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A novel grapevine badnavirus is associated with the Roditis leaf discoloration disease

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ABSTRACT

Roditis leaf discoloration (RLD), a graft-transmissible disease of grapevine, was first reported in Greece in the 1980s. Even though various native grapevine viruses were identified in the affected vines, the etiology of the disease remained unknown. In the present study, we used an NGS platform for sequencing siRNAs from a twenty-year old Roditis vine showing typical RLD symptoms. Analysis of the NGS data revealed the presence of various known grapevine viruses and viroids as well as a hitherto uncharacterized DNA virus. The circular genome of the new virus was fully reassembled. It is 6988 nts long and includes 4 open reading frames (ORFs). ORF1, ORF2 and ORF4 code for proteins with unknown functions while ORF3 encodes a polyprotein with motifs related to the replication, encapsidation and movement of the virus. Phylogenetic analysis classified the novel virus within the genus *Badnavirus*, with closest relationship to *Fig badnavirus 1*. Further studies showed that the new badnavirus is closely related with the RLD disease and the provisional name grapevine Roditis leaf discoloration-associated virus (GRLDaV) is proposed. Our findings extend the number of DNA viruses identified in grapevine, further drawing attention to the potential importance of this virus group on grapevine pathology.

1. Introduction

In the early 1980s a new disease of grapevine (*Vitis vinifera* L.), designated as "Roditis leaf discoloration" (RLD), was observed in central Greece, primarily in the local cultivar "Roditis" and to a lower extent in cv. "Savastiano" (Rumbos and Avgelis, 1989). RLD symptoms appear in late summer and include yellow and/or reddish discolorations and deformations of the young leaves. Moreover, grape berries are reduced in number and size and have lower sugar content (Fig. 1). Disease symptoms were reproduced by grafting onto *V. vinifera* cv. "Mission". Even though different known grapevine viruses were serologically detected in the affected vines, the causal agent of RLD remained unknown (Avgelis and Rumbos, 1990; Avgelis et al., 2006; Rumbos and Avgelis, 1993).

The advent of unbiased powerful next-generation sequencing technologies (NGS) broadened the potentials in diagnostics and increased our knowledge of the viral pathogens infecting grapevine (Alabi et al., 2012; Al Rwahnih et al., 2012; Coetzee et al., 2010; Glasa et al., 2014; Pantaleo et al., 2010; Zhang et al., 2011). More specifically, sequencing of the accumulated 21–24 nt virus-derived siRNAs generated by Dicer enzymes upon recognition of viral dsRNA was shown to be a powerful tool for the detection of both DNA and RNA viruses, allowing the reconstruction of partial or even complete viral genomes (Kreuze et al., 2009; Seguin et al., 2014).

During the last years the application of NGS technologies also facilitated the determination of the etiology of unknown infectious diseases, often disclosing the existence of novel viral agents (Al Rwahnih et al., 2013; Giampetruzzi et al., 2012; Zhang et al., 2011). Interestingly, so far only RNA viruses were known to infect grapevine, but using NGS technologies two grapevine-infecting DNA viruses have been recently identified (Martelli, 2014). Indeed, a new geminivirus has been associated with a set of symptoms

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Fig. 1. Roditis leaf discoloration (RLD) disease symptoms on leaves (A–C) and grapevine bunches (D and E) of the Roditis variety. The bunch on the right side in (D) is from a healthy plant.

known as "Red Blotch Disease" (RBD) in the USA (Al Rwahnih et al., 2013) and a new badnavirus was correlated with grapevine vein-clearing and vine decline syndrome (Zhang et al., 2011).

In the present study, in order to identify the etiological agent of the disease, a deep sequencing approach was followed, using the 318 chip of the Ion Torrent PGM platform for the analysis of siRNAs from a twenty year old vine exhibiting typical RLD symptoms. As expected, the analysis revealed the presence of several viruses and viroids already known to be prevalent in grapevine (Al Rwahnih et al., 2009; Alabi et al., 2012; Zhang et al., 2011). However, a hitherto unrecorded circular DNA virus, putative member of the genus *Badnavirus*, was also identified. Further studies revealed that the new agent is closely associated with RLD symptoms and it has therefore been given the provisional name grapevine Roditis leaf discoloration-associated virus (GRLDaV).

2. Materials and methods

2.1. Plant material used for NGS and for the RLD association study

A self-rooted twenty-year old grapevine plant of cv. Roditis (W4), showing RLD symptoms was selected for the deep sequencing analysis. The vine was grown in a pot under a net house at the Institute of Viticulture of Heraklion and it was established from cuttings of a diseased vine plant from an old vineyard located in Central Greece (Nea Aghialos, Magnesia). Tissue from fully expanded leaves and stems was used for the NGS analysis.

For the association study of the RLD disease with the newly identified badnavirus, shoots of 20 symptomatic, six asymptomatic cv. Roditis vines and one asymptomatic progeny of cv. Italia vine graft inoculated onto infected Roditis, grown in the same net house were collected during the summer of 2012. All stocks came from Roditis vines grown in the diseased viticultural area of Nea Aghialos and were maintained in pots.

Total RNAs were extracted from bark tissue using a modification of the in-house extraction protocol A developed by Chatzinasiou et al. (2010). Briefly, plant tissue was grounded 1/10 w/v in "lysis buffer" (8 M GuHCl, 25 mM EDTA, 1% Sarcosyl, 2% Triton X-100, 25 mM sodium citrate, 0.2 M sodium acetate, pH adjusted to 5.2 with acetic acid). The lysate was incubated at 65 °C for 10 min and then centrifuged at 16,000 × *g* for 10 min. 500 μl of the upper phase were transferred to a new tube and 625 μl of absolute ethanol was added (to obtain 55.5% final concentration). Then, the mixture was passed through a silica column (FT-2.0 Filter-Tube Spin-Column System, G. Kisker GbR, Steinfurt, Germany) by centrifugation at 1500 × *g* for 10 min. The column was washed once with 700 μl "wash buffer 1" (4 M GuHCl, 25 mM Tris–HCl pH 6.6, and 60% ethanol) and twice (700 and 400 μl respectively) with "wash buffer 2" (2 mM Tris–HCl pH 7.0, 20 mM NaCl, and 80% ethanol) by centrifugation at 8000 × *g* for 1 min. RNA was finally recovered in 50 μl of preheated (80 °C) nuclease-free elution buffer (10 mM Tris–HCl, pH 8.0).

DNA was extracted using the same protocol with the following modifications: (a) ethanol was not added in the lysate before its passage through the silica column and (b) "wash buffer 2" included 10 mM Tris–HCl pH 7.4, 100 mM NaCl, 25% ethanol and 25% isopropanol.

2.2. NGS and analysis of siRNA reads

siRNAs were isolated from a pool of grapevine leaves using the mirPremier microRNA Isolation Kit (Sigma–Aldrich, St. Louis, USA). Optimal quality of the extracted RNAs was verified by checking the OD_{260}/OD_{280} values using a NanoDrop (Thermo Scientific, Wilmington, USA). RNA was quantified using a Qubit® 2.0 Fluorometer (Invitrogen, Grand Island, USA). Subsequently siRNAs library construction and sequencing were performed by Life Sequencing S.L. (Paterna, Spain). *De novo* genome assembly was performed using the CLC Genomics Workbench (CLC bio, Aarhus, Denmark) software. The resulting contigs were blasted (Blastn, BlastX, TBlastX) against Genbank nr nucleotide and protein databases at NCBI. Each resulting contig from the previous step was used as reference in an iterative mapping and contig extension process using Geneious (Biomatters Ltd., Aukcland, New Zealand) and different custom assembly options.

2.3. Determination of the new DNA virus full-length genome by Sanger sequencing

In order to confirm the complete sequence assembled from the siRNA data, eleven primer pairs spanning the complete virus