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High-throughput small RNA sequencing for evaluation of grapevine sanitation efficacy

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ABSTRACT

This study describes the application of high-throughput sequencing of small RNA analysis of the efficacy of using Ribavirin to eliminate *Grapevine leafroll-associated virus 1*, *Grapevine fleck virus* and *Grapevine rupestris stem pitting-associated virus* from *Vitis vinifera* cv. Riesling. The original plant used for sanitation by Ribavirin treatment was one naturally infected with all the viruses mentioned above as confirmed by RT-PCR. A tissue cultures of the plant were established and plantlets obtained were sanitized using Ribavirin. Three years after sanitation, a small RNA sequencing method for virus detection, targeting 21, 22 and 24 nt-long viral small RNAs (vsRNAs), was used to analyze both the mother plant and the sanitized plants. The results showed that the mother plant was infected by the three mentioned viruses and additionally by two viroids - *Hop stunt viroid* and *Grapevine yellow speckle viroid 1*. After Ribavirin treatment, the plants contained only the two viroids, with the complete elimination of all the viruses previously present.

1. Introduction

Grapevines are very sensitive to viral infections. More than 70 viral pathogens can invade grapevine tissues (Meng et al., 2017). Among the most important causal agents producing serious symptom manifestations include: *Grapevine leafroll-associated virus 1* (GLRaV-1); *Grapevine fanleaf virus* (GFLV); *Arabis mosaic virus* (ArMV); *Grapevine fleck virus* (GfkV); *Grapevine virus A* (GVA); *Grapevine rupestris stem pitting-associated virus* GRSPaV; *Grapevine virus B* (GVB) and, recently, the *Grapevine Pinot gris virus* (GPGV) reported for the first time in Italian vineyards of the Trentino region (Giampetruzzi et al., 2012; Saldarelli et al., 2015).

GLRaV-1 (genus *Ampelovirus*, family *Closteroviridae*) commonly occurs in the Czech Republic (Komínek, 2008). It is the first virus discovered to be associated with the grapevine leafroll disease (GLRD) (Habili et al., 2007) which has gained a worldwide reputation as the most severe disease of grapevines (*Vitis vinifera* L.). Currently, GLRD accounts for approximately 60% of the global loss of grape production due to virus diseases (Martelli, 2000).

GFkV (genus *Maculavirus*, family *Tymoviridae*) produces a widespread disease, latent in all European grapevine cultivars (*Vitis vinifera* L.) and in most American rootstocks. The prevalence of the virus is very high in both single as well as in mixed infections with other more harmful viruses (Cretazzo et al., 2010; Eichmeier et al., 2016b). GFkV is also a common grapevine virus in the Czech Republic according to previous surveys (Komínek, 2008; Eichmeier et al., 2016a,b).

GRSPaV (*Grapevine rupestris stem pitting-associated virus*, genus *Foveavirus*, family *Betaflexiviridae*) causes the rupestris stem pitting disease and is a common grapevine pathogen occurring worldwide (Martelli, 1993). It has also been reported in the Czech Republic, but without typical symptoms (Komínek et al., 2009; Eichmeier et al., 2016b).

Hop stunt viroid (HSVd) and *Grapevine yellow speckle viroid 1* (GYSVd-1) are very widely distributed in grapevines (Al Rwahnih et al., 2009; Ward et al., 2011; Giampetruzzi et al., 2012). Vineyards in the Czech Republic also showed a high level of occurrence of these viroids (Eichmeier et al., 2016b).

Ribavirin (1-β-D ribofuranosyl-1H-1,2,4-triazole-3-carboxamide) is a chemical drug used in the elimination of viruses in various horticultural species. In grapevines, Ribavirin has been used successfully for the elimination of GVA (Panattoni et al., 2007), GFLV (Weiland et al., 2003), GRSPaV (Skiada et al., 2013; Komínek et al., 2016) and GPGV (Komínek et al., 2016). Ribavirin can be incorporated into RNA during replication as a base analogue, inducing mutations, which are lethal for RNA viruses (Crotty et al., 2002). This is also the basis of its effect in grapevine viruses because most of the viruses invading grapevine

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tissues have a single-stranded positive RNA genome (Komínek et al., 2016).

Currently, one of the most sensitive methods for the detection of viruses is small RNA sequencing (Pantaleo et al., 2010; Pecman et al., 2017; Pooggin, 2018), and this method was also used in our previous work on characterising grapevine virome in Czech Republic (Eichmeier et al., 2016b).

In this study there is described an application of high-throughput sequencing of small RNA for evaluation of grapevine sanitation efficacy.

2. Material and methods

2.1. Plant material and RT-PCR detection

A grapevine plant of cv. Riesling was selected for *in vitro* chemotherapy during the grapevine sanitation program at the Crop Research Institute (CRI), Prague, Czech Republic (Komínek et al., 2016). The plant was tested by RT-PCR for the presence of GFkV, GLRaV-1, GRSPaV, GFLV, ArMV, GVA and GVB before sanitation started. The presence of GLRaV-1, GFkV and GRSPaV was ascertained (Komínek et al., 2016). Primers used in this study are listed in Table 1. The original non-sanitated plant is still maintained *in vitro* and it was used in the present work as a mother plant (sample RR).

The plant was sanitated by repeated cultivation *in vitro* in a medium containing Ribavirin as reported by Komínek et al. (2016). After the sanitation, plants were maintained *in vitro* for three years. After this period, it was used in the present work as sanitated grapevine, labelled as sample RR10.

2.2. Small RNA sequencing

Three years after the last treatment with Ribavirin, both the mother plant (sample RR) and Ribavirin-treated plant (sample RR10) were sampled. Small RNAs were extracted from *in vitro* cultivated plants, starting with one g of leaf material from every plant and using a mirVana miRNA Isolation Kit, with phenol (TFS, Waltham, USA). The amount and quality of RNA were determined using the Agilent Small RNA kit (Agilent, Santa Clara, USA) and the Modulus™ Single Tube Multimode Reader (Turner Biosystems, Sunnyvale, USA) was used to measure the fluorescence. A small RNA library was constructed using the TruSeq small RNA library preparation kit (Illumina, San Diego, USA) and purification was done with the TailorCut Gel Extraction Tool Set (SeqMatic, Fremont, USA). The quality and quantity of the library were determined using the Agilent High Sensitivity DNA Kit (Agilent, Santa Clara, USA). All the kits were used according to the manufacturer's instructions. For the sequencing run, final pooled library of small RNAs consisted of 4 samples, sample RR was labelled with index 7 (CAGATC), sample RR10 with index 8 (ACTTGA) and two others. The libraries were sequenced with the MiSeq instrument (Illumina, San Diego, USA) using the MiSeq Reagent Kit v2 sequencing kit, 50-cycles (Illumina, San Diego, USA) providing 50 nucleotides long reads.

2.3. Sequencing data analysis

The sequence quality was checked using the FastQC-0.10.1 program (Andrews, 2010). A FASTX-Toolkit Clipper (http://hannonlab.cshl.edu/fastx_toolkit/), specifying the Q33 parameter, was used to remove the adaptors (TGGAATTC), sequences shorter than 15 nucleotides were discarded. Contigs of individual reads were assembled *de novo* using the Velvet-1.2.10 assembler (Zerbino and Birney, 2008) with a k-mer of 15. Previously, k-mer values of 15 and 17 were used for data evaluation, but k-mer 15 was selected as suitable based on a more accurate blastn algorithm used through the GenBank/NCBI reference database (version 2.2.30+). Created contigs were screened for homology to known viruses by Nucleotide-Nucleotide BLAST 2.2.31+ in the unix environment, using the newest version of viral.1.1. genomic database of NCBI. The e-value threshold was set as 10^{-5} (Eichmeier et al., 2016b). A list of the potential viruses and viroids present in the analyzed samples was created, and respective reference sequences were selected for the mapping of sequencing reads. First, sequences from the RefSeq database (NCBI) were used, because they contain full-length sequences of particular viruses and viroids. A list of reference sequences is presented in Table 2. Subsequently, sequencing reads were aligned with the CLC Genomics Workbench 6.5.1 (CLC Bio, Aarhus, Denmark) on the reference sequences with the following parameters: Mismatch cost = 2 (The cost of a mismatch between the read and the reference sequence), Insertion cost = 3 (The cost of an insertion in the read-causing a gap in the reference sequence), and Deletion cost = 3 (The cost of having a gap in the read). The parameters were used for Global alignment, and the reads were matched randomly. For the results of the mapping, see Table 2 and Fig. 1.

2.4. Virus spectrum determination

On a base of mapping the reads on the reference sequences, the presence of viruses in the grapevine samples were determined. Those viruses whose genome coverage were at least 53%, and with a sequencing depth greater than 5, were expected to be present in the sample. Further confirmation of the presence of a virus or viroid was done by RT-PCR as a second method, and a positive reaction in RT-PCR was the important criterium for estimating the presence of a virus or a viroid (Eichmeier et al., 2016b).

The genome coverage threshold of > 50% was used according to the author's experience (Eichmeier et al., 2016b). The sequencing depth of 5 has been used in previously published works (Mascher et al., 2013; Broeckx et al., 2015; Valenzano et al., 2015; Eichmeier et al., 2016b).

The presence of viruses and viroids detected by small RNA sequencing was confirmed by RT-PCR, as has been published by Eichmeier et al. (2016b).

Table 1
Description of the primers and PCR conditions used for the detection of grapevine viruses.

Virus	Primers	Expected product size (bp)	PCR cycling conditions	Reference
GLRaV-1	5´ TGGCATCGTTGCTAAATTGAG 3´ A 5´ AATCCTATGCGTCAGTATGC 3´ S	175	95 °C/15 min, 40 × (94 °C/30 s, 53 °C/30 s, 72 °C/1 min), 72 °C/10 min	Komínek et al. (2009)
GRSPaV	5´ CACATRTCATCVCCYGCAAA 3´ A 5´ AGRYTTAGRGTRGCTAARGC 3´ S 5´ ATTGCGGAGTTGCCTTCAAG 3´ S	476	95 °C/15 min, 40 × (94 °C/55 s, 50 °C/55 s, 72 °C/55 s), 72 °C/10 min	Terlizzi et al. (2011)
GFkV	5´ CGACGCAGGCGTCATTGCG 3´ A 5´ CCGTCTGCTGACCAGCCTG 3´ S	520	95 °C/15 min, 40 × (94 °C/55 s, 55 °C/55 s, 72 °C/55 s), 72 °C/10 min	Glasa et al. (2011)
HSVd	5´ GTTGGAAGACGAACCGAGAG 3´ A 5´ GGGCAACTCTTCTCAGAATCC 3´ S	171	95 °C/15 min, 40 × (94 °C/40 s, 55 °C/40 s, 72 °C/50 s), 72 °C/10 min	Eichmeier et al. (2016b)
GYSVd-1	5´ ACTTCATGGTGGTGCCGGTG 3´ A 5´ CCAATGGGTCGCACTTGTTG 3´ S	374	95 °C/15 min, 40 × (94 °C/55 s, 55 °C/55 s, 72 °C/55 s), 72 °C/10 min	Ward et al. (2011)

Legend: Primers: A antisense S sense.

Table 2
Results of analyzes – list of reference sequences used for individual viruses and viroids, number of contigs, genome coverage, and RT-PCR detections.

Grapevine	Accession Nr.	Virus Detected	Reads identified by blastn (1e-5)	Contigs (velvet, k-15) identified by blastn (1e-5)	Reads assembled by CLC Genomics WB 6.5.1	Average seq depth	Genome coverage %	RT-PCR
RR	NC_016509.1	Grapevine leafroll-associated virus 1	36	76	23689	26.91	53	+
	NC_003347.1	Grapevine fleck virus	28	40	16365	44.27	65	-
	NC_001351.1	Hop stunt viroid	6	4	6969	495.32	100	+
	NC_001948.1	G. rupestris stem pitting-associated virus	7	20	3154	7.62	90	+
RR10	NC_001920.1	Grapevine yellow speckle viroid 1	0	3	2715	155.15	66	+
	NC_001920.1	Grapevine yellow speckle viroid 1	9	0	5070	295.11	74	+
	NC_001351.1	Hop stunt viroid	15	5	4662	331.07	100	+

3. Results

3.1. Viruses and viroids detection by small RNA sequencing

Sequenced libraries represented the sRNA populations extracted from grapevines *in vitro*: RR (Riesling before sanitation, maintained *in vitro*), and RR10 (Riesling after sanitation, maintained also *in vitro*). They were sequenced by the SBS (sequencing by synthesis) approach - contained 1,947,842 reads (RR), and 2,394,132 reads (RR10), respectively. *De novo* assembly of sequenced reads, and a blastn search for homologies of the obtained contigs led to the identification of three different viruses and two viroids in the set of the two grapevines (Table 2 and Fig. 1). Two viroids were present in both tested plants, while the three viruses were detected in the non-sanitated plant only. The results of small RNA sequencing were checked by RT-PCR detection and therefore, indicating that all detected viruses and viroids had their presence proven in the tested materials by at least two methods (see discussion and Table 2).

3.2. Descriptions of detected viruses

3.2.1. Grapevine leafroll-associated virus 1

GLRaV-1 was detected in the mother plant of the cv. Riesling. The virus had 23,689 reads mapped on reference genome NC_016509, and 36 reads and 76 contigs were identified by blastn as belonging to GLRaV-1; the average sequencing depth was 26.91, and the genome coverage 53%. The Ribavirin treated variant was virus free. The results were proven also by RT-PCR.

3.2.2. Grapevine fleck virus

This virus had 16,365 reads mapped on NC_003347, and 28 reads and 40 contigs were identified by blastn as belonging to GFkV; the average sequencing depth was 44.27, and the genome coverage was 65%. The untreated variant RR was tested by RT-PCR with negative result for this virus. The treated variant was virus free, and this result was confirmed by RT-PCR.

3.2.3. Grapevine rupestris stem pitting-associated virus

GRSPaV was detected with 3154 reads mapped on NC_001948, and 7 reads and 20 contigs were identified by blastn as belonging to GRSPaV. The average sequencing depth was 7.62, and the genome coverage was 90%. The treated variant was virus free. The presence of the virus was confirmed in the mother plant by RT-PCR.

3.2.4. Hop stunt viroid and grapevine yellow speckle viroid

HSVd was detected in the mother plant through 6969 reads mapped on reference NC_001351, with 6 reads and 4 contigs identified by blastn. The average sequencing depth was 495.32, and the genome coverage was 100%. GYSVd-1 was found in 2715 reads mapped on NC_001920, 3 contigs were identified by blast as belonging to GYSVd-1; average sequencing depth was 155.15, and the genome coverage was 66%. The plant treated by Ribavirin was found to be still infected by GYSVd-1 and HSVd. The mapping showed 5070 reads on NC_001920, and 4662 reads on NC_001351. The blastn also confirmed the presence of GYSVd-1 when 9 reads were identified - with a sequencing depth of 295.11, and genome coverage of 74%; while the blastn revealed 15 reads and 2 contigs belonged to HSVd - with a sequencing depth of 331.07, and a genome coverage of 100%. RT-PCR confirmed the presence of both viroids in the mother plant, as well as in the sanitized plants, thus confirming the results obtained using small RNA sequencing. Both viroids remained in the grapevine tissues.

In the Riesling mother plant (sample RR) maintained *in vitro*, they were obtained identical results of RT-PCR detection of the present viruses as in the case of the source plant growing in a vineyard.

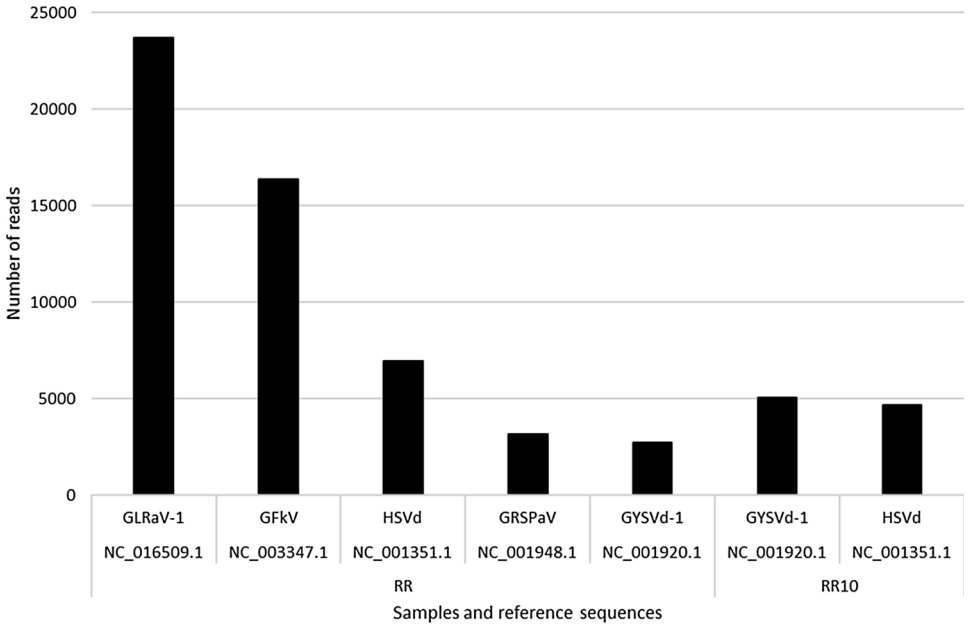

Fig. 1. Numbers of reads mapped on reference viral and viroid sequences. Reference genomes were used in relation to blast analysis. CLC Genomics Workbench 6.5.1 (CLC Bio, Aarhus, Denmark) was used.

4. Discussion

Plant health organizations are being urged to develop and implement rapid and robust methods to eliminate virus-infected plant propagation materials, in order to reduce the losses caused to agronomically important genotypes. In addition, *in vitro* chemotherapy methods are an important focus for the deployment of control measures, especially for the viruses consisting of a single-stranded positive RNA genome (Komínek et al., 2016). The effectiveness of chemotherapy for the elimination of grapevine viruses was ascertained also in the present work.

The only pathogens remaining in grapevine plants after chemotherapy, detected in the present work, were both viroids (HSVd and GYSVd-1). However, in the case of grapevines, it is questionable if viroids really represent causal agents of the symptoms, and if they are real pathogens of grapevines. Some viroids produce serious disease, others may multiply without symptoms. There is no direct relationship between the symptoms and replication (Astier et al., 2001). Viroids were not the subject of detection in the previous work on grapevine sanitation (Komínek et al., 2016), but their presence was detected in the current work using the small RNA sequencing approach.

An interesting observation in the results obtained is the presence of GFkV, confirmed by small RNA sequencing, but not by RT-PCR in the mother plant tissue of the RR sample. This could be caused by the fact that the primers used targeted a not very well conserved nor covered part of the GFkV genome. The divergence of the GFkV genome could be an important factor for the unsuccessful RT-PCR detection (Terlizzi et al., 2011; Glasa et al., 2011). This work proves that small RNA sequencing can be a more reliable method for the detection of viruses and viroids, which was already proven by Santala and Valkonen (2018).

Use of high-throughput sequencing of sanitized grapevines for evaluation of health status proved to be highly suitable, and also highly reliable for the proper evaluation of their health status. One further achievement of the current work is the analysis of the plants three years after completion of the sanitation procedures, proving the stability of sanitation from viruses in the Riesling grapevine cultivar.

The results obtained showed that: (1) The high-throughput small RNA sequencing was a suitable and a powerful method for revealing viruses and viroids in grapevine tissues; (2) Ribavirin treatment could eliminate plant viruses but not viroids and (3) The high-throughput small RNA sequencing procedure significantly enhanced the characterization and detection of pathogens diversity compared to the traditional methods.

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References

Al Rwahnih, M., Daubert, S., Golino, D., Rowhani, A., 2009. Deep sequencing analysis of RNAs from a grapevine showing Syrah decline symptoms reveals a multiple virus infection that includes a novel virus. Virology 387, 395–401.

Andrews, S., 2010. FastQC: A Quality Control Tool for High throughput Sequence Data. Available online at:. http://www.bioinformatics.babraham.ac.uk/projects/fastqc.

Astier, S., Albouy, J., Maury, Y., Lecoq, H., 2001. Principles of Plant Virology: Genome, Pathogenicity, Virus Ecology. Institut National de la Recherche Agronomique, Paris, pp. 444.

Broeckx, B.J., Hitte, C., Coopman, F., Verhoeven, G.E., De Keulenaer, S., De Meester, E., 2015. Improved canine exome designs, featuring ncRNAs and increased coverage of protein coding genes. Sci. Rep. 2015 (5), 12810.

Cretazzo, E., Tomás, M., Padilla, C., Rosselló, J., Medrano, H., Padilla, V., Cifre, J., 2010. Incidence of virus infection in old vineyards of local grapevine varieties from Majorca: implications for clonal selection strategies. Spanish J. Agric. Res. 8, 409–418.

Crotty, S., Cameron, C., Andino, R., 2002. Ribavirin’s antiviral mechanism of action: lethal mutagenesis? J. Mol. Med. 80, 86–95.

Eichmeier, A., Peňázová, E., Pavelková, R., Mynarzová, R., Saldarelli, P., 2016a. Detection of Grapevine Pinot gris virus in certified grapevine stocks in Moravia, Czech Republic. J. Plant Pathol. 98, 155–157.

Eichmeier, A., Komínková, M., Komínek, P., Baránek, M., 2016b. Comprehensive virus detection using next generation sequencing in grapevine vascular tissues of plants obtained from the wine regions of Bohemia and Moravia (Czech Republic). PLoS One 11, e0167966.

Giampetruzzi, A., Roumi, V., Roberto, R., Malossini, U., Yoshikawa, N., La Notte, P., Saldarelli, P., 2012. A new grapevine virus discovered by deep sequencing of virus- and viroid-derived small RNAs in Cv Pinot gris. Virus Res. 163, 262–268.

Glasa, M., Predajňa, L., Komínek, P., 2011. Grapevine Fleck Virus isolates split into two distinct molecular groups. J. Phytopathol. 159, 805–807.
Habili, N., Komínek, P., Little, A., 2007. Grapevine leafroll-associated virus 1 as a common grapevine pathogen. Plant Viruses 1, 63–68.
Komínek, P., 2008. Distribution of grapevine viruses in vineyards of the Czech Republic. J. Plant Pathol. 90, 357–358.
Komínek, P., Glasa, M., Komínková, M., 2009. Analysis of multiple virus-infected grapevine plant reveals persistence but uneven virus distribution. Acta Virol. 53, 281–285.
Komínek, P., Komínková, M., Jandová, B., 2016. Effect of repeated Ribavirin treatment on grapevine viruses. Acta Virol. 60, 400–403.
Martelli, G.P., 1993. Rugose wood complex. In: Martelli, G.P. (Ed.), In Graft Transmissible Diseases of Grapevines, Handbook for Detection and Diagnosis 45-54. FAO, Rome, Italy.
Martelli, G.P., 2000. Major graft-transmissible diseases of grapevines: nature, diagnosis, and sanitation. Proc. 50th Anniversary Annu. Meet. ASEV 231–236.
Mascher, M., Richmond, T.A., Gerhardt, D.J., Himmelbach, A., Clissold, L., Sampath, D., 2013. Barley whole exome capture: a tool for genomic research in the genus *Hordeum* and beyond. Plant J. 76, 494–505.
Meng, B., Martelli, G.P., Golino, D.A., Fuchs, M. (Eds.), 2017. Grapevine Viruses: Molecular Biology, Diagnostics and Management. Springer International Publishing.
Panattoni, A., D'Anna, F., Cristani, C., Triolo, E., 2007. Grapevine vitivirus A eradication in Vitis vinifera explants by antiviral drugs and thermotherapy. J. Virol. Methods 146, 129–135.
Pantaleo, V., Saldarelli, P., Miozzi, L., Giampetruzzi, A., Gisel, A., Moxon, S., Burgyan, J., 2010. Deep sequencing analysis of viral short RNAs from an infected Pinot Noir grapevine. Virology 408, 49–56.
Pecman, A., Kutnjak, D., Gutiérrez-Aguirre, I., Adams, I., Fox, A., Boonham, N., Ravnikar, M., 2017. Next generation sequencing for detection and discovery of plant viruses and viroids: comparison of two approaches. Front. Microbiol. 8, 1998.
Pooggin, M., 2018. Small RNA-omics for plant virus identification, virome reconstruction, and antiviral defense characterization. Front. Microbiol. 9, 2779.
Saldarelli, P., Giampetruzzi, A., Morelli, M., Malossini, U., Pirolo, C., Bianchedi, P., Gualandri, V., 2015. Genetic variability of Grapevine Pinot gris virus and its association with grapevine leaf mottling and deformation. Phytopathology 105, 555–563.
Santala, J., Valkonen, J.P., 2018. Sensitivity of small RNA-based detection of plant viruses. Front. Microbiol. 9, 939.
Skiada, F.G., Maliogka, V.I., Katis, N.I., Eleftheriou, E.P., 2013. Elimination of Grapevine rupestris stem pitting-associated virus (GRSPaV) from two Vitis vinifera cultivars by in vitro chemotherapy. Eur. J. Plant Pathol. 135, 407–414.
Terlizzi, F., Li, C., Ratti, C., Qu, W., Credi, R., Meng, B., 2011. Detection of multiple sequence variants of Grapevine rupestris stem pitting-associated virus using primers targeting the polymerase domain and partial genome sequencing of a novel variant. Ann. Appl. Biol. 159, 478–490.
Valenzano, D.R., Benayoun, B.A., Singh, P.P., Zhang, E., Etter, P.D., Hu, C.K., 2015. The African turquoise killifish genome provides insights into evolution and genetic architecture of lifespan. Cell 163, 1539–1554.
Ward, L.I., Burnip, G.M., Liefting, L.W., Harper, S.J., Clover, G.R.G., 2011. First report of Grapevine yellow speckle viroid 1 and hop stunt viroid in grapevine (Vitis vinifera) in New Zealand. Plant Dis. 95 617-617.
Weiland, C.M., Cantos, M., Troncoso, A., Perez-Camacho, F., 2003. Regeneration of virus-free plants by *in vitro* chemotherapy of GFLV (*Grapevine fanleaf virus*) infected explants of *Vitis vinifera* L. cv "Zalema". Acta Hortic. 652, 463–466.
Zerbino, D., Birney, E., 2008. Velvet: algorithms for de novo short read assembly using de Bruijn graphs. Genome Res gr-074492.