Genome-wide identification of viral and host transcripts targeted by viral siRNAs in *Vitis vinifera*

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SUMMARY

In plants, RNA silencing is a surveillance mechanism against invading viruses. It involves the production of virus-derived small interfering RNAs (vsiRNAs), which guide the RNA-induced silencing complex (RISC) to inactivate viruses. vsiRNAs may also promote the silencing of host mRNAs in a sequence-specific manner. In this work, vsiRNAs derived from two grapevine-infecting viruses (*Grapevine fleck virus* and *Grapevine rupestris stem pitting-associated virus*) were selected from cDNA libraries of short RNAs and were cross-referenced with the remnants of both cleaved host transcripts and viral RNAs from a degradome dataset. We identified dozens of host transcripts targeted by vsiRNAs. Among them, several encode putative proteins involved in ribosome biogenesis and in biotic and abiotic stresses. Moreover, we identified vsiRNAs which explain the cleavage sites in viral genomes. A consistent fraction of vsiRNAs did not apparently account for cleavage, suggesting that only a low percentage of vsiRNAs are involved in the antiviral response.

INTRODUCTION

RNA silencing is a natural defence mechanism against invading viruses in plants and entails the production of virus-derived small interfering RNAs (vsiRNAs), which guide specific Argonaute (AGO)-containing effector complexes to inactivate viral genomes. As a response to this defence system, viruses encode viral suppressors of RNA silencing (VSRs) that affect the accumulation and spread of viruses in infected plants (Csorba *et al.*, 2009). In addition to antiviral defence, RNA silencing includes pathways that target endogenous mRNAs or transposons. Small RNA silencing that targets endogenous mRNAs may have a distinct biogenesis and involve microRNAs (miRNAs) or other endogenous siRNAs (Baulcombe, 2004).

vsiRNAs are generated by DICER-like (DCL) proteins that recognize and cleave perfect double-stranded RNAs (dsRNAs) or

dsRNA-like molecules of viral origin. In virus-infected plants, two distinct classes of vsiRNA have been identified: primary siRNAs, which result from DCL-mediated cleavage of an initial trigger RNA, and secondary siRNAs, whose biogenesis requires an RNA-dependent RNA polymerase (RDR) enzyme (Ding, 2010; Dunoyer *et al.*, 2005; Wang *et al.*, 2011; Wassenegger and Krczal, 2006).

DCL4 and DCL2 are the most important plant DICERs involved in virus-induced RNA silencing, and produce 21- and 22-nucleotide siRNAs, respectively. Although DCL4 is the most important player in vsiRNA production, in the absence of DCL4, DCL2 is known to produce 22-nucleotide vsiRNA (Deleris *et al.*, 2006; Fusaro *et al.*, 2006). However, recent findings have suggested that 22-nucleotide viral siRNAs alone do not guide efficient silencing (Wang *et al.*, 2011). vsiRNAs are associated with distinct AGO-containing effector complexes to guide them to their RNA target molecules (Ding and Voinnet, 2007; Hutvagner and Simard, 2008; Vaucheret, 2008). In plants, the loading of siRNAs into a particular AGO complex is preferentially, but not exclusively, dictated by their 5'-terminal nucleotides (Mi *et al.*, 2008). AGO1 is the major slicer in plants, but other AGO paralogues are likely to be involved, potentially also mediating translational repression (Mallory and Vaucheret, 2010). Previous studies using plant viruses have shown that RNA silencing-related viral inactivation is based on the endonucleolytic cleavage of viral RNAs, although antiviral mechanisms based on translational arrest could not be excluded (Brodersen *et al.*, 2008; Omarov *et al.*, 2007; Pantaleo *et al.*, 2007; Zhang *et al.*, 2006). In addition to the *cis*-action of vsiRNAs (i.e. targeting viral RNAs from where they originate), vsiRNAs can act *in trans*, for example by targeting subviral satellite RNA (Pantaleo and Burgyan, 2008). Moreover, vsiRNAs can function to inhibit homologous cellular transcripts at the post-transcriptional level, as reported for vsiRNA derived from the *Cauliflower mosaic virus* 35S leader sequence, which specifically down-regulates the *Arabidopsis* At1g76950 transcript (Moissiard and Voinnet, 2006). In addition, vsiRNAs from *Tobacco mosaic virus* Cg specifically cleave At1g30460 and At2g16595 transcripts (Qi *et al.*, 2009); however, the effect, if any, of the vsiRNA host gene targeting is poorly understood. Moreover, vsiRNA from the Y-satellite of *Cucumber mosaic virus* specifically cleaves the *Nicotiana benthamiana* Chll mRNA (Shimura *et al.*, 2011; Smith *et al.*,

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2011) resulting in bright yellow symptoms. It is likely that some vsiRNAs might create a cellular environment more suitable for viral proliferation (i.e. targeting host defence factors) and may be positively selected (Ruiz-Ferrer and Voinnet, 2009).

AGO-mediated cleavage of mRNA targets often occurs between the 10th and 11th nucleotides in the target sites of si/miRNAs. The resulting upstream fragments of the cleaved target RNA rapidly degrade (Souret *et al.*, 2004), although the 5'-uncapped and polyadenylated fragment is sufficiently stable to be identified and characterized by 5'-rapid amplification of cDNA ends (5'-RACE) or by genome-wide mapping of uncapped transcripts (termed degradome analysis) (Addo-Quaye *et al.*, 2008; German *et al.*, 2008; Llave *et al.*, 2002; Pantaleo *et al.*, 2010b). It is widely assumed that the same rules are valid for vsiRNA-mediated RNA cleavage.

Recently, the *Vitis vinifera* genome has been entirely sequenced (Jaillon *et al.*, 2007; Velasco *et al.*, 2007) and high-throughput analysis of short RNAs and the degradome has been reported (Pantaleo *et al.*, 2010b). The latter has allowed the identification of several miRNAs and their targeted transcripts. Moreover, the analysed plant was infected by several viruses, and vsiRNAs were isolated and characterized (Pantaleo *et al.*, 2010a).

In this article, we further analyse the datasets by integrating the vsiRNAs and degradome libraries, and we identify a pool of vsiRNAs responsible for the cleavage of both host (*V. vinifera*) transcripts and viral RNAs. A consistent fraction of vsiRNAs did not account for cleavage, suggesting that relatively few vsiRNAs are involved in the RNA silencing of host mRNAs and in the antiviral response. Selected vsiRNAs are characterized with regard to size, viral origin and sequence, and aspects of the vsiRNA-driven viral RNA targeting are discussed.

RESULTS

vsiRNA from Grapevine fleck virus (GFKV) and Grapevine rupestris stem pitting-associated virus (GRSPaV)

Previously, we carried out a deep-sequencing analysis of short RNAs from *V. vinifera* clone ENTAV115 tissues. The analysis revealed a large number of vsiRNAs derived from several grapevine-associated (+)-strand RNA viruses (Pantaleo *et al.*, 2010a). GFKV and GRSPaV datasets were separated and filtered for vsiRNA sequences of 21, 22 and 24 nucleotides (Pantaleo *et al.*, 2010a). The population of GFKV vsiRNAs contained 718 unique sequences (2303 redundant), which covered c. 38% of the viral genome. Most of the GFKV vsiRNAs were of 21 and 22 nucleotides (63% and 35.8%, respectively), mainly of antisense polarity (89%) and showed a strong bias for having a G or C at their 5'-termini (37% and 29.2%, respectively) (Pantaleo *et al.*, 2010a). In the case of GRSPaV, we selected 562 vsiRNA unique sequences (990 redundant), predominantly 21 nucleotides in size

(78.6%), covering c. 50% of the viral genome. They were mainly of sense polarity (62%) and were poorly represented by G at the 5'-termini (12.1%) (Pantaleo *et al.*, 2010a).

Plant transcripts targeted by vsiRNAs

CleaveLand is a bioinformatics tool developed to identify mRNAs cleaved by miRNA (Addo-Quaye *et al.*, 2008, 2009). Previously, we used this tool to integrate miRNAs from short RNA libraries with a genome-wide map of uncapped transcripts (degradome) from the same *V. vinifera* plant. Indeed, we were able to identify several grapevine mRNAs targeted by conserved and novel grapevine miRNAs (Pantaleo *et al.*, 2010b). In the present study, we adopted the same method to identify grapevine mRNAs targeted by vsiRNAs from GFKV or GRSPaV. Therefore, we integrated the population of unique sequences of vsiRNAs (see previous paragraph) with the degradome database (Fig. 1A). Targets were considered to be significant when the estimated *P* value was ≤ 0.05 . The cleavage sites were classified according to the categories defined in the CleaveLand pipeline and described in Experimental procedures.

Our analysis resulted in 24 different grapevine transcripts targeted by 27 vsiRNAs derived from the GFKV genome (Table 1A). Distinct size classes of vsiRNAs were found; 13 of 27 were 21 nucleotides in length, nine were of 22 nucleotides and five were identified in both 21- and 22-nucleotide size classes. Most were of antisense polarity (20 of 27). Moreover, the nucleotide at their 5'-ends was G in 12 cases, C in eight and A in seven. Surprisingly none of the identified host mRNA targeting vsiRNAs bore a U at the 5'-terminus, despite the rule that the major slicer, Ago1, preferentially incorporates siRNAs with a 5'-terminal U.

Eleven of the 24 targeted transcripts were annotated with putative functions in the *V. vinifera* genome (Gene Index Project annotation; <http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=grape>) and BLAST2GO annotation (Conesa *et al.*, 2005) (Table 1A). In 16 cases, the highest frequency of degradome tags mapped on the host transcripts in the expected positions of vsiRNA cleavage sites (i.e. three were of category 0 and 13 were of category 1; see Experimental procedures) (Table 1A and Appendix S1, see Supporting Information). vsiR1378 targets the transcript TC107032 coding for a putative S2P metalloprotease. Proteins belonging to this family are implicated in the control of regulated intramembrane proteolysis (RIP) (Chen and Zhang, 2010). vsiR4655 and vsiR6038 target transcripts TC121043 and TC105673, respectively. These transcripts encode putative proteins involved in stress responses [i.e. the stress-associated protein containing a A20/AN1 zinc finger domain and a thaumatin-like protein (TLP), respectively] (Vij and Tyagi, 2008; Liu *et al.*, 2010).

Table 1B lists the 30 vsiRNAs from GRSPaV that target 26 different grapevine transcripts (Table 1B). Contrary to that found in

Fig. 1 Pipeline of the bioinformatics analysis used for the identification of virus-derived small interfering RNAs (vsiRNAs) driving the cleavage of grapevine targets (A) and viral genomes (B).

the case of GFKV, vsiRNAs were found exclusively in 21- or 22-nucleotide size classes and none were present in both 21- and 22-nucleotide size classes. Moreover, both sense and antisense polarities were equally represented among vsiRNAs. Twelve of 30 GRSPaV vsiRNAs had a U at the 5'-terminus, 10 a C, three a G and five an A.

vsiR6978 and vsiR6977 target two transcripts (TC109537 and EE081111, respectively) which encode two putative proteins

belonging to the vacuolar protein-sorting 55 (VPS55) and possessing membrane-spanning domains (Table 1B). The yeast VPS55 protein has been shown to be involved in the regulation of the endosomal trafficking of proteins (Schluter *et al.*, 2008). GRSPaV vsiR8652 targets the transcript TC107235 coding for a putative signal peptide peptidase-like 3 (SPPL3) protein (Table 1B). It is interesting to note that, like the S2P metalloprotease (target of GFKV vsiR1378), the SPPL3 protein is also involved in controlling

Table 1 Grape mRNA targets of *Grapevine fleck virus* (GFKV)-derived virus-derived small interfering RNA (vsiRNA) (A) and *Grapevine rupestris stem pitting-associated virus* (GRSPaV)-derived vsiRNA (B)†.

vsiRNA sequence	vsiRNA ID	Size	Strand	Target ID	Target description	Cleavage site	Alignment score	Category	P value
A									
GGCGUUGGCUUGGAUGGUGG (4)	vsiR5070(–)	21	–	TC109765*	PsbY precursor (psbY)	227	7	2	0.04
GUCCAAAGGCGUGAGUUCGAC (1)	vsiR1605(+)	22	+	TC130967* / TC109765*	PsbY precursor (psbY)	407	6	1	0.01
GUCCAAAGGCGUGAGUUCGA (2)	vsiR1607(+)	21	+			407	5	1	0.02
CCAAAGGCGUGAGUUCGACU (1)	vsiR1607(+)	21	+			405	7	2	0.03
AGCGUUGGCUUGGAUGGUGG (27)	vsiR5071(–)	22	–	TC107930	60S ribosomal protein l18a-1	485	7	1	0.02
GGAAGGGGAGUUGGAGUUCUAC (3)	vsiR6577(–)	22	–	TC118037	60S ribosomal protein l18a-1	37	5.5	1	<0.01
GGAAGGGGAGUUGGAGUUCUUA (2)		21	–			37	4.5	1	<0.01
AGGACGAGAGGCGUGAGAGC (5)	vsiR1172(–)	21	–	EE103699	60S ribosomal protein l10a-1	240	7	1	0.04
AGAACAGACUC GAGACGGU (18)	vsiR1378(–)	22	–	TC107032	S2P-M50 metalloprotease	1807	6.5	1	0.01
AGAACAGACUC GAGACGG (43)		21	–			1807	6.5	1	0.01
AAGAGGGGCGGUGAGGGAAG (3)	vsiR6038(–)	22	–	TC105673	Thaumatin-like protein	1171	7	0	0.01
GGGUAGACGGUCUCGAGGUGG (2)	vsiR4655(–)	22	–	TC121043	A20/AN1 zinc finger domain-containing protein	607	6	1	<0.01
GGGUAGACGGUCUCGAGGUG (1)		21	–			607	6	1	0.02
AGAGCUAGAGACAGCGGCG (1)	vsiR6477(–)	21	–	TC119185	β-Lectin and protein kinase domain-containing protein	2361	4.5	4	0.05
CUGGAAGGGGAGUUGGAGUUCU (10)	vsiR6579(–)	22	–	TC125978	Transporter-related protein	404	5	4	<0.01
GCCAAAGGCUUCGCGUCGAC (1)	vsiR5062(+)	21	+	TC110551	nc domain-containing protein	701	6.5	1	0.01
GUACUGGUGUAGAGGAGGCG (4)	vsiR5577(–)	21	–	TC130492	Conserved protein	215	5.5	4	0.02
GAGGAUGGAGCGCAGAGUGGC (1)	vsiR7002(–)	21	–	TC130492	Conserved protein	203	5	4	0.05
GGAAGGCAAGAGAGAGAG (3)	vsiR246(–)	21	–	TC123683	Hypothetical protein	675	3.5	4	0.05
CAUGUUCAGAGAGGUGCGAA (1)	vsiR906(+)	21	+	TC131095	Hypothetical protein	267	7	0	0.01
CAGAGCUCGCGGCGGCGUACCA (1)	vsiR4258(+)	22	+	TC109019	Hypothetical protein	606	7	1	0.01
GGAGUAGACGAACUCACGCG (1)	vsiR3709(–)	21	–	TC107597	Hypothetical protein	615	6.5	1	<0.01
GGGAGGCUACACAGGAGGCG (2)	vsiR6377(–)	22	–	TC129481	Hypothetical protein	370	7	1	0.01
GGGAGGCUACACAGGAGGCG (2)		21	–			370	6	1	0.01
CUGAAGGAGGAGGAGGAGGA (1)	vsiR7504(–)	21	–	TC134829	Hypothetical protein	136	5	1	0.01
GUGAUGGAGGAGGUGUGAGC (12)	vsiR1331(–)	22	–	TC134829	Hypothetical protein	136	7	1	0.04
AUCGACAGGUCUACAGGAGGCG (1)	vsiR3628(+)	22	+	TC126203	Hypothetical protein	340	6.5	2	<0.01
GAUGGAGCGCGAGUUGGCGG (6)	vsiR6999(–)	22	–	TC133601	Hypothetical protein	59	7	1	0.05
ACAGAACAGGAGAGUCCCAA (1)	vsiR2167(–)	21	–	TC133769	Hypothetical protein	233	7	0	0.01
CAGAGUACGGUUGUACACCGU (1)	vsiR2230(–)	22	–	TC124977	Hypothetical protein	776	4.5	4	0.02
CCAUCACCGCGGAGGAGGAG (1)	vsiR1325(+)	22	+	TC136319	Hypothetical protein	53	4	4	<0.01
CAGUUGUUGCGGAGAGAGAU (1)	vsiR4739(–)	21	–	EC986867	Hypothetical protein	114	4.5	4	0.05

Table 1 Continued.

vsRNA sequence	vsRNA ID	Size	Strand	Target ID	Target description	Cleavage site	Alignment score	Category	P value
B									
CAGGAGUUGAGGCGAAGAAG (1)	vsR5570(+)	21	+	TC130967* [†] /TC109765*	PsbY precursor (psbY)	365	6	2	0.02
CUGGGAUUAUAGGAGGAGUGA (1)	vsR180(+)	21	+	TC130967* [†] /TC109765*	PsbY precursor (psbY)	451	5.5	2	<0.01
UCUGGGAACUUGAAACGCUU (1)	vsR2121(-)	21	-	TC130967* [†] /TC109765*	PsbY precursor (psbY)	320	7	2	<0.01
CUGAAAGCGUGCAAUUGAUG (1)	vsR8057(+)	21	+	TC130967* [†] /TC109765*	PsbY precursor (psbY)	469	6	1	0.04
AAUUCUUC AACAGCAGCGCU (1)	vsR7746(-)	21	-	EE105806	60S ribosomal protein 118a-1	374	6.5	1	0.04
UAGAAACAGAUUAGGUC CAA (1)	vsR3036(+)	21	+	TC111405	60S ribosomal protein 126	689	6	1	0.04
UGAGAACUCUCAAGAAGCCAG (1)	vsR1062(+)	21	+	TC112924	40S ribosomal protein s19	612	6	2	<0.01
AAUUAGAGCGCUUUCGACAG (1)	vsR5439(-)	22	-	TC110069	eif6	739	4.5	4	0.03
UUUAGGUUAGCAGUGACGGU (6)	vsR6977(+)	21	+	EE081111	Vacuolar protein sorting 55 domain-containing protein	381	4.5	4	0.02
UUAGGUUAGCAGUGACGGUU (4)	vsR6978(+)	21	+	TC109537	Vacuolar protein sorting 55 domain-containing protein	511	6.5	1	0.01
UUUUAAGGUUAGCAGUGACGG (1)	vsR6976(+)	21	+			513	4	4	<0.01
CUUGGUUGGCAACGCUCCAAA (6)	vsR657(-)	21	-	TC107140	Peroxisomal membrane protein	1084	6	1	0.03
CAGCUCUACAGGCAUUAUAGUAG (1)	vsR141(+)	21	+	TC105502	acd3 amino acid-binding protein	1206	6	2	<0.01
GAAAGGGAUUAUUAUAGUAC (1)	vsR8652(-)	21	-	TC107235	Signal peptide peptidase-like 3	1216	6	1	0.03
AACAUUGGUUUUAAGACAGGC (1)	vsR2249(+)	22	+	TC109075	RNA recognition motif (RRM)-containing protein	499	6.5	1	0.01
CACAUCAACUUCACGCGCUUCC (1)	vsR3661(-)	21	-	TC125753	Succinate dehydrogenase flavoprotein α subunit	1441	5.5	1	<0.01
UUAGUACGGUUAUCCAGCGAAC (1)	vsR8638(-)	22	-	TC110936	Aconitase C-terminal domain-containing protein	698	7	1	0.01
GGCAUAGCAUUCUUCUCCAA (1)	vsR3445(-)	21	-	TC118235	Protein kinase interacting protein	1002	6.5	2	0.01
UUUGGUCAAGCUAAGCAGAGC (2)	vsR1957(-)	21	-	TC127300	Kinesin-like protein	265	7	1	0.05
UGGUUUUAGAGAAUUAUAGC (1)	vsR2305(-)	21	-	TC117153	F-box family protein	99	5	4	0.04
GAGUGGGUGUCGGAAGCUGC (1)	vsR2139(-)	21	-	TC128610	Hypothetical protein	222	6.5	1	0.01
UUGUCACUAGAACUUAUAAACG (1)	vsR492(-)	21	-	TC136128	Hypothetical protein	341	4.5	4	0.03
UCAAAGGCGUCAAACUUAUUA (1)	vsR8327(-)	21	-	TC110065	Hypothetical protein	461	4	4	0.03
CAAGAUGAGAUUGUGAAGUUG (1)	vsR5135(+)	22	+	TC125040	Hypothetical protein	88	7	1	0.01
CAGGUUUAAGCAAGCGUUAUC (2)	vsR2089(+)	21	+	TC128929	Hypothetical protein	916	7	0	<0.01
AUUAAGAAGAUUGAGGAGGAGU (1)	vsR3098(+)	21	+	TC132885	Hypothetical protein	251	7	1	0.03
CACAGGAAGUACUUAAGCC (1)	vsR6760(+)	21	+	TC133677	Hypothetical protein	637	7	1	0.05
CAUUUAUCUGGAGGAGGGAUA (1)	vsR6320(+)	21	+	TC137781	Hypothetical protein	71	4.5	4	0.04
AAGGCACAAAUAUUCAGAUU (1)	vsR7486(-)	22	-	CB915267	Hypothetical protein	24	5	4	0.05
UGGGAUUGGAAACAAACUUAAC (1)	vsR6272(-)	22	-	EE073671	Hypothetical protein	138	6	1	<0.01

*Indicates targets common to both viruses.

†Values in parentheses indicate the number of reads.

the RIP process (Schroder and Saftig, 2010; Weihofen *et al.*, 2002). vsiR2249 targets transcript TC109075, which encodes a putative RNA recognition motif (RRM)-containing protein (Table 1B).

vsiR5071 and vsiR6577 from GfKV and vsiR7746 from GRSPaV target the transcripts TC107930, TC118037 and EE105806, respectively (Table 1). These transcripts code for the putative 60S ribosomal protein l18a-1, one constituent of the large ribosomal subunit. Moreover, other transcripts encoding constituents of cytoplasmic ribosomal subunits are targeted by vsiRNAs from both GfKV and GRSPaV. Indeed, GfKV vsiR1172 targets transcript EE103699, which encodes the putative 60S ribosomal protein l10a (Table 1A). The GRSPaV vsiR3036 and vsiR1062 target grapevine transcripts encoding the putative 60S ribosomal protein l26 and the 40S ribosomal protein s19, respectively (Table 1B). In addition, GRSPaV vsiR5439 targets the transcript TC110069 coding for a putative protein annotated as similar to translation initiation factor 6 (eIF6). The protein eIF6 has been shown previously to bind specifically the 60S ribosomal subunit and, in yeast, it appears to be required for 60S subunit biogenesis and maturation (Basu *et al.*, 2001; Si and Maitra, 1999).

Strikingly, vsiRNAs from the two viruses can target the same grapevine transcripts. This is the case for transcripts TC130967 and TC109765 encoding for a putative protein similar to a photosystem II precursor protein (PsbY) from *Arabidopsis* (At1g67740). Indeed, these transcripts are targeted by vsiR5070, vsiR1605 and vsiR1607 from GfKV and by vsiR5570, vsiR8180, vsiR2121 and vsiR8057 from GRSPaV in different sites (Table 1). PsbY is a thylakoid membrane protein, which is translated in the cytosol and then directed to the chloroplasts, where it takes part in the core of photosystem II (Gau *et al.*, 1998). Table 1 also includes 23 targeted transcripts encoding for putative proteins not yet annotated.

Endogenous siRNAs, including miRNAs, are known to down-regulate host mRNAs at the post-transcriptional level. Our analysis likewise suggests that discrete species of vsiRNAs from both GfKV and GRSPaV can act as bona fide siRNAs promoting the cleavage of the transcripts in Table 1. To examine whether GfKV and GRSPaV infection is associated with a lower accumulation of transcripts listed in Table 1, we carried out quantitative real-time polymerase chain reaction (qRT-PCR). Both GfKV and GRSPaV are known to widely infect *V. vinifera* clones; this renders difficult to find an appropriate virus-free negative control (including the clone ENTAV115 under analysis). Thus, we performed qRT-PCR analysis using *V. vinifera* 'Brachetto', a clone of *V. vinifera* which was available infected by both viruses under analysis, as well as free of viruses (Gribaudo *et al.*, 2006). Transcripts encoding for putative annotated proteins and having a CleaveLand category of 0, 1 or 2 were selected for qRT-PCR. Among the grapevine transcripts targeted by the GfKV vsiRNAs, TC109765, TC107930 and TC107032 were found to be significantly down-regulated to levels of c. 56%, 60% and 63%, respectively, of the corresponding genes in healthy plants (Fig. 2A). The levels of the transcripts EE103699, TC118037,

TC105673 and TC110551 were not affected significantly by the viral infection (Fig. 2A). Among the grapevine transcripts targeted by GRSPaV, TC110069, TC107140 and TC127300 were found to be significantly down-regulated to levels of c. 43%, 80% and 66%, respectively, of the corresponding transcripts in uninfected plants (Fig. 2A). Levels of TC112924, TC109537, TC111405, TC109075, TC10723 and TC125753 remained unaffected, whereas those of transcripts TC110936 and TC118235 were found to be up-regulated (Fig. 2A).

To further provide evidence supporting the idea that the down-regulation of transcripts can be associated with vsiRNA-mediated cleavage at the expected sites, as reported in Table 1, a 5'-RACE analysis was carried out on the RNA used for qRT-PCR. The transcripts were selected within those whose sequence was sufficiently represented in the grapevine expressed sequence tag (EST) dataset (see Experimental procedures) and, in addition, those significantly reduced in expression level (i.e. TC127300, TC110069, TC109765 and TC107930; Fig. 2A). The 5'-RACE approach revealed gene-specific products of the expected size for only TC107930 and TC110069, targeted by GRSPaV vsiR5071(-) at position 485 and by GfKV vsiRNA5439(-), respectively (Table 1), only in the infected plant. 5'-RACE analysis succeeded in identifying uncapped ends at the expected positions for both transcripts (grey arrows in Fig. 2B,C). Moreover, we identified additional uncapped 5'-ends downstream to the expected vsiRNA-mediated cleavage sites (black arrows), which are discussed later.

Analysis of vsiRNAs targeting viral genomes

Both GfKV and GRSPaV possess single positive (+)-stranded genomic RNAs with a poly(A) tail at their 3'-ends. In addition, they are thought to be M7G capped at their 5'-ends (Ghanem-Sabanadzovic *et al.*, 2003; Martelli and Jelkmann, 1998; Martelli *et al.*, 2002; Meng *et al.*, 1998; Sabanadzovic *et al.*, 2001). These characteristics prompted us to search for virus-derived uncapped 5'-remnants within the degradome database (Fig. 1B).

We found 69 and 362 5'-end unique uncapped sequences (995 and 3778 redundant) for GfKV and GRSPaV, respectively. As shown in Fig. 3, the sequences were nonuniformly distributed along the viral genomes, but were mainly concentrated in the 3'-proximal region; however, this was probably the consequence of the method used for the identification of 5'-end unique uncapped sequences (degradomes), as we analysed only viral RNA fragments with a poly(A) tail. Viral RNA fragments without poly(A) have not been seen using this approach.

After the identification of 5'-end uncapped sequences, we used the CleaveLand pipeline to correlate the pool of vsiRNAs (both 21 and 22 nucleotides) with the datasets of GfKV and GRSPaV degradomes in order to identify the putative vsiRNA-mediated cleavage sites (Fig. 1B). In the case of GfKV, 15 of 69 unique cleavages were

Fig. 2 Quantitative real-time polymerase chain reaction (qRT-PCR) analysis of the expression of transcripts targeted by virus-derived small interfering RNAs (vsiRNAs) originating from *Grapevine fleck virus* (GFkV) and/or *Grapevine rupestris stem pitting-associated virus* (GRSPaV) in healthy (white bars) and infected plants (grey bars). For each gene, asterisks mark significant differences ($*P < 0.05$; $**P < 0.01$) of infected relative to healthy plants (A). 5'-Rapid amplification of cDNA ends (5'-RACE) analysis in the context of the infected plant revealing the 5' uncapped remnants of the transcripts TC107930 (B) and TC110069 (C) targeted by vsiRNAs from GFkV and GRSPaV, respectively. Grey arrows indicate the expected cleavage sites. The symbol '0' indicates a noncanonical pairing G and U.

Fig. 3 Distribution of 5'-uncapped ends along the viral genomes of *Grapevine fleck virus* (GFkV) (A) and *Grapevine rupestris stem pitting-associated virus* (GRSPaV) (B).

explained by 23 different vsiRNAs (Fig. 4A); these vsiRNAs represented only 3.2% of the GFkV-derived siRNAs. The 15 selected cleavage sites were located in nine different loci along the viral genome (Fig. 4A). For GRSPaV, we identified 21 of 362 unique cleavage sites located in 12 different loci (Fig. 4B), and the corresponding 27 vsiRNAs (3.7% of the total GRSPaV vsiRNAs), which are probably responsible for these cleavages. As we analysed the positive strand of viral genomes, only antisense vsiRNAs were identified to mediate cleavages. The procedure was accompanied by an estimation of the false positives (see Experimental procedures), which resulted in values of 0.3 for GFkV and 0.36 for GRSPaV. Despite the presence of several vsiRNA hotspots, only a few associated with cleavage sites.

5' -Terminal nucleotide bias of the cleaving vsiRNAs

vsiRNA-driven cleavage events require the incorporation into AGO-containing cleavage effector complexes. In *A. thaliana*, the sorting of siRNAs into AGOs is mainly determined by the 5'-terminal nucleotide: AGO1 preferentially incorporates RNAs bearing a 5'-terminal U, whereas AGO2 and AGO4 select those having a terminal A, and AGO5 those with a terminal C (Mi *et al.*, 2008; Takeda *et al.*, 2008). Thus, given a conserved mechanism of siRNA incorporation into AGOs among different plant species (i.e. *A. thaliana* and *V. vinifera*), the analysis of the 5'-terminal nucleotides of those vsiRNAs cleaving viral genome or plant transcripts would provide an indication of the AGOs involved in such phe-

Fig. 4 Distribution of virus-derived small interfering RNA (vsiRNA)-directed cleavages along the viral genomes of *Grapevine fleck virus* (GfKv) and *Grapevine rupestris stem pitting-associated virus* (GRSPaV) (A and B, respectively). Bottom: alignments of vsiRNAs with viral target sequences. Arrows indicate positions of cleavage sites. vsiRNAs are named with the position of the first base at their 5'-end. nt, nucleotide.

nomena. vsiRNAs from GfKv and GRSPaV driving cleavage on the viral genome (Fig. 4) or targeting grapevine transcripts (Table 1) were grouped by the first nucleotide (U, C, G and A), and their relative abundance was compared with values in the input dataset of vsiRNAs. In the case of viral genome targets, vsiRNAs from the viral region including cleavage sites (i.e. region 6450–7564 for

GfKv and 7050–8744 for GRSPaV) were taken into consideration. The significance of the enrichment was estimated using Fisher's exact test.

Among the pool of vsiRNAs from GfKv targeting *V. vinifera* transcripts, a significant enrichment of vsiRNAs bearing a 5'-terminal adenosine was observed ($P = 8 \times 10^{-4}$) (Fig. 5A).

Fig. 5 (A,B) 5'-Terminal nucleotide enrichment of virus-derived small interfering RNAs (vsiRNAs) originating from *Grapevine fleck virus* (GfKV) (A) and *Grapevine rupestris stem pitting-associated virus* (GRSPaV) (B) and targeting the *Vitis vinifera* transcripts. (C,D) 5'-Terminal nucleotide enrichment of vsiRNAs originating from GfKV (C) and GRSPaV (D) and targeting the viral genomes. Asterisk indicates statistically significant differences (Fisher's exact test); black bars, all vsiRNAs; grey bars, targeting vsiRNAs.

Conversely, among the pool of vsiRNAs from GRSPaV, a significant enrichment of vsiRNAs bearing a 5'-terminal uridine was observed ($P = 8 \times 10^{-4}$) (Fig. 5B).

Among the pool of vsiRNAs targeting the GfKV viral genome, a significant enrichment of vsiRNAs with a U at their 5'-ends ($P = 5.2 \times 10^{-5}$) was observed (Fig. 5C), although the enrichment for G ($P = 8.3 \times 10^{-15}$) at the 5'-end was even higher than for U (Fig. 5C). Conversely, in the case of GRSPaV, a significant enrichment for C at the 5'-end ($P = 1.1 \times 10^{-8}$) was observed (Fig. 5D). Given that different AGO proteins may have a 5'-nucleotide-based affinity for siRNAs, our findings suggest that grapevine-targeting siRNAs might incorporate into AGO complexes different from those involved in antiviral activity.

DISCUSSION

High-throughput technologies are thought to facilitate the identification of authentic RNA targets of siRNAs on a genome-wide scale. Indeed, recently, we have generated a 5'-RACE uncapped mRNA library (degradome library) and short RNA libraries from *V. vinifera* tissues. The analysis of the libraries with the computational algorithm CleaveLand version 2 allowed us to reveal several mRNAs targeted by conserved and novel *V. vinifera* miRNAs (Pantaleo *et al.*, 2010b). *Vitis vinifera*, a woody plant cultivated and selected together with its viruses for centuries, approximates to a natural plant–virus system, and vsiRNAs from several viruses, including GfKV and GRSPaV, have been characterized recently from our libraries (Pantaleo *et al.*, 2010a).

The availability of databases containing the uncapped transcripts (degradome) and the dataset of vsiRNAs generated from the same clone of *V. vinifera* (Pantaleo *et al.*, 2010a,b) allowed us to investigate further whether some vsiRNAs could be associated with the presence of uncapped transcripts. To this end, we adopted the CleaveLand pipeline (Addo-Quaye *et al.*, 2009) and *V. vinifera* degradome data to find cleaved vsiRNA targets. From the viruses identified in our previous study (Pantaleo *et al.*, 2010a), we restricted the investigation to the vsiRNAs derived from the two positive-strand RNA viruses GfKV and GRSPaV.

Importantly, we identified several vsiRNAs from both GfKV and GRSPaV which probably target specific grapevine transcripts. Indeed, transcripts encoding components of the large 60S ribosomal subunit (i.e. proteins l18a-1, l10a and l26) or of the small 40S ribosomal subunit (i.e. protein s19), and transcripts encoding other factors linked to ribosome biogenesis (i.e. eIF6), were found to be uncapped in the degradome database. Strikingly, these uncapped transcripts were probably generated by specific vsiRNAs targeting ribosomal protein (RP) mRNAs (Table 1).

Similar to miRNA-mediated post-transcriptional regulation of mRNAs, vsiRNA-mediated cleavage results in down-regulated levels of the targeted mRNAs. As for miRNAs, this assumes that miRNA and target expression domains coincide in the sampled tissues. These requirements may make it difficult to validate experimentally vsiRNA-mediated cleavages for plant systems in which viruses are not uniformly distributed (i.e. GfKV– or GRSPaV–grapevine) or for nonstable cleavage products. Such obstacles may be overcome by a wide genomics approach, such as

the degradome used in this work. Accordingly, the traditional 5'-RACE approach was able to reveal the 5'-remnants of vsiRNA-mediated cleavage only for some transcripts. The 5'-uncapped species at position 485 of TC107930 (encoding for the 60S ribosomal protein l18a-1, Table 1A) and at position 739 of TC110069 (encoding for the elongation factor 6) were identified (grey arrows in Fig. 2B,C). In Fig. 2B,C, black arrows indicate the positions found downstream of the vsiRNA-mediated cleavages for both transcripts. These findings can be explained by the cellular exonuclease activity after the vsiRNA-mediated cleavages; alternatively, the generation of these uncapped RNA ends may not be related to the vsiRNA-generated cleavages (Gy *et al.*, 2007; Souret *et al.*, 2004). With regard to the cleavage sites, and given the vsiRNA/mRNA target alignments (Fig. 2B,C), we observe what is expected, i.e. a vsiRNA-guided cleavage site between the nucleotides pairing to residues 10 and 11 of the siRNA (Elbashir *et al.*, 2001; Hutvagner and Zamore, 2002; Kasschau *et al.*, 2003; Pantaleo and Burgyn, 2008; Shimura *et al.*, 2011).

In addition to the so-called 'tuning targets' of miRNAs, i.e. miRNA-mediated cleavage results in a down-regulation of the mRNA, there are also 'neutral targets' for which down-regulation by miRNAs is tolerated or, at least, negated by feedback processes (reviewed in Bartel, 2004 and Voinnet, 2009). Therefore, we can speculate whether transcripts TC110936 and TC118235 belong to such classes of mRNAs when targeted by vsiRNAs. However, we cannot exclude the possibility that either GFKV or GRSPaV, once infecting grapevine cells, could induce an over-expression of these transcripts in a non-RNA silencing-related pathway.

The plant-invading viral RNAs compete with other cellular mRNA translation machinery (i.e. cap-binding complexes, ribosomes and other factors) to be translated into viral proteins, including RNA replication factors. The fact that both GFKV and GRSPaV can produce vsiRNAs targeting specific components of cytoplasmic ribosomes seems to be incongruous with the translational step of viral genomic RNA, which is required, at least, for viral replication. However, deletions of yeast genes *YOR312C* (RPL20B, similar to 60S RP l18a-1 from grapevine transcript TC107930), *YGR034W* (RPL26B, similar to 60S RP l26B from grapevine transcript TC111405), *YMR242C* (RPL20A, similar to 60S RP l18a-1 from grapevine transcript EE105806) and *YOL121C* (RPL19S, similar to 40S RP s19 from grapevine transcript TC112924) have been shown previously to stimulate *Brome mosaic virus* (BMV)-directed reporter gene expression (Kushner *et al.*, 2003). Furthermore, a previous gene expression study has shown that, in response to *Turnip mosaic virus* (TuMV) infection, most *Arabidopsis* RP mRNAs (including *Arabidopsis* homologues to *V. vinifera* RPl18a-1, RPl10a-1 and RPs19) are strongly induced (Yang *et al.*, 2007). These findings suggest that the replicating virus tries to down-regulate RPs using vsiRNAs, which may lead to a decrease in the expression of plant proteins involved in the antiviral response (i.e. AGO1, which is up-regulated on virus

infection, as reported recently) (Varallyay *et al.*, 2010; Yang *et al.*, 2007).

The RPl18 protein in mammals has been proposed to prevent protein kinase R (PKR) activation by dsRNA (Kumar *et al.*, 1999), and dsRNAs from viruses stimulate the phosphorylation of PKRs, thus representing a possible triggering event in virus pathogenesis in plants and mammals (Langland *et al.*, 1995). This possible virus-induced down-regulation of RPl18 may represent a new layer of plant-virus interaction, and can be considered functional to facilitate viral infection.

The annotation of putative proteins from grapevine transcripts is still underway and is still a limiting factor in order to obtain an exact classification into functional categories. However, among the proteins encoded by grapevine transcripts targeted by GFKV and GRSPaV vsiRNAs, many contain conserved motifs in common with protein factors involved in signalling or resistance responses to viruses (Table 1).

Recent discoveries strongly suggest that host gene targeting by vsiRNAs does not occur fortuitously and, instead, represents a bona fide viral strategy to control host gene expression. Our analysis suggests that many plant factors can be targeted by vsiRNAs, including resistance proteins, kinase proteins, transcription factors, F-box proteins and pathogen-related (PR) proteins (reviewed by Carr *et al.* 2010). They can have a direct or indirect effect on viral fitness, and viruses can, in turn, modulate these factors through the harnessing of plant RNA silencing machinery to usurp host cells. These results indicate the multiple layers of plant-virus interaction. In particular, our findings show that RNA silencing has an important role not only in the antiviral response, but also in the facilitation of virus invasion through the control of plant gene expression.

EXPERIMENTAL PROCEDURES

Datasets used for analysis

Grapevine transcripts (VVGI release 7.0) were downloaded through the DFCI Gene Index Project (<http://compbio.dfci.harvard.edu/tgi/>). The viral short RNA datasets were retrieved from GEO (<http://www.ncbi.nlm.nih.gov/geo/>) via accession number GSE18405 (Pantaleo *et al.*, 2010a). The uncapped 5'-end RNA dataset originating from plant tissues, described previously in Pantaleo *et al.* (2010b), was retrieved from the GEO database, accession number GSE18406 (Pantaleo *et al.*, 2010a,b).

The PatMaN alignment tool (Prufer *et al.*, 2008) was used to select short RNAs and uncapped 5'-end sequences exactly matching the genomic sequences of GFKV (GenBank accession number NC_003347) and GRSPaV (GenBank accession number NC_001948).

Identification of short RNA targets

The identification of grapevine mRNAs targeted by vsiRNAs was performed using CleaveLand 2.0.0 (Addo-Quaye *et al.*, 2009). Input datasets

Table 2 Oligonucleotides used for quantitative real-time polymerase chain reaction (qRT-PCR).

Target ID	Target description	Primer	Primer sequence 5'–3'
EE103699	60S ribosomal protein l10a-1	F	AGCGTTTTAGTGGGTCTGTGAAG
		R	AGCATCACCAAGCATGCAGAT
TC105673	Thaumatin-like protein	F	CATCATCAATTCGCTGCCTCTT
		R	TAACAGAACGATGGGCTACAAGTAA
TC107032	S2P-M50 metalloprotease	F	CATTGGGAGATGACAGGTATGC
	protein family	R	GAGAATGTCCCTCCACCATAGG
TC107930	60S ribosomal protein l18a-1	F	TCTTCTCTCATCACACCACACA
		R	ACCATTCTGGAGGGGAGAGA
TC118037	60S ribosomal protein l18a-1	F	TGGGTGCGGTAGTGTCTCCTTT
		R	GGACTCTCCCAATTGTCTAGGTT
TC109765	PsbY precursor (psbY)	F	AAGCATTTGGCGTTGAGCAT
		R	AAAGCACACAAGCAACATCATCA
TC110069	Eukaryotic translation initiation	F	AGCTACTGCACCTTTTCCAACAG
	factor 6	R	CCTGAAGGAGTGTGAAAGTTCATC
TC111405	60S ribosomal protein l26	F	AACCCGTCTAAGGTGGTGATCA
		R	GAGGAGGCTCTTGCGATCCT
TC112924	40S ribosomal protein s19	F	GGTGAAGCAAGAGGAATGG
		R	TGTGGCGAGCAATAGAACCA
TC107140	Peroxisomal membrane protein	F	GGAGAGAGTCGCCAAGAAAGTG
		R	CTGTAGAATCTGAAGAAGCGTGCTA
TC109537	Vacuolar protein sorting 55	F	GATTGAGTTCTTCTCTTCTCGTA
	domain-containing protein	R	GCTCGTTCACCATTCATCTTCA
TC107235	Signal peptide peptidase-like 3	F	GGAGTTGTCTGCGGTAA
		R	ATAGCCATGTACCAAGACCAA
TC109075	Elav-like protein4	F	GATGCCGCTGAAGGTATAAAGG
		R	CTCTGCAATATCACCAACCA
TC110936	Aconitase C-terminal	F	CCATGGCGGCTTCAACAG
	domain-containing protein	R	AAGACGTGAGGTAGGGTTTCCT
TC125753	Succinate dehydrogenase	F	CAGTCCATGGTGCCAAATCG
	flavoprotein α subunit	R	CCGACCAAAAACAACATGTCA
TC127300	Kinesin-like protein	F	CCGTCAATGGAGGCACTGA
		R	GAATTGGGTGCCGAAGCTT
TC118235	Protein kinase-interacting	F	CGATGCCACCGACACTTTT
	protein	R	GCTGAGGGCGTTTTGGTAGT
TC110551	nc domain-containing (putative	F	CCCATCAACCTGTTCAACCTTT
	chaperone)	R	GGACAACCCCACTGTTTGGT
XM_002282480	Actin	F	GCCCCTCGTCTGTGACAATG
		R	CCTTGGCCGACCCACAATA
XM_002273532	Ubiquitin	F	TCTGAGGCTTCGTGGTGGTA
		R	AGGCGTGCATAACATTGCG

F, forward primer; R, reverse primer.

were the grapevine transcripts (VVGI release 7.0) and the pool of vsiRNAs of 21 and 22 nucleotides in length (Fig. 1A).

The same pipeline was used to identify cleavage sites along the viral genomes targeted by vsiRNAs. In this case, the input datasets were the population of vsiRNAs and the genomic sequence of the corresponding virus (Fig. 1B).

The significance of each cleavage site was evaluated according to the procedure implemented in CleaveLand 2.0, with a number of 100 random shuffles (default setting). As the *P* value obtained is linked to the alignment score, we defined a cut-off on the *P* value. Only cleavage sites with $P \leq 0.05$ were considered to be statistically significant. Each cleavage site was categorized according to the categorization system implemented in CleaveLand 2.0: category 0 when the number of raw degradome reads at the cleavage position was equal to the maximum on the transcript and there was only one maximum position on the transcript; category 1 if the number of raw reads was equal to the maximum, but there was more than one maximum on the transcript; category 2 when the number of raw reads was less than the maximum, but higher than the

median; category 3 if the number of reads was equal to or less than the median; category 4 if only one read was detected at the cleavage position.

Evaluation of the percentage of false positives (PFP)

The PFP among the cleaving vsiRNAs was estimated using datasets of vsiRNAs randomly originated from the viral genomes. From each viral genome, a list of all possible virus-derived siRNAs was originated, and randomized datasets of vsiRNAs containing the same number of short sequences as the true dataset were originated independently. As a result of the high computational time needed for each run, only 10 randomized datasets were used. For each random dataset, the number of cleaving vsiRNAs was recorded. PFP was then calculated as the ratio between the number of cleaving vsiRNAs obtained from the randomized datasets and the true cleaving vsiRNAs. The overall PFP was calculated as the average over all the obtained PFPs.

Other bioinformatics analyses

Further analyses were performed by a mix of *ad hoc* custom Perl scripts. Visualization tools 'short_read_gbrowse_image.pl' and 'short_read_his_togram.pl', developed by T. J. Hardcastle (Department of Plant Science, University of Cambridge, UK) and available at the webpage <http://people.pwf.cam.ac.uk/tjh48/>, were used to visualize the distributions of the reads along the viral genomes.

Relative qRT-PCR and 5'-RACE analysis

Three healthy plants from somatic embryos and three grapevines co-infected by GfKV and GRSPaV were analysed (Gribaudo *et al.*, 2006). The viral status of these plants was confirmed by multiplex RT-PCR (Gambino and Gribaudo, 2006). Primers for qRT-PCR (Table 2) were designed using the software Primer Express 3.0 (Life Technologies Corporation, Carlsbad, CA, USA) corresponding to the cleavage sites predicted (TC109537, TC105673, EE103699), downstream of the cleavage site (TC118037) or upstream of the cleavage site (the remaining). Relative qRT-PCR was performed as described previously (Gambino *et al.*, 2011). Specific annealing of the primers was controlled on the dissociation kinetics performed at the end of each PCR run. Gene expression was expressed as the mean and standard error calculated over three biological and three technical replicates. Expression data were subjected to *t*-test statistical analysis.

5'-RACE analysis was performed as described previously (Shimura *et al.*, 2011) on the polyadenylated fraction obtained from total RNA. Polyadenylated RNA was obtained with an Oligotex mRNA mini kit (Qiagen, Hilden, Germany) following the manual instructions. Gene-specific reverse oligonucleotides for PCR and nested PCR were designed downstream of the cleavage sites on the TC107930 and TC110069 sequences, and their sequences are reported here: GSP TC107930 rev 5'-CAAGTTGGGCC TTGATGCTTTGTAGGTGG-3'; GSP TC107930 rev nested 5'-TAGGTGG TCTTGAGCTTCCTGGTTGG-3'; GSP TC110069 rev 5'-CAGACATAGCTGTC AATCAACGATTCTCTC-3'; GSP TC110069 rev nested 5'-TCCTCATCTCATC CACAATGGCAC-3'.

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