shown). None of the samples tested positive for GYSVd-2 and other viruses and viroids. These results indicated the presence of GLRaV-3 only in GLRD+ve vines and HpSVd and GYSVd-1 in GLRD+ve and GLRD–ve vines.

sRNAs in grapevine samples

Two sRNA libraries generated separately from leaves of GLRD+ve and GLRD–ve grapevines were subjected to high-throughput sequencing using Illumina sequencing technology. Totals of 6 850 066 and 5 108 298 raw reads were obtained from GLRD–ve and GLRD+ve libraries, respectively (Table 1). Of these, 2 303 758 and 1 583 128 reads of 18–28 nucleotides in size were obtained from GLRD–ve and GLRD+ve libraries, respectively, after removing reads of low quality, reads without reliable 3' adaptor sequence and reads smaller than 18 or longer than 28 nucleotides. The sRNAs corresponding to repeat elements and known noncoding RNAs (rRNAs, tRNAs, small nuclear RNAs and small nucleolar RNAs) were removed and the remaining high-quality reads from

Table 1 Classification and abundance of small RNAs from grapevine leafroll disease (GLRD)-affected (GLRD+ve) and unaffected (GLRD–ve) grapevine leaves.

Category of small RNA*	Reads	
	GLRD–ve library	GLRD+ve library
Reads between 18 and 28 nucleotides	4546308	3525170
Reads corresponding to repetitive elements and noncoding RNAs (tRNA, rRNA, snRNA, etc.)	355435	201101
Host-derived sRNAs:		
Known miRNA homologues	1010327	1078224
New and candidate miRNAs	5966	2021
mRNAs and sRNA reads caused by RNA editing or splicing	830329	291696
GLRaV-3-derived sRNAs (vsRNAs)	0	2299
Viroid-derived sRNAs (vd-sRNAs)		
HpSVd-derived vd-sRNAs	6118	5413
GYSVd-1-derived vd-sRNAs	3121	1338
GYSVd-2-derived vd-sRNAs	692	332
18–28-nucleotide sRNAs that cannot be mapped to host or pathogen genome	91770	704
Total raw reads	6850066	5108298

*GLRaV-3, *Grapevine leafroll-associated virus 3*; GYSVd-1, *Grapevine yellow speckle viroid 1*; GYSVd-2, *Grapevine yellow speckle viroid 2*; HpSVd, *Hop stunt viroid*; miRNA, microRNA; sRNA, small RNA; snRNA, small nuclear RNA; vd-sRNA, viroid-derived small RNA; vsRNA, virus-derived small interfering RNA.

both libraries were mapped to the recently published genome of *V. vinifera* cv. 'Pinot Noir' derived line PN40024 (Jaillon *et al.*, 2007). This analysis yielded total sRNA reads of 1 846 622 (642 462 unique reads) and 1 371 941 (465 524 unique reads) from GLRD–ve and GLRD+ve libraries, respectively. The majority of sRNAs from both libraries were specific to grapevine (Table 1). sRNA reads specific to GLRaV-3 (vsRNAs) were found only in GLRD+ve libraries, whereas sRNAs specific to HpSVd, GYSVd-1 and GYSVd-2 (vd-sRNAs) were found in both libraries. These results correlated with RT-PCR data in that GLRaV-3 was detected only in GLRD-affected leaves, whereas HpSVd and GYSVd-1 were detected in both types of leaves. In contrast with RT-PCR data, sRNAs specific to GYSVd-2 were recovered in GLRD+ve and GLRD–ve libraries. On a comparative basis, vd-sRNAs specific to HpSVd were more abundant in both libraries than were sRNAs specific to the other two viroids. Conversely, very low vd-sRNA reads specific to GYSVd-2 were recovered from both libraries (Table 1). Further analysis indicated that HpSVd-specific vd-sRNAs were present in more or less equal amounts in both libraries, and twice the amount of vd-sRNA population specific to GYSVd-1 and GYSVd-2 was recovered from the GLRD–ve library (Table 1). An analysis of the size classes of vsRNAs and vd-sRNAs showed that the 21-nucleotide size class of sRNAs was the most abundant in both GLRD+ve and GLRD–ve leaves, regardless of the virus or viroid species (Fig. 1).

Composition of GLRaV-3-derived vsRNAs in symptomatic leaves

A total of 2299 reads represented by 1373 unique reads showed perfect homology to the GLRaV-3 genome sequence (Fig. 2a, Table 1). A smaller number of reads (~0.07% of total reads) is probably the result of a low concentration of GLRaV-3, as it is phloem limited and known to occur in lower quantities in infected tissue. None of the reads showed any similarity to genome sequences of other currently known grapevine-infecting viruses. These vsRNAs ranged in length between 18 and 28 nucleotides, with the most abundant (~72%) being in the 21-nucleotide size class (Fig. 1b), suggesting that a significant majority of vsRNAs were processed by the DCL4 homologue, although other grapevine DCLs may also participate in this process. The vsRNAs of all size classes were mapped throughout the GLRaV-3 genome in both sense and antisense orientations (Fig. 2; Table S1, see Supporting Information). About 47% of the total vsRNAs cloned were of positive polarity, whereas 53% were of negative polarity, suggesting a slight bias towards antisense vsRNAs in GLRD-affected leaves (Fig. S1a, see Supporting Information). On a genome-wide scale, the density of vsRNAs of both polarities and sizes showed a biased distribution with relatively fewer reads mapping to the 5'-terminal region corresponding to nucleotide positions 1 to approximately 5500 (encompassing the 5' nontranslated region and a portion of the polymerase/helicase domain of the viral polymerase gene) than to other portions of the viral genome (Fig. 2a and Table S1). A similar pattern was observed when only the 21-nucleotide size class vsRNAs of both polarities were mapped to the virus genome (Fig. 2b). As a result of this uneven distribution pattern along the GLRaV-3 genome, multiple vsRNA-generating hotspots [based on normalized raw reads (TPM, transcripts per million) of vsRNAs of both polarities] were located in the replicase [open reading frame (ORF) 1a&b], heat shock protein 70h (HSP70h), HSP90h, coat protein (CP), diverged coat protein (CPd) and the 3' noncoding region (Fig. 2; Table S1).

As the most abundant class of GLRaV-3-derived vsRNAs is 21 nucleotides in size, this class of vsRNAs was chosen to determine whether the 5' end nucleotide was biased to any specific base. The proportion of vsRNA reads containing a 5'-terminal U was greater (about 37%) than the other three nucleotides (Fig. S1b). This biased accumulation of GLRaV-3-derived vsRNAs with a 5'-terminal U suggested preferential loading of vsRNAs into the grapevine homologue of argonaute-1 (AGO1), known to have preference for a 5'-terminal U (Kim, 2008).

sRNAs specific to three viroid species are present in both symptomatic and asymptomatic leaves

We analysed sRNA libraries from both samples for the presence of vd-sRNAs perfectly matching the reference sequences of five

Fig. 1 Size distribution of small RNAs (sRNAs) in libraries prepared from grapevine leafroll disease (GLRD)+ve and GLRD-ve grapevine leaves. Bar graphs for: (a) grapevine-specific sRNAs; (b) vsRNAs specific to *Grapevine leafroll-associated virus 3*; (c) vd-sRNAs specific to *Grapevine yellow speckle viroid 1*; (d) vd-sRNAs specific to *Grapevine yellow speckle viroid 2*; (e) vd-sRNAs specific to *Hop stunt viroid*. vd-sRNA, viroid-derived sRNA; vsRNA, virus-derived small interfering RNA.

viroid species [HpSVd (X06873), GYSVd-1 (GQ995473), GYSVd-2 (DQ377124), CEVd (DQ831486) and AGVd (FJ940923)]. As shown in Table 1, vd-sRNAs perfectly matching the genomes of HpSVd, GYSVd-1 and GYSVd-2 were identified from GLRD+ve and GLRD-ve libraries. None of the sRNA sequences in the two libraries matched the genomes of CEVd and AGVd, indicating their absence in GLRD+ve and GLRD-ve samples. Further analysis indicated that amounts of vd-sRNA reads specific to HpSVd, GYSVd-1 and GYSVd-2 were greater in GLRD-ve than in GLRD+ve samples

(Table 1), suggesting possible antagonistic interactions between GLRAV-3 and the three viroid species. As vd-sRNA reads specific to GYSVd-1 and GYSVd-2 were about two-fold higher in the GLRD-ve library and HpSVd-specific vd-sRNA reads were almost equal in both libraries (Table 1), it is likely that antagonistic influences may be greater on vd-sRNA populations specific to GYSVd-1 and GYSVd-2 than on populations specific to HpSVd.

Examination of vd-sRNA reads revealed a distribution pattern in which higher proportions of sRNAs from corresponding viroid

Fig. 2 Genome-wide view of *Grapevine leafroll-associated virus 3* (GLRaV-3)-specific virus-derived small interfering RNAs (vsRNAs) from grapevine leafroll disease (GLRD)+ve leaves. The numbers of unique hits at each genomic position are represented by red (+sense reads) or black (–sense reads) bars. Each bar shows normalized raw reads (transcripts per million, TPM) of vsRNAs at each genomic position from the total pool of unique vsRNAs. Total of all size classes [18–28-nucleotide (nt) vsRNAs] captured by high-throughput sequencing (a) and 21-nt vsRNA class (b) are shown. The GLRaV-3 genome and the location of different open reading frames (ORFs) (based on accession number EU259806) were drawn to scale and shown above each graph. MET, methyl transferase; HEL, helicase; RdRp, RNA-dependent RNA polymerase; HSP70h, heat shock protein 70 homologue; HSP90h, heat shock protein 90 homologue; CP, coat protein; CPd, diverged coat protein; p21, 21-kDa protein; p20, 19.6-kDa protein; p20, 19.7-kDa protein; p4, 4-kDa protein; p7, 7-kDa protein.

species were of the 21-nucleotide size class, followed by the 24-nucleotide size class, and significantly lower proportions of vd-sRNAs were of other size classes (Fig. 1c–e). A genome-wide view of vd-sRNAs (Fig. 3) revealed hotspots similar to those reported previously (Navarro *et al.*, 2009). Interestingly, vd-sRNA hotspots were similar in GLRD+ve and GLRD–ve leaves (Fig. 3) for each of the three viroid species. With regard to polarity, the ratios of positive to negative polarity of HpSVd-specific vd-sRNAs in the GLRD+ve and GLRD–ve libraries were roughly 2:1 and 1:1, respectively (Fig. S2c, see Supporting Information). For GYSVd-1, the ratios were roughly 1:1 and 1:2 and, for GYSVd-2, they were roughly 2:1 and 1:1, in GLRD+ve and GLRD–ve libraries, respectively (Fig. S2a,b). These results indicate that HpSVd- and GYSVd-2-derived vd-sRNAs are predominantly of positive polarity in GLRD+ve samples, whereas GYSVd-1-derived vd-sRNAs are pre-

dominantly of negative polarity in GLRD+ve samples. Analysis of the 5′-terminal position of the most abundant vd-sRNAs (21 and 24 nucleotides) revealed the following: the 21-nucleotide species of GYSVd-1 have a dominant U, those of GYSVd-2 present a dominant C and HpSVd have a dominant A, regardless of the source library (Fig. S3, see Supporting Information). In contrast, the 24-nucleotide species of GYSVd-1 present a dominant G, those of GYSVd-2 present a dominant A and HpSVd have a dominant C (Fig. S3). Although this trend was similar for the 24-nucleotide species of GYSVd-2 and HpSVd, GYSVd-1 showed distinct differences; with a dominant G in GLRD–ve and A and U of similar numbers in GLRD+ve samples (Fig. S4, see Supporting Information). These results suggest that different AGO proteins may be involved in the biogenesis of distinct vd-sRNA classes of each of the three viroid species.

Fig. 3 Viroid-derived small RNAs (vd-s RNAs) from grapevine leafroll disease (GLRD)+ve and GLRD-ve leaves. The numbers of unique hits at each genomic position are represented by red (+sense reads) or blue (–sense reads) bars. Each bar shows the number of vd-s RNAs specific to the respective viroid species at each genomic position from the total pool of unique vd-s RNAs. GYSVd-1, *Grapevine yellow speckle viroid 1*; GYSVd-2, *Grapevine yellow speckle viroid 2*; HpSVd, *Hop stunt viroid*.

New and conserved miRNAs identified in grapevine cv. Merlot

Sequence analysis, coupled with the fold-back structure predictions for potential novel miRNAs (Fig. 4), led us to identify 39 new candidate miRNAs from grapevine. Based on the presence of miRNA* (miRNA star) reads in the sRNA sequencing data, 10 of the candidate miRNAs (Table 2) were annotated as novel Vvi-miRs in line with established criteria (Meyers *et al.*, 2008). All 10 novel

Vvi-miRs were amplified from total RNA extracts of grapevine by poly(A)-tailed RT-PCR (Fig. 5), and the specificities of the PCR amplicons were determined by cloning and sequencing (GenBank accession numbers JQ989166–185) to further confirm the results. The PCR amplicons specific to known grapevine miRNA (Vvi-miR162) and U6 RNA, used as positive controls, were similarly cloned and sequenced (GenBank accession numbers JQ989186–189). We did not recover miRNA* sequences for the remaining 29 candidate miRNAs (Table 3), despite the fact that reliable

Fig. 4 Predicted fold-back structures of novel microRNAs (miRNAs) cloned from grapevine small RNA (sRNA) libraries. A total of 10 novel grapevine microRNAs (Vvi-miRs) were identified in this study. Nine were detected in both grapevine leafroll disease (GLRD)+ve and GLRD-ve leaves, whereas s49410 was found only in GLRD+ve samples. Sequences indicated in red and blue correspond to mature miRNAs and predicted miRNA*, respectively.

fold-back structures could be predicted (Fig. S5, see Supporting Information) and some of them are abundantly expressed on grapevine (Table 3). Twenty-four grapevine mRNAs were predicted as potential targets for six of the 10 novel Vvi-miRs (Table S2, see

Supporting Information). These putative targets, belonging to different gene families, are associated with diverse biological functions (Table S2). Multiple mRNAs of grapevine were observed as potential targets for four novel Vvi-miRs (s466917, s675359,

Table 2 Novel grapevine microRNAs (Vvi-miRs) and corresponding miRNA* sequences cloned from grapevine leaves affected (GLRD+ve) and unaffected (GLRD–ve) by grapevine leafroll disease (GLRD).

miRNA	Sequence (5'–3')†	Size (nt)	Number of reads ^b		Corresponding miRNA* sequence (5'–3')	Number of reads‡	
			GLRD–ve	GLRD+ve		GLRD–ve	GLRD+ve
s49410	AAGUACUUUUUCUUGCUUUUGAAAG	24	0	4	UCCAAAAGUAGGAAAAGUACUUGC	2	0
s56731	AAUUAUUGUCUGCUUUAGGUCCACU	24	74	44	UGGGUUCAAAGUAGACAAUUAUUA	2	1
s409712	AUAAAUGCAUUUUAAAGUCGUGAG	24	19	9	CAUGGCUUUACAAUGUGUUUAUA	0	1
s466917	AUGUAUUUGAGGGAAAGCAAA	21	5	5	UUUGUUUUCCCUCAAAAACAUG	0	2
s675359	CCAGAACCAACUGCACAUCAA	21	10	1	AUGUGCAGUUAGUUCGCGCAA	0	1
s840122	CUAGGGGUGGACCGGUUGACUAGC	24	1	1	GGCAGGUGACCGUUGGGCCUACG	0	1
s935122	GACCAGUGAGAUAGUCCUCUA	21	3	3	GGGGAUUAUCACUGGUCCU	1	1
s1222800	UAGGAGUUGUUUGAUAGGAUUAUC	24	2	3	AAUCCUAUCAAACAACUCCUAUUU	0	1
s1434076	UUAACAGGAGUGGGUGGUCUUUCC	24	2	1	AGAGACCACCUAGUCCUGUUAAGA	2	1
s1565394	UUUUGUGACACAUGAAUCCAAGUU	24	3	1	CUUGGAUUAUGUGUCACAAAUC	3	2

†Each novel Vvi-miR was validated by poly(A)-tailed reverse transcriptase-polymerase chain reaction (RT-PCR), cloning and sequencing (GenBank accession numbers JQ989166–185).

‡Numbers of reads are based on normalized raw reads (TPM, transcripts per million).

Fig. 5 Validation of novel grapevine microRNAs (Vvi-miRs). Amplicons specific to the Vvi-miRs were amplified by poly(A)-tailed reverse transcriptase-polymerase chain reaction (RT-PCR), cloned and the sequences were obtained (GenBank accession numbers JQ989166–185). Recombinant plasmids carrying the Vvi-miR inserts were then used as templates in PCR to obtain the gel image presented. Plasmids carrying a known grapevine microRNA (miRNA) (Vvi-miR162) and U6 RNA were used as positive controls, and a 25-bp DNA ladder (Invitrogen, Carlsbad, CA, USA) was used as the size marker. NTC, no template control.

s935122 and s1565394), whereas only a single mRNA target could be predicted for two novel Vvi-miRs (s840122 and s1434076). Conversely, no mRNA targets could be predicted for the other four novel Vvi-miRs (s49410, s56731, s409712 and s1222800; Table S2).

In earlier reports, a total of 169 Vvi-miRs belonging to 49 known miRNA families (version 13; <http://microrna.sanger.ac.uk/>; accessed on 6 March 2012) were computationally predicted (Jaillon *et al.*, 2007; Velasco *et al.*, 2007) or experimentally recovered (Carra *et al.*, 2009; Pantaleo *et al.*, 2010b; Wang *et al.*, 2011b) from grapevine. In the present study, we identified Vvi-miRs belonging to 36 known miRNA families in libraries derived from GLRD–ve and GLRD+ve leaves of grapevine cv. Merlot (Table 4). Thirty-four of these 36 miRNA families were present in sRNA libraries derived from both GLRD–ve and GLRD+ve leaves.

The other two, Vvi-miR1151 and Vvi-miR477, were found only in GLRD+ve and GLRD–ve libraries, respectively (Table 4). We did not find homologues belonging to 13 known grapevine miRNA families in leaf tissues of cv. Merlot used in this study. Rather, we found miRNA reads belonging to seven families (Vvi-miR472, Vvi-miR529, Vvi-miR530, Vvi-miR827, Vvi-miR894, Vvi-miR1507 and Vvi-miR1511) that have not been reported previously from grapevine. The predicted grapevine gene targets for the 36 miRNA families are provided in Table 4.

Expression profile of conserved miRNAs in symptomatic and asymptomatic leaves

Sequence analysis of the sRNA libraries indicated that conserved miRNAs are differentially expressed in GLRD+ve and GLRD–ve leaves (Table 4). To further validate the sequencing-based profiling data, quantitative real-time RT-PCR experiments were performed for selected primary miRNA transcripts (Vvi-MIR156f, Vvi-MIR156g, Vvi-MIR156h, Vvi-MIR166d, Vvi-MIR166e, Vvi-MIR166h, Vvi-MIR167a and Vvi-MIR167b) belonging to four miRNA families (Vvi-miR156, Vvi-miR162, Vvi-miR166 and Vvi-miR167). The specificity of primers for individual pre-miRNAs was ascertained by single melting peaks for each pre-miRNA (Fig. S6, see Supporting Information) and by sequencing the amplified DNA fragments (Table S3, see Supporting Information). Sequencing profiles indicated that Vvi-miR156, Vvi-miR162 and Vvi-miR167 were down-regulated (Table 4). Quantitative real-time PCR analysis for Vvi-MIR156g, Vvi-MIR156f, Vvi-MIR162, Vvi-MIR167a and Vvi-MIR167b also showed decreased expression on GLRD+ve leaves, which is in agreement with the sequencing data. However, sequencing profiles indicated that Vvi-miR166 levels were up-regulated in GLRD+ve leaves. Similarly, quantitative real-time PCR analysis for Vvi-MIR166h indicated up-regulation, although the other two loci (Vvi-MIR166d and Vvi-MIR166e) were either

miRNA	Sequence (5'–3')	Size (nt)	Number of reads*	
			GLRD–ve	GLRD+ve
s23442	AACAAGGAUCUCUUAAGAAAGGAC	24	5	3
s38258	AAGACUUAAGGACGAUAAGGUUGC	24	36	54
s397544_1	AGUAAUAGACAUGGUAGAAACACU	24	13	2
s397544_2	AGUAAUAGACAUGGUAGAAACACU	24	13	2
s450244	AUGACAUGAGUUGGAACUAAAAGA	24	6	5
s480274	AUUGAACUCAUGUGCAAGCUCAA	24	5	3
s501879_1	CAAAGCGGACAAUAUCUACAU	21	22	24
s501879_2	CAAAGCGGACAAUAUCUACAU	21	22	24
s501879_3	CAAAGCGGACAAUAUCUACAU	21	22	24
s612169	CAGUCUCUGAAGUAGCUCCAU	21	2	6
s710066	CCCUUUGGUCAAAAGCGGACAAU	24	22	18
s766416	CGACGAGAUAGGUCCACCCUA	21	1	1
s774661	CGAUACCAUGUGGAAAAGAGGAA	23	3	0
s836623	CUACUGAUAAACUUGAAUAGCAGC	24	1	3
s848638	CUCAAUGAGUAUAGGCAGCAAA	22	1	0
s885197_1	CUGUUGACAUUAUCCAAUAUA	21	1	1
s885197_2	CUGUUGACAUUAUCCAAUAUA	21	1	1
s1129393	GUCUCUUGAGGAUUGUAAAGGGUG	24	2	1
s1233315	UAUAAUUGGUUCAAGAUUGGUU	21	23	6
s1346974_1	UCUUCAAGAGACGAGUCCAUAGAA	24	6	6
s1346974_2	UCUUCAAGAGACGAGUCCAUAGAA	24	6	6
s1410609_1	UGGUCAUGUGACCGUUGGAGGCUU	24	4	4
s1410609_2	UGGUCAUGUGACCGUUGGAGGCUU	24	4	4
s1428123_1	UGUUGACAUCAUCCAAUAUA	20	178	149
s1428123_2	UGUUGACAUCAUCCAAUAUA	20	178	149
s1428123_3	UGUUGACAUCAUCCAAUAUA	20	178	149
s1428127	UGUUGACAUCCGUCCAACAUA	21	6	6
s1428710_1	UGUUGGAUGAUGUCAACAUGU	21	10	6
s1428710_2	UGUUGGAUGAUGUCAACAUGU	21	10	6
s1428712	UGUUGGAUGAUGUCAAAUAAA	20	486	188
s1428721	UGUUGGAUGAUGUCAAAUAAU	21	503	225
s1453071	UUAUUGGAGUGAGGAUUAU	21	89	8
s1497218	UUGACAGUGAUUUUAGGAAGU	24	13	6
s1497299	UUGACAUCCGUCCAACAUAU	20	5	6
s1512811	UUGGAUGAUGUCAACAUGUACUU	24	1	0
s1523093	UUGUCUUAUACUCAUUUAUUU	21	16	6
s1547786_1	UUUGACAGUGAUUUUAGGAAG	21	8	4
s1547786_2	UUUGACAGUGAUUUUAGGAAG	21	8	4
s1547786_3	UUUGACAGUGAUUUUAGGAAG	21	8	4
s1554162_1	UUUGGUAGUGAUUUUAGGAAG	21	1	0
s1554162_2	UUUGGUAGUGAUUUUAGGAAG	21	1	0

*Numbers of reads are based on normalized raw reads (TPM, transcripts per million).

down-regulated or unaltered. Overall, there was a good correlation between the sequencing-based profiling data (Table 4) and quantitative real-time RT-PCR experiments (Fig. 6), with a few exceptions (Vvi-miR156h, Vvi-miR166d and Vvi-miR166e). These results also indicated that individual members of certain miRNA family were differentially regulated in GLRD+ve leaves. For instance, pre-miRNAs Vvi-MIR156f and Vvi-MIR156g were down-regulated in GLRD+ve leaves, whereas Vvi-MIR156h was up-regulated (Fig. 6). Likewise, Vvi-MIR166d was down-regulated in GLRD+ve leaves, and Vvi-MIR166h was up-regulated. It should be noted, however, that sequence-based profiling data refer to mature miRNAs, whereas the quantitative real-time RT-PCR data refer to pre-miRNAs of the corresponding mature miRNAs. Thus, it is plausible that pre-miRNAs for certain miRNAs could be present

in the cell without processing into mature miRNAs, as observed previously (Nogueira *et al.*, 2009).

DISCUSSION

Previous studies related to sRNAs in virus- and viroid-infected plants have been conducted in model systems under defined environmental conditions, and very little information is available on sRNA profiles in virus-infected perennial crops grown under field conditions. A few recent studies have shown that viroids can elicit RNA-silencing pathways in perennial crops, leading to an accumulation of vd-sRNAs in infected plants (Bolduc *et al.*, 2010; Di Serio *et al.*, 2009; Martinez *et al.*, 2010; Navarro *et al.*, 2009; St-Pierre *et al.*, 2009). In addition, vsRNAs specific to viruses belonging to

Table 3 Candidate grapevine microRNAs (Vvi-miRs) cloned from grapevine leaves with (GLRD+ve) and without (GLRD–ve) grapevine leafroll disease (GLRD).

Table 4 Known grapevine microRNAs (miRNAs) in grapevine leafroll disease (GLRD)-affected (GLRD+ve) and unaffected (GLRD–ve) leaves.

miRNA family	Number of reads*		Target gene annotation†
	GLRD–ve	GLRD+ve	
Vvi-miR156	12580	6331	SPL transcription factor
Vvi-miR159	694	442	SPL transcription factor
Vvi-miR162	720	446	Dicer-like (DCL)
Vvi-miR164	60	234	NAC domain transcription factor
Vvi-miR166	357697	604010	HD-ZIPIII transcription factor
Vvi-miR167	11668	9562	Auxin response factor (ARF)
Vvi-miR168	3882	786	Argonaute (AGO)
Vvi-miR169	114	32	HAP2 transcription factor
Vvi-miR171	289	256	SCL
Vvi-miR172	1992	8205	Apelata2-like transcription factor
Vvi-miR319	14	21	MYB transcription factor
Vvi-miR390	54	36	TAS3
Vvi-miR393	634	26	F-box protein
Vvi-miR394	231	84	F-box protein
Vvi-miR395	22334	23790	APS, AST
Vvi-miR396	2845	2847	GRF
Vvi-miR397	12	6	LAC
Vvi-miR398	17	10	CSD
Vvi-miR399	59	13	E2-UBC
Vvi-miR403	846	510	AGO
Vvi-miR408	54	45	LAC, PLC
Vvi-miR472	2	3	CC-NBS-LRR
Vvi-miR477	2	0	Unknown
Vvi-miR479	8	1	Unknown
Vvi-miR482	82	85	Unknown
Vvi-miR529	1	3	Squamosa promoter binding protein-like (SPL) family of transcription factors
Vvi-miR530	18	6	Zinc knuckle (CCHC-type) family protein, homeobox transcription factor KN3
Vvi-miR535	1665	5910	SPL transcription factor, nucleoside phosphatase
Vvi-miR827	3825	4609	Protein with SPX domain
Vvi-miR828	34	56	MYB, TAS4
Vvi-miR858	883	655	MYB transcription factor
Vvi-miR894	13908	10647	Unknown
Vvi-miR1507	453	991	Mycolyl transferase-like protein, putative nitrate transporter
Vvi-miR1511	0	1	Unknown
Vvi-miR3626‡	6	10	chr 19 open reading frame 29
Vvi-miR3631‡	45	20	Protein

*Numbers of reads are based on normalized raw reads (TPM, transcripts per million).

†Target gene annotations are based on mRNA orthologues from *Arabidopsis* (published online at: <http://asrp.cgrb.oregonstate.edu/db/microRNAfamily.html>).

‡Target gene annotations are based on Pantaleo *et al.* (2010b).

APS, ATP-sulfurylase; AST, Sulfate transporter; CC-NBS-LRR, coiled-coil nucleotide-binding site leucine-rich repeat; CSD, copper/zinc superoxide dismutase; GRF, Growth regulating factor; HAP, HAP2 transcription factor; HD-ZIPIII: homeodomain-Leu zipper (HD-ZIP); LAC, Laccase; MYB, MYB (Myeloblastosis) transcription factor; NAC, N-terminal DNA-binding domain and a variable C-terminal domain transcription factor; PLC, Plantacyanin-like; SCL: Scarecrow-like transcription factor; TAS, tasiRNA-generating locus; UBC, E2 ubiquitin-conjugating protein.

the genera *Foveavirus*, *Maculavirus*, *Marafivirus* and *Nepovirus* have been characterized recently from grapevine cv. Pinot Noir by high-throughput sequencing (Pantaleo *et al.*, 2010a). In this context, our study provided information on vsRNAs specific to a grapevine-infecting member of the genus *Ampelovirus* in the family *Closteroviridae*, thus expanding current knowledge on the sRNA landscape in grapevines co-infected with viruses and viroids.

Among the known Vvi-miRs that were found at relatively high frequencies [>100 normalized raw reads (TPM)] in both GLRD–ve and GLRD+ve libraries, six miRNA families (Vvi-miR164, Vvi-miR166, Vvi-miR172, Vvi-miR535, Vvi-miR827 and Vvi-miR1507) were present in higher numbers, whereas 11 families (Vvi-miR156, Vvi-miR159, Vvi-miR162, Vvi-miR167, Vvi-miR168, Vvi-miR169, Vvi-miR393, Vvi-miR394, Vvi-miR403, Vvi-miR858 and Vvi-miR894) were present in lower numbers, in the GLRD+ve library. The relative expression of pre-miRNAs specific to selected miRNA families (Vvi-MIR156f, Vvi-MIR156g, Vvi-MIR156h, Vvi-MIR162, Vvi-MIR166d, Vvi-MIR166e, Vvi-MIR166h, Vvi-MIR167a, Vvi-MIR167b and Vvi-MIR168), known to play a critical role in different aspects of plant development (Mallory and Vaucheret, 2006), showed modulation in GLRD+ve leaves (Fig. 6). It is likely that these subsets of Vvi-miRs play a role in host–virus interactions leading to the development of symptoms associated with GLRD.

Recent studies have shown the modulation of miR162 and miR168 in virus-infected plants (Csorba *et al.*, 2007; Váralay *et al.*, 2010). These two miRNAs negatively regulate the miRNA pathway by targeting DCL1 and AGO1, respectively (Vaucheret *et al.*, 2004; Xie *et al.*, 2003). Such a regulatory relationship between the two miRNAs and their targets has been shown to be crucial for appropriate plant development, as decreasing the complementarity of AGO1 mRNA with miR168 results in increased accumulation of AGO1 mRNA and associated developmental defects in plants (Vaucheret, 2006; Vaucheret *et al.*, 2004, 2006; Zhang *et al.*, 2006). In a recent study, the abundance of DCL1 and AGO1 transcripts was shown to be significantly increased in tomato plants inoculated with different strains of *Cucumber mosaic virus* (CMV) compared with mock-inoculated plants (Cillo *et al.*, 2009).

Members of the miR156 family target a group of transcription factors, called SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) (Wang *et al.*, 2011b), which are known to affect a broad range of developmental processes in *Arabidopsis* (Gou *et al.*, 2011). Transgenic *Arabidopsis* plants overexpressing miR156 showed enhanced accumulation of anthocyanins and reduced SPL activity relative to wild-type plants (Gou *et al.*, 2011). Likewise, overexpression of miR156 in poplar (*Populus* spp.) trees resulted in decreased leaf size, increased leaf initiation rate and reduced apical dominance (Wang *et al.*, 2011b). Members of the miRNA families miR166 and miR167 are known to target transcriptional

Fig. 6 Expression patterns of pre-microRNAs corresponding to certain known microRNAs in grapevine leafroll disease (GLRD)+ve and GLRD-ve leaves by quantitative real-time reverse transcriptase-polymerase chain reaction. Data represent the mean $\pm$ standard error (SE) (vertical bars) of two biological replicates, with each replicate an average of three technical replicates. Significant differences between GLRD+ve and -ve leaves, determined by SigmaPlot 11.0 software using Student's *t*-test, are indicated by asterisks (* $P < 0.05$).

factors regulating plant development. Studies have shown that increased levels of miR166 inversely affect the expression of the HD-ZIPIII target, leading to alterations in primary and secondary vascular tissue pattern formation, as well as lateral organ and cambial polarity, in herbaceous annual plants (Côté *et al.*, 2010). Members of the miR167 family are known to target auxin response factors (ARFs). ARFs are involved in plant development through the regulation of auxin signalling, and their perturbations in *Arabidopsis* have been correlated directly with various developmental defects (Mallory *et al.*, 2005). In another recent study, an increase in the expression levels of certain miRNAs, including miR172, was reported in tomato affected by tomato leaf curl disease, and it was suggested that miR172 could be associated with *Tomato leaf curl New Delhi virus* infection and pathogenesis (Naqvi *et al.*, 2010). Likewise, higher levels of Vvi-miR172 in GLRD+ve grapevine leaves (Table 4) could suggest its role in certain aspects of leafroll disease. It is also noteworthy that Vvi-miR393, which showed lower TPM reads in GLRD+ve leaves, targets F-box proteins, including auxin receptor, TIR1 (Navarro *et al.*, 2006; Sunkar and Zhu, 2004). Our attempts to measure the relative expression of pre-miRNAs of members belonging to Vvi-miR families 172 and 393 were unsuccessful.

The above interpretations are compatible with growing evidence that perturbations of miRNA biology occur in virus-infected plants, and altered miRNA accumulation levels have been correlated with disease symptoms and associated developmental abnormalities or morphological defects (Chapman *et al.*, 2004; Chen *et al.*, 2004; Cillo *et al.*, 2009; Dunoyer *et al.*, 2004; Kasschau

et al., 2003; Naqvi *et al.*, 2010; Shibolet *et al.*, 2007; Silhavy and Burgyn, 2004; Tagami *et al.*, 2007). Recent studies have also indicated that virus infection could interfere with miRNA pathways at the transcriptional level, and imbalances in plant hormones caused by virus infection could alter directly or indirectly plant miRNA biogenesis (Bazzini *et al.*, 2009). Accordingly, it is reasonable to propose that negative regulation of certain Vvi-miRs (Table 4, Fig. 6) could result in the perturbation of grapevine metabolism, leading to GLRD symptoms. Detailed studies on the spatio-temporal dynamics of Vvi-miRs at different phenological stages of grapevine will be useful in elucidating the functions and regulatory mechanisms of miRNAs in various aspects of the disease.

Plants infected by chloroplast-replicating (family *Avsunviroidae*) or nuclear-replicating (family *Pospiviroidae*) viroids can accumulate 21–24-nucleotide vd-sRNAs (Bolduc *et al.*, 2010; Di Serio *et al.*, 2009; Martinez *et al.*, 2010; Navarro *et al.*, 2009; St-Pierre *et al.*, 2009). In a recent study, the prevalence of 21, 22 and 24-nucleotide species of vd-sRNAs specific to HpSVd and GYSVd-1 was reported in grapevines (Navarro *et al.*, 2009). The profile of vd-sRNAs obtained in our study differs somewhat from these reports in that, although the predominant species obtained in this study were 21 nucleotides in size, they were followed, to a lesser extent, by 24-nucleotide species. Together, these results support the view that HpSVd, GYSVd-1 and GYSVd-2, belonging to nuclear-replicating viroids in the family *Pospiviroidae*, are targeted by diverse DCL homologues present in grapevine (Margis *et al.*, 2006). Lower vd-sRNA reads specific to GYSVd-1 and GYSVd-2

than those specific to HpSVd in both GLRD+ve and GLRD–ve libraries may indicate antagonistic interactions between these viroid species when present together in the same plant.

Antagonistic outcomes of interactions between co-infecting viroids have been documented previously (Vernière *et al.*, 2006; and references cited therein). As the three viroid species share common accumulation sites and employ similar host-encoded polymerases (Flores and Pallas, 2006), decreased replication of GYSVd-1 and GYSVd-2 could lead to reduced availability of dsRNA presenting the template for the production of vd-sRNAs specific to the two viroids. It is also likely that other factors, such as viroid-specific conformational changes and/or specific host interaction of each viroid species, independent of their replication, could be responsible for the observed differences in vd-sRNA reads specific to the three viroid species. Another likely possibility is that a network of events could be occurring in mixed infections of viroids that involves vd-sRNAs from one viroid priming the host RNA silencing pathway to target the genome of other viroids (Flores *et al.*, 2005). Further studies are required to discriminate which of the three viroid species is the primary effector of antagonistic interactions at the molecular level.

As a result of their clonal propagation, grapevine cultivars harbour many viruses and viroids, and co-infections of two or several unrelated viruses and viroids are frequent in grapevines (Coetzee *et al.*, 2010). Co-infections are often known to result in synergistic effects, as shown between disparate viruses, viroids, viruses and their satellite RNAs or viruses, and between a virus and a viroid in plants other than grapevines (Pruss *et al.*, 1997; Scholthof, 1999; Valkonen, 1992; Vernière *et al.*, 2006). It is likely that mixed infections of GLRaV-3 and the three viroids could lead to a complex network of interactions at the molecular level, and the extent of these multiple combinatory interactions—antagonism or synergism—could reflect on the sRNA biosynthetic and functional pathways. The low frequency of reads of vd-sRNAs specific to GYSVd-1 and GYSVd-2 (Table 1) retrieved from GLRD+ve leaves, as opposed to the higher reads in GLRD–ve leaves, suggests an antagonistic influence of GLRaV-3 on vd-sRNA biogenesis of specific viroid species. It has been hypothesized that the RNA silencing machinery targets nuclear viroids at several key points in their infectious cycle, including replication and cytoplasmic trafficking (Navarro *et al.*, 2009), thus providing avenues for such antagonistic influences. In addition, the role of RNA silencing suppressors of GLRaV-3 in single or mixed infections is yet to be explored. It is likely that one or more proteins may function as silencing suppressors for this virus, and they, either alone or in concert, may play an active role in post-transcriptional gene silencing-mediated antiviral defence in grapevines. Nevertheless, as viroids and GLRaV-3 have a contrasting replication strategy and diverse subcellular replication sites, the results obtained in this study open up the fascinating possibility to dissect the RNA

silencing pathways in plants regulated by disparate pathogens in mixed infections.

To our knowledge, this study provides the first high-resolution genome map of vsRNAs for an ampelovirus in the family *Closteroviridae*. The depth of vsRNAs obtained in this study (Fig. 2) and their alignment along the entire genome of GLRaV-3 (Fig. 2; Table S1) reveal the power of the sRNA high-throughput sequencing approach in retrieving almost the entire sequence or major portion of a large-sized plant virus genome. The sequencing and assembly of total sRNAs isolated from an infected plant are more amenable for the generation of information on an 'infectious' virus, as sRNAs are produced as a consequence of virus infection and subsequent replication. Furthermore, the identification of vd-sRNAs for HpSVd, GYSVd-1 and GYSVd-2 in GLRD+ve and GLRD–ve leaves by high-throughput sequencing technology, and the detection of only HpSVd and GYSVd-1 by RT-PCR, indicate the potential benefit of the high-throughput Solexa sequencing platform in the detection of viroid sequences present in quantities undetectable by molecular-based diagnostic assays. In addition, sRNA technology also has the potential for the discovery of hitherto unknown viral and subviral pathogens replicating in grapevine and other perennial plants that occur at low concentration, show an uneven distribution in the host plant and sometimes cause seemingly symptomless infections (Al Rwahnih *et al.*, 2009; Pantaleo *et al.*, 2010a).

In addition to conserved miRNAs in grapevine, we have identified 10 novel and several candidate Vvi-miRs, thereby increasing the repertoire of miRNAs documented to date in grapevine. Using high-throughput sequencing technology, Mica *et al.* (2009) and Pantaleo *et al.* (2010b) have recently described several conserved and novel miRNAs from the grapevine cultivars Corvina and Pinot Noir, respectively, whereas another study described newer miRNAs from the interspecific table grape cultivar 'Summer Black' (Wang *et al.*, 2011a). One explanation for the discovery of additional novel and candidate Vvi-miRs in our study could be the characteristics of the specific developmental stages of the leaves sampled for the construction of sRNA libraries, as the spatial expression pattern of mature miRNAs and their targets are regulated at different stages of leaf development from initiation to senescence (Pulido and Laufs, 2010). Alternatively, the differences in miRNAs between wine grape cultivars Pinot Noir, Corvina and Merlot could be, at least in part, a result of their different genetic backgrounds (Boursiquot *et al.*, 2009; This *et al.*, 2006). Thus, it is plausible that each wine grape cultivar grown across different viticultural regions may exhibit developmental plasticity with regard to the expression of miRNAs as a consequence of the geographical location of plantings. In this context, it is also likely that the spatial and temporal expression patterns of miRNAs may vary in a perennial crop such as grapevine in response to endogenous cues and/or changing environmental conditions during the growing season and as a function of the viticultural practices.

EXPERIMENTAL PROCEDURES

Plant material

Leaf samples were collected from 10-year-old, own-rooted grapevines (cv. Merlot) planted in a commercial vineyard block near Prosser in Washington State (46.2°N latitude, 119.8°W longitude). Two grapevines showing GLRD symptoms (GLRD+ve) and two adjacent nonsymptomatic, virus-free grapevines (GLRD–ve), grown under standard viticultural practices, were selected for this study. The GLRD+ve and adjacent GLRD–ve grapevines were tested for the presence of different viruses and viroids by single-tube RT-PCR (Naidu *et al.*, 2006). Leaves at the basal portion of canes showing typical symptoms of GLRD from GLRD+ve grapevines and comparable leaves from adjacent GLRD–ve grapevines were collected at the same time in mid-September to minimize the influence of the developmental age of leaves on sRNA profiles between the two samples. The leaves were frozen immediately in liquid N₂ and stored at –80 °C until required for total RNA extraction. Anecdotal evidence suggested that GLRD was introduced into the vineyard block via planting of virus-infected cuttings.

Extraction of total RNA from grapevine leaves

Total RNA was extracted from frozen leaf tissues of GLRD–ve and GLRD+ve grapevines using a Spectrum Plant Total RNA kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. On-column DNase I digestion (Qiagen Inc., Valencia, CA, USA) of total RNA preparations was performed to remove any genomic DNA. The integrity of RNA was verified by resolving in GelRed-stained 1% formaldehyde–agarose gels, and the purity was assessed by measuring an absorbance ratio of 1.8–2.0 at 260/280 nm using a NanoDrop 2000c spectrophotometer (NanoDrop Products, Wilmington, DE, USA).

Cloning of sRNA and Illumina sequencing

sRNAs were isolated from frozen leaf tissues of GLRD+ve and GLRD–ve grapevines using the mirPremier™ microRNA isolation kit (Sigma-Aldrich) according to the manufacturer's protocol. sRNAs of the desired size range (18–28 nucleotides) were gel purified by resolving in denaturing 15% polyacrylamide gel. The isolated sRNAs were then sequentially ligated to 5' and 3' RNA oligonucleotide adaptors, reverse transcribed and amplified by PCR (Jagadeeswaran *et al.*, 2010). High-throughput sequencing of the small cDNA libraries was performed using the Sequencing-By-Synthesis Technology (Illumina Inc., San Diego, CA, USA).

Analyses of sRNA sequences from grapevine cDNA libraries

Computational analyses of sRNA reads obtained from grapevine sRNA libraries were performed as reported previously (Jagadeeswaran *et al.*, 2010). Briefly, all sRNA reads without perfect matches to the most proximal 11 nucleotides of the 5' adaptor sequences were first removed. The unique sRNAs were aligned to REPBASE (version 13.04, obtained from <http://www.girinst.org>), the TIGR Plant Repeats DB (<http://plantrepeats.plantbiology.msu.edu/downloads.html>) and known noncod-

ing RNAs (rRNAs, tRNAs, small nuclear RNAs, small nucleolar RNAs, etc.) obtained from RFAM (<http://www.sanger.ac.uk/Software/Rfam/ftp.shtml>) with National Center for Biotechnology Information (NCBI) BLASTn (Altschul *et al.*, 1990). Following the removal of sRNAs corresponding to repeat elements and known noncoding RNAs, unique sequences between 18 and 28 nucleotides were utilized to map to the recently published complete genome of *V. vinifera* cv. 'Pinot Noir' derived line PN40024 (Jaillon *et al.*, 2007), the complete genome of GLRaV-3 (Maree *et al.*, 2008) and the complete genomes of HpSVd (accession number X06873), GYSVd-1 (GQ995473) and GYSVd-2 (DQ377124) using BLASTn searches. Thus, the reads were sorted into sRNAs of host, viral and viroid origins (Table 1). The host-derived sRNAs were used to scan miRBase (version 13; <http://microrna.sanger.ac.uk/>) and resulted in the identification of conserved miRNA homologues in grapevine (Vvi-MiRs). For the identification of novel miRNAs, unique sRNAs with more than 10 genomic hits were removed from further analysis. The flanking regions of the remaining genome-matched sequences were cut out, and the fold-back structures were predicted using the RNAfold program (Hofacker, 2003). Next, we examined the resulting folding structures to choose those that had at least 18 base pairs, one central loop and a folding energy no greater than 18 kcal/mol. We then applied the MIRCHECK program (Jones-Rhoades and Bartel, 2004) to choose sequences that had six or less mismatches, two or less bulged or asymmetrically unpaired nucleotides and two or less continuous mismatches in the regions of the sRNA reads. Subsequently, a program was developed in house to check the existence of the miRNA* sequence of the selected mature miRNA, based on the criterion that there were 2-nucleotide overhang(s) at the 3' end(s) of either miRNA or miRNA*. Then, the distributions of unique sRNAs on the putative pre-miRNAs were examined manually. Pre-miRNAs without a clear accumulation of reads in the selected mature miRNA regions were removed from the putative pre-miRNAs, because mature miRNAs were expected to be cut out precisely from the pre-miRNAs based on the annotation criteria of plant miRNAs (Meyers *et al.*, 2008). Host-derived sRNAs that showed no match to existing miRNAs in miRBase, but had an accompanying miRNA* sequence coupled with a predictable fold-back structure (Zuker, 2003), were designated as novel miRNAs. Those without an accompanying miRNA* sequence, but with reliable fold-back structures for their precursor sequences, were designated as candidate miRNAs. The normalized reads (TPM) were used to identify the differences in sRNA profile between GLRD+ve and GLRD–ve leaves.

Target prediction

The Hitsensor (Zheng and Zhang, 2010) and PATSCAN (Dsouza *et al.*, 1997) search algorithms were used to predict putative targets for the newly identified miRNAs. The known *V. vinifera* ORFs and their annotations were downloaded from the *V. vinifera* Genome Annotation Database (http://www.genoscope.cns.fr/externe/Download/Projets/Projet_ML/data) and used for host-derived miRNA target predictions. For the selection of putative miRNA–target pairs, only three mismatches were allowed between an mRNA target and the miRNA in our prediction (Jones-Rhoades and Bartel, 2004; Rhoades *et al.*, 2002), and the Pfam database (<http://pfam.sanger.ac.uk/>) was used to retrieve the annotation of the predicted targets. The GLRaV-3- and viroid-derived sRNAs were mapped to the complete genome of their respective virus and viroid species using BLASTn

and Vmatch (<http://www.vmatch.de>) in order to determine the presence of 'hotspots' and 'coldspots' for the biogenesis of these sRNAs.

Validation of novel grapevine miRNAs by poly(A)-tailed RT-PCR, cloning and sequencing

The poly(A)-tailed RT-PCR was performed for each of the 10 novel grapevine miRNAs using the All-in-One™ miRNA quantitative RT-PCR Detection Kit (GeneCopoeia, Inc., Rockville, MD, USA) according to the manufacturer's instructions. To make first-strand cDNAs, poly(A) polymerase was used in a 25-μL reaction to add poly(A) tails to the 3' end of miRNAs using 2 μg of total RNAs extracted from grapevine leaves with the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich) as template. At the same time, M-MLV RTase and a unique oligo-dT adaptor reverse primer were used to transcribe the poly(A) miRNAs by incubating the mix at 37 °C for 60 min after a brief centrifugation, followed by incubation at 85 °C for 5 min to inactivate the enzyme. The amplification of miRNAs with poly(A) tails was performed using each novel miRNA sequence (Table 2) as the forward primer and the Universal Adaptor PCR Primer (GeneCopoeia, Inc.) as the reverse primer. RT-PCR assays were performed in a final reaction volume of 25 μL containing 1 × PCR buffer (Roche Applied Sciences, Indianapolis, IN, USA), 200 μM of each deoxynucleoside triphosphate (dNTP), 0.2 μM of each forward and reverse primer, 1 U Taq Polymerase (Roche Applied Sciences) and a 1:50 dilution of first-strand cDNA (prepared above). Separate PCR assays for individual novel miRNAs were performed in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). Cycling conditions were as follows: initial denaturation at 94 °C for 5 min, followed by 36 cycles of 94 °C for 10 s, 55 °C for 20 s and 72 °C for 10 s, and a final extension step at 72 °C for 7 min. The PCR amplicons were stained with bromophenol blue dye and run on GelRed-stained 3% agarose gel with 1% Tris-acetate-EDTA buffer. Cloning, sequencing and sequence analysis of the miRNA-specific amplicons were performed as described previously (Alabi *et al.*, 2011).

Expression profiling of pre-miRNAs in grapevine leaves by quantitative real-time RT-PCR

Pre-miRNA sequences were retrieved from the grapevine miRNAs deposited with miRBase (version 13; <http://microrna.sanger.ac.uk/>) and primers specific to each pre-miRNA were designed (Table S3) with the aid of PrimerQuestSM software (Integrated DNA Technologies, Inc, Coralville, IA, USA). One microgram of total RNA was used to prepare cDNA specific to each pre-miRNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany). The cDNA was used to PCR amplify DNA fragments specific to each pre-miRNA and internal control genes, and the resulting DNA was cloned and sequenced (GenBank accession numbers JQ989190–222).

Equal amounts of first-strand cDNA made from total RNA of GLRD+ve and GLRD–ve leaves were used for quantitative real-time RT-PCR amplification of pre-miRNA-specific sequences with SYBR green chemistry (Gutha *et al.*, 2010). cDNA from two biological replicates (two pairs of GLRD–ve and two pairs of GLRD+ve grapevines, with each pair of grapevines located adjacent to each other in the vineyard block) were used for quantitative real-time RT-PCR analysis. Three technical replicates were

included in each assay for every biological replicate. Each technical replicate, in turn, was the mean of duplicate values. Aliquots from the same cDNA were used in all technical replications. All assays were performed in 384-well plates using a LightCycler® 480 real-time PCR instrument (Roche Diagnostics) according to the guidelines established in the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE; Bustin *et al.*, 2009). The Cq values were calculated using LightCycler® 480 software. The relative expression of pre-miRNAs in GLRD+ve and GLRD–ve samples was analysed with the three most stable reference genes using the methodologies described in Gutha *et al.* (2010).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Polarity distribution and 5'-terminal nucleotide abundance of different size classes of *Grapevine leafroll-associated virus 3* (GLRaV-3)-specific virus-derived small interfering RNAs (vsRNAs). Bar graphs show the distribution of different size classes of vsRNAs specific to the genome of GLRaV-3 (a) and the relative abundance of GLRaV-3-derived vsRNAs of the 21-nucleotide (nt) size class with distinct 5'-terminal nucleotides (b).

Fig. S2 Polarity distribution of different size classes of viroid-specific small (sRNAs). The viroid-derived small RNAs (vd-sRNAs) (positive and negative polarity) specific to the genomes of *Grapevine yellow speckle viroid 1* (a), *Grapevine yellow speckle viroid 2* (b) and *Hop stunt viroid* (c) derived from grapevine leafroll disease (GLRD)-affected and unaffected leaves are shown. For each viroid, normalized raw reads (transcripts per million, TPM) of vd-sRNAs from GLRD+ve and GLRD-ve libraries (a1, b1 and c1), the

GLRD–ve library (a2, b2 and c2) and the GLRD+ve library (a3, b3 and c3) are indicated.

Fig. S3 Relative abundance of viroid-derived small RNAs (vd-sRNAs) of the 21-nucleotide (nt) size class with distinct 5' terminal nucleotides. The vd-sRNAs specific to *Grapevine yellow speckle viroid 1* (a), *Grapevine yellow speckle viroid 2* (b) and *Hop stunt viroid 1* (c) are shown. For each viroid, normalized raw reads (transcripts per million, TPM) of vd-sRNAs of the 21-nt size class from grapevine leafroll disease (GLRD)+ve and GLRD–ve libraries (a1, b1 and c1), the GLRD–ve library (a2, b2 and c2) and the GLRD+ve library (a3, b3 and c3) are indicated.

Fig. S4 Relative abundance of viroid-derived small RNAs (vd-sRNAs) of the 24-nucleotide (nt) size class with distinct 5' terminal nucleotides. The vd-sRNAs specific to *Grapevine yellow speckle viroid 1* (a), *Grapevine yellow speckle viroid 2* (b) and *Hop stunt viroid 1* (c) are shown. For each viroid, normalized raw reads (transcripts per million, TPM) of vd-sRNAs of the 24-nt size class from grapevine leafroll disease (GLRD)+ve and GLRD–ve libraries (a1, b1 and c1), the GLRD–ve library (a2, b2 and c2) and the GLRD+ve library (a3, b3 and c3) are indicated.

Fig. S5 Predicted fold-back structures for candidate microRNAs (miRNAs) cloned from grapevine small RNA (sRNA) libraries. The sRNA libraries were constructed from grapevine leafroll disease

(GLRD)-affected and unaffected leaves. The mature miRNA sequences are shown in red font.

Fig. S6 Melting curve analysis of pre-microRNA- and reference gene-specific amplicons. The blue/red coloured curves indicate dissociation curves for each pre-microRNA and the three reference genes (actin, Nad5 and EF1-a).

Table S1 Genome-wide abundances of *Grapevine leafroll-associated virus 3* (GLRaV-3)-specific virus-derived small interfering RNAs (vsRNAs) based on the complete genome of GLRaV-3 (GenBank Accession number EU259806; Maree *et al.*, 2008).

Table S2 Predicted targets of novel grapevine microRNAs (Vvi-miRs) derived from grapevine leaves with (GLRD+ve) and without (GLRD–ve) grapevine leafroll disease (GLRD).

Table S3 A list of primers used for the amplification of pre-microRNAs from grapevine leafroll disease (GLRD)–ve and GLRD+ve grapevine leaves, their amplification efficiencies and product sizes.

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