DEEP SEQUENCING ANALYSIS OF VIRAL SHORT RNAS FROM PINOT NOIR CLONE ENTAV 115

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Summary

Short interfering RNAs (siRNAs) isolated from *Vitis vinifera* Pinot noir clone ENTAV 115 were sequenced by high throughput techniques. Viral (v)siRNAs of several grapevine viruses belonging to *Fovea-, Macula-, Marafi- and Nepoviruses* genera were identified. v-siRNAs were dominated by 21 and 22 nt species spanning the entire or being discontinuously distributed throughout *Grapevine rupestris stem-pitting associated virus* (GRSPaV) and *Grapevine fleck virus* (GFkV) genomic RNAs, respectively. v-siRNAs derived from positive and negative viral RNA strands for RSPaV or, abundantly, from negative viral RNA strands for GFkV.

INTRODUCTION

In plants RNA silencing is a complex of related pathways which drives, among the other functions, the defense against viral parasites. This process relies on the activity of RNAse III Dicer enzymes (DCL) that cleave RNAs with double stranded (ds) features into 21 to 24 nt duplex siRNAs or microRNAs (miRNAs). Evidence from the model plant *Arabidopsis thaliana*, showed that RNA viruses are mainly targeted by DCL4, DCL2 and DCL3 to generate primary v-siRNAs of 21, 22 and 24 nt, respectively, which are followed, in a pathway controlled by host RNA-dependent RNA polymerase (RDR) enzymes, by secondarily generated v-siRNAs. v-siRNAs seem to derive either by genome-lenght dsRNAs or highly structured, single-stranded viral RNAs, which act as silencing trigger (Ding S.W. & Voinnet O., 2007).

Grapevine is affected by several viruses, some of which cause symptomless infections or express symptoms in specific genetic backgrounds or graft combinations (Martelli & Boudon-Padieu, 2006). This is the case of GRSPaV, a foveavirus associated with stem-pitting symptoms in *Vitis rupestris* and with vein necrosis in the rootstock 110R, and GFkV, a maculavirus which is latent in *V. vinifera* but induces specific foliar symptoms in *V. rupestris*. Both viruses, together with sequences related to the grapevine maculavirus Grapevine red globe virus (GRGV), the marafiviruses Grapevine rupestris vein feathering virus (GRVFV) and Grapevine asteroid mosaic associated virus (GAMaV), and *Grapevine fanleaf virus* (GFLV), were found in a subset of siRNAs obtained by deep sequencing total small RNAs from the grapevine clone ENTAV 115. The analysis of these data is the object of the present abstract.

MATERIAL AND METHODS

RNA isolation and RT-PCR detection. Total RNA was extracted from leaf or phloem tissues with the silica capture protocol (Rott & Jelkmann, 2001). Two-step RT-PCR was carried out as previously described (Saldarelli *et al.,* 2006). PCR primers were from Al Rwahnih *et al*. (2009) and Abou Ghanem-Sabanadzovic *et al.* (2003).

Cloning and sequencing of siRNAs. Total RNA was extracted from leaves of the Pinot noir clone ENTAV 115, by guanidine thyocianate buffer (Rott & Jelkmann, 2001), followed by phenol/chloroform extractions. 19–24 nt small RNAs were isolated from 15% denaturing polyacrylamide gel and 15 µg were ligated to Solexa adaptors (Illumina Inc.). Short RNAs were converted to DNA by RT-PCR and the DNA was sequenced by a Solexa machine (Illumina Inc.).

Analysis of vsiRNAs. v-siRNAs reads were analyzed by BLASTN software against a viral genomic sequences database from NCBI (http://www.ncbi.hlm.nih.gov).

RESULTS AND DISCUSSION

Composition of the v-siRNA population in grapevine ENTAV clone 115. Initial BLASTN analysis of the source library against the viral genomic (refseq_rna) database identified v-siRNAs having sequence homologies with several plant viruses (Table 1 refseq), and showed the largest reads for GFkV and GRSPaV. A further group contained several "non-grapevine" tymovirids belonging to the *Tymo-* and *Marafivirus* genera. Since genomic variations among grapevine tymovirids may account for these erroneus BLASTN identifications (Abou Ghanem-Sabanadzovic *et al.*, 2003), the original siRNAs data were filtered against a new database containg all the available sequences of GFkV, GRSPaV GAMaV, RGRV and GRVFV. This approach allowed the identification of v-siRNAs related to these new viruses whose presence, together to GFkV and GRSPaV, was ascertained by RT-PCR (Figure 1). Moreover, selected GRSPaV and GFkV viral genomic regions were sequenced from the original plant, confirming the trueness of the v-siRNAs sequences obtained.

Table 1. BLASTN reads of v-siRNAs against a viral genomic sequences (refseq) and a selected database.

Organism name	database	
	refseq	*selected*
Grapevine fleck virus	3307	-
Grapevine rupestris stem-pitting associated virus	1125	-
Oat blue dwarf virus	74	-
Turnip yellow mosaic virus	55	-
Onions yellow mosaic virus	51	-
Okra mosaic virus	43	-
Maize rayado fino virus	28	-
Citrus sudden death-associated virus	16	-
Grapevine red globe virus	-	2856
Grapevine asteroid mosaic associated virus	-	33
Grapevine rupestris vein feathering virus	-	68
Grapevine fanleaf virus	27	-

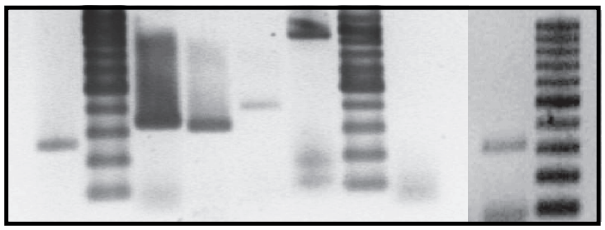

Figure 1. Electrophoretic analysis of specific virus PCR products amplified from total RNAs extracted from *P. noir* ENTAV115. 1: GFkV; 2: GRSPaV; 3: GAMaV(nt); 4: RGRV(nt); 5: rRNAs control; 6: GSyV-1; 7:GFLV; L:DNA ladder.

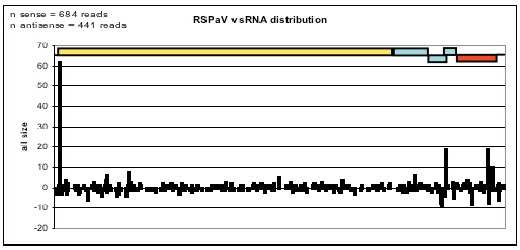

Figure 2. Distribution of v-siRNA species along GRSPaV genome.

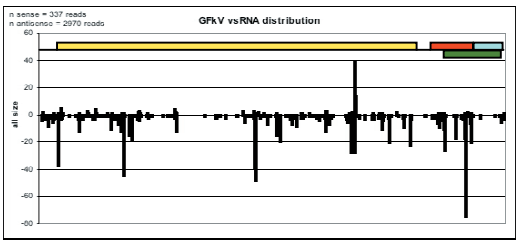

Figure 3. Distribution of v-siRNA species along GFkV genome.

Analysis of v-siRNA population in grapevine. v-siRNAs size ranged between 18 and 26 nt, prevalent lengths being 21 and 22 nt. The majority of v-siRNAs were sequenced once or twice. When all v-siRNAs species were mapped on viral genomes two different pictures emerged for RSPaV and GFkV. GRSPaV v-siRNAs originated from sense (60,8%) and antisense (39,2%) orientations (Fig. 2), suggesting that both polarities had contributed v-siRNAs. Three hotspots were identified in the 5' and 3' genomic regions, being mainly composed of 21 nt species.

GFkV v-siRNAs had a different distribution (Fig. 3), as they were mainly of negative polarity (89.8%) and did not span the entire genome length, thus showing that several genomic regions did not contribute v-siRNA. Alignments of v-siRNA sequences at the identified hotspots showed that both 21 and 22 nt species overlapped and were shifted by one nucleotide. GFKV genome has a cytosine content (49.8%) higher than that of the type member of its family, *Turnip yellow mosaic virus* (TYMV) (Sabanadzovic *et al*., 2001), which would lead to a large proportion of unpaired cytosine residues in the viral RNA. According to Hellendoorn *et al.* (1996), TYMV RNA secondary structure consists of long cytosine-rich tracts interacting with the protein capsid and separated by weak stem-loop elements. By analogy, for GFkV, the complete lack of v-siRNAs from diverse genomic regions that have a relatively higher *C* content (75-80%), could be explained by the absence of secondary structures. Intriguing is the prevalence of negative-strand v-siRNAs originated from GFkV (and other related viruses), which does not find counterpart in the literature. Site (phloem tissue) and modality (vesicles originating from the invaginations of plastidial and/or mitochondrial bounding membranes) of GFkV replication differ from those of GRSPaV, and may have a bearing on the observed behaviour. As recently pointed out (Al Rwahnih *et al*., 2009), high throughput sequence analysis is a valuable tool for unraveling the complex interactions between the grapevine and viral pathogens in multiple infections. This information may assist in understanding the origin of mild or latent infections for a disease condition results from altered and/or synergic interactions between viral agents and the host.

LITERATURE

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