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viral silencing suppressors: Tools forged to fine-tune host-pathogen coexistence

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ABSTRACT

RNA silencing is a homology-dependent gene inactivation mechanism that regulates a wide range of biological processes including antiviral defense. To deal with host antiviral responses viruses evolved mechanisms to avoid or counteract this, most notably through expression of viral suppressors of RNA silencing. Besides working as silencing suppressors, these proteins may also fulfill other functions during infection. In many cases the interplay between the suppressor function and other "unrelated" functions remains elusive. We will present host factors implicated in antiviral pathways and summarize the current status of knowledge about the diverse viral suppressors' strategies acting at various steps of antiviral silencing in plants. Besides, we will consider the multi-functionality of these versatile proteins and related biochemical processes in which they may be involved in fine-tuning the plant-virus interaction. Finally, we will present the current applications and discuss perspectives of the use of these proteins in molecular biology and biotechnology.

Contents

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Plant viruses

Plant viruses are amongst the most important pathogens causing huge economic losses worldwide by reducing crop quality and quantity. A better understanding of the viral infection processes and plant defense strategies is important for crop improvement.

Based on their genome organization viruses can be classified into positive-sense-, negative-sense-, double-stranded-RNA viruses and single-stranded or double-stranded DNA viruses (Hull, 2002). Difference in the genome organization implies difference in the replication strategy. Generally, the genetic information embedded into the viral RNA or DNA encode for a surprisingly restricted number of proteins that coordinate the infection process. Viral proteins interact with host factors to manipulate biochemical events and molecular interactions required for the virus replication and movement. Viruses can spread within the plants through plasmodesmata on short distance (cell-to-cell movement) or through phloem (systemic movement). During host-pathogen co-evolution a set of complex interactions involving virus attack and host defense has been developed. These include hypersensitive reaction (HR) (Mandadi and Scholthof, 2013), systemic acquired resistance (SAR) (Kachroo and Robin, 2013), activation of ubiquitin/26S proteasome system (UPS) (Dielen et al., 2010) or RNA silencing (RNA interference, RNAi) (Pumplin and Voinnet, 2013).

RNA silencing pathways in plants

RNA silencing is a fundamental genetic regulatory mechanism conserved in eukaryotic organisms. RNAi can act at transcriptional (Transcriptional Gene Silencing, TGS) or at post-transcriptional levels (Post-Transcriptional Gene Silencing, PTGS), and has many diverse roles including developmental regulation, stress response or defense against invading nucleic acids like transposons or viruses. The antiviral function of RNA silencing was demonstrated in plants and invertebrates (Bronkhorst and van Rij, 2014; Pumplin and Voinnet, 2013), however recent reports have further provided evidence for a similar function in mammals (Cullen et al., 2013; Li et al., 2013; Maillard et al., 2013).

Mechanistically, the RNA silencing process consists of initiation phase, effector phase and amplification phase. During silencing initiation double-stranded RNAs (dsRNA) of different origins are processed by an RNase III type enzyme Dicer (DCR, in plants DICER-LIKE proteins, DCLs) into short, 21–24 nt long, small RNA (sRNA) duplexes (Bernstein et al., 2001; Hamilton and Baulcombe, 1999; Hutvagner et al., 2001). DICERs require DOUBLE-STRANDED RNA BINDING (DRB) proteins for accurate sRNA production (Eamens et al., 2012a,b; Hiraguri et al., 2005). The sRNAs are stabilized at their 3′ end by the HUA Enhancer 1 (HEN1)-dependent methylation (a process found in plants and flies so far (Boutet et al., 2003; Yang et al., 2006) and exported from nucleus by HASTY (HST) (Park et al., 2005; Peragine et al., 2004) to be loaded onto Argonaute proteins (Fagard et al., 2000; Hammond et al., 2001; Liu et al., 2004), the effectors of the RNA-Induced Silencing Complex (RISC) (Lee et al., 2004; Pham et al., 2004; Tomari et al., 2004) or RNA Induced Transcriptional Silencing complex (RITS) (Ekwall, 2004). Guided by the sRNA sequence, RISC induces slicing or translational repression of its target RNAs (during PTGS) in a sequence-specific manner (Kim et al., 2014), whereas RITS complex causes histone and/or DNA methylation, resulting in transcriptional gene silencing (TGS) of the homologous gene (Creamer and Partridge, 2011). In plants and worms the effector step can result in amplification of silencing response involving RNA-dependent RNA polimerases (RDRs) proteins (Mourrain et al., 2000; Dalmay et al., 2000; Sijen et al., 2001; Vaistij et al., 2002; Voinnet et al., 1998). Amplification of RNA silencing has been implicated in the spread of an RNA silencing signal, a non-cell-autonomous process (Kalantidis et al., 2008; Schwach et al., 2005).

The best studied plant model, *Arabidopsis thaliana* genome encodes 4 members of DCLs (DCL1-4) (Bologna and Voinnet, 2014), five DRBs (HYL1/DRB1, DRB2, 3, 4, 5) (Hiraguri et al., 2005), 10 AGOs (AGO1-10) (Mallory and Vaucheret, 2010) and 6 RDRs (RDR1, 2, 3a, 3b, 3c and 6) (Wassenegger and Krczal, 2006). These proteins have partially redundant roles and combine with each other to result in divers classes of small RNAs and different effector outputs of the RNA silencing pathways. The small RNA classes identified in plants include microRNAs (miRNAs), trans-acting small interfering RNAs (ta-siRNAs), natural-antisense RNAs (nat-siRNAs), repeat-associated siRNAs (ra-siRNAs), viral siRNAs (vsiRNAs) and virus-activated siRNAs (vasiRNAs). These classes possess specialized roles during development, stress responses, heterochromatic silencing, viral infection and host-pathogen interplay, respectively (Bologna and Voinnet, 2014).

Antiviral silencing host factors

Initiation of antiviral silencing

The hallmark of the antiviral silencing response is the Dicer-dependent production of viral siRNAs (vsiRNAs) (Hamilton and Baulcombe, 1999). In uninfected cells long dsRNA is not detectable, however upon virus infection, viral dsRNA molecules of different sources becomes available. Highly structured regions of viral single-stranded RNAs (ssRNA), replicative intermediates (RI) or overlapping bidirectional read-through transcripts from DNA virus genome may all contribute to vsiRNA production (Aregger et al., 2012; Blevins et al., 2011; Donaire et al., 2008; Molnar et al., 2005). Although the viral dsRNA structures are likely accessible to all of the DCLs, a strong hierarchy exists between them regarding vsiRNA production: RNA virus infections are mainly affected by DCL4, while DCL2 becomes critical in a *dcl4* mutant background (Deleris et al., 2006; Donaire et al., 2008; Garcia-Ruiz et al., 2010; Qu et al., 2008) (Fig. 1). DCL3 has only a minor role against RNA viruses (Qu et al., 2008; Raja et al., 2008). Recent report suggests additional functional diversity between DCL4 and DCL2, such as that DCL2 stimulates transitivity and secondary siRNA production, while DCL4 is sufficient for silencing on its own (Parent et al., 2015). The fact that silencing suppressors of RNA viruses interfere with DCL3 pathway suggests it could have important antiviral gene regulatory functions (Azevedo et al., 2010; Hamera et al., 2012; Lacombe et al., 2010). DCL3 is essential against DNA viruses (Akbergenov et al., 2006) and works presumably by inducing DNA methylation (Blevins et al., 2006; Raja et al., 2014) (Fig. 1). Finally, DCL1 acts as a positive regulator in the production of vsiRNAs by making viral dsRNAs available to other DCLs both in RNA and DNA virus infections (Blevins et al., 2006; Moissiard and Voinnet, 2006) but also as a

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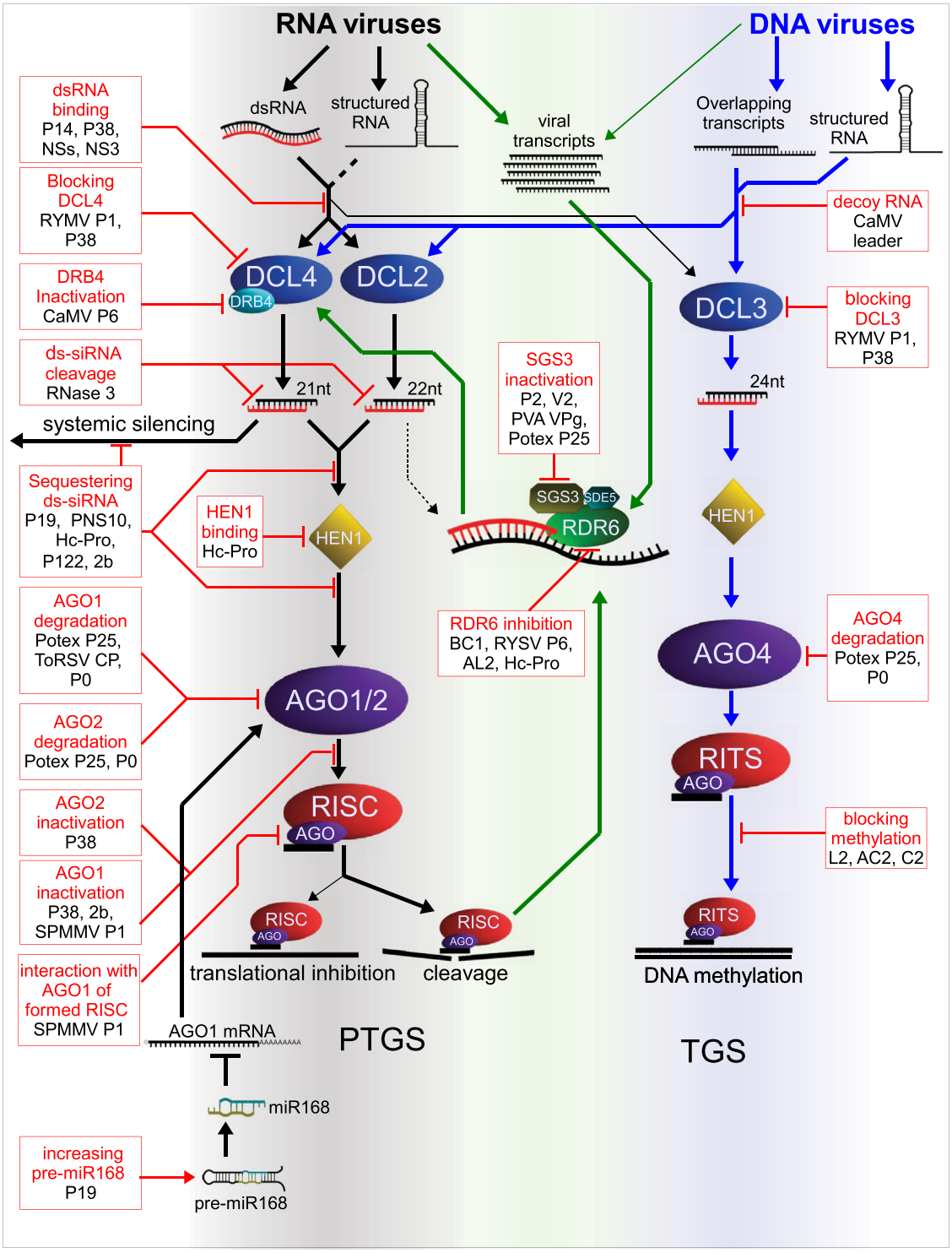

Fig. 1. The model of antiviral RNA silencing and the diverse suppressor strategies that interfer with the pathway. Silencing response against RNA viruses is initiated by DCL4 and DCL2 through production of 21–22 nt viral siRNAs and to a lesser extent DCL3 (DCL1 that also has roles in antiviral defense was omitted for simplicity, please see main text). Viral siRNAs are methylated by HEN1 and subsequently loaded into AGOs. AGO1 and AGO2 are the two main effectors, but AGO5 and 7 may also have antiviral roles. Loaded-AGO containing RISC complexes complete the effector step of post-transcriptional gene silencing (PTGS) through viral RNA cleavage or translational inhibition (left side). AGO-sliced products, aberrant RNAs or aborted viral transcripts serve as templates for RDR complexes to amplify the antiviral response. SGS3 and SDE5 cofactors are required for RDR6 activity. (RDR1 and RDR2 also have antiviral functions, omitted for simplicity). RDR activities may be primed by DCL2-derived 22 nt vsiRNAs (middle). In DNA virus infections DCL4 and DCL3-derived viral siRNA initiate PTGS or transcriptional gene silencing (TGS) respectively. (DCL1 recognizes highly folded structures like the CaMV 35S leader, omitted for simplicity). DCL3-generated 24 nt vsiRNA following the HEN1-methylation are loaded into AGO4. AGO4-RITS causes the hypermethylation of viral genome to complete TGS (right side). DNA-virus transcripts are poor templates for RDR-based amplification process. The diverse viral suppressors inhibiting PTGS and/or TGS are shown in black boxes along the sides of the figure. The dashed lines point to the interference place where the VSRs interact with the antiviral pathway.

negative regulator limiting DCL4 and DCL3 through miRNA pathway (Azevedo et al., 2010; Qu et al., 2008). DCLs interact with DRBs to produce small RNAs. DCL4 partner DRB4 is important in antiviral defense against RNA viruses *Turnip crinkle virus* (TCV) (Curtin et al., 2008) or *Turnip yellows mosaic virus* (TYMV) (Jakubiec et al., 2012). Evidence show that *Cauliflower mosaic virus* (CaMV) suppressor P6

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Table 1
The summary of identified VSRs, their mode of suppression and alternative functions. For suppressors encoded by different viruses within the genus and having the same name (*e.g.* cucumoviral 2b, potyviral HC-Pro etc.) the alternative functions are displayed in a combined manner. For details on each individual function please see the references within.

Genus	Species	VSR	Function	Reference
Phytoreo virus	RDV	PNS10	siRNA binding, RDR6 downregulation	Ren et al. (2010) and Cao et al. (2005)
	RGDV	PNS11	Unknown, miRNA pathway interference	Shen et al. (2012) and Liu et al. (2008)
	RGDV	PNS12	Unknown (nucl. localization)	Guo et al. (2011) and Wu et al. (2011)
Oryza virus	RRSV	PNS6	Unknown	Wu et al. (2010)
Tospo virus	TSWV, GBNV	NSs	siRNA, miRNA and dsRNA binding, avirulance factor	Zhai et al. (2014), Ronde et al. (2014), Schnettler et al. (2010) and Goswami et al. (2012)
Cucumo virus	CMV, TAV	2b	sRNA binding, AGO1 binding, AGO4 binding, aphid interaction, RDR6 downregulation, AGO1 downregulation via miR168 upregulation, downregulation of AGOs and DCL1, impairing 5′ secondary siRNA genesis, cell-to-cell movement, interaction with CAT3, induction of host drought resistance, SA/JA pathway disruption, HR elicitor	Nemes et al. (2014), Du et al. (2014), Gonzalez et al. (2012), Duan et al. (2012), Diaz-Pendon et al. (2007), Lewsey et al. (2007), Ahn et al. (2010), Chen et al. (2008), Zhang et al. (2006), Feng et al. (2013), Gonzalez et al. (2012), Hamera et al. (2012), Westwood et al. (2013a,b), Ziebell et al. (2011), Zhang et al. (2008), Inaba et al. (2011), Lewsey et al. (2010), Várallyay and Havelda (2013), Zhou et al. (2014), Ji and Ding (2001) and Li et al. (1999)
Ilarvirus	AV-2	2b	Unknown (no local suppression)	Shimuran et al. (2013)
Como virus	CPMV	CP (S)	Unknown	Canizares et al. (2004)
Nepo virus	ToRSV	CP	Mediating AGO1 degradation	Karran and Sanfacon (2014)
Rymo virus	AgMV, HoMV	Hc-Pro	unknown	Young et al. (2012)
Poty virus	TEV, ZYMV, PVY, TuMV, SCMV, SMV, TVY, PVA, PRSV	Hc-Pro	ds-siRNA binding, blocking HEN1 methyltransferase, HEN1 binding, blocking primary siRNA biogenesis by RAV2 interaction, RDR6 downregulation, impairs 3′ secondary siRNA genesis, JA pathway disruption, SAHH-interaction, Hip2 interaction, PaCRT interaction, ferredoxin-5 interaction, NtMinD interaction, interfering with miRNA pathway, interference with host gene expression levels, AGO1 downregulation via miR168 upregulation	Sahana et al. (2014), Torres-Barcelo et al. (2010), –Ruiz et al (2010), Torres-Barcelo et al. (2008), Goto et al (2007), Shiboleth et al. (2007), Yu et al. (2006), Merai et al. (2006), Dunoyer et al. (2004), Llave et al. (2000), Mallory et al. (2001), Lozsa et al. (2008), Ebhardt et al. (2005), Jamous et al. (2011), Endres et al. (2010), Zhang et al. (2008), Seo et al. (2010), Westwood et al. (2014), Canizares et al. (2013), Haikonen et al. (2013a), Shen et al. (2010), Cheng et al. (2008), Jin et al. (2007), Chapman et al. (2004), Kasschau et al. (2003), Soitamo et al. (2011) and Várallyay and Havelda (2013)
	PPV	HcPro-P1	Unknown	Valli et al. (2006)
	PVA	VPg	SGS3 interaction	Rajamäki et al. (2014) and Rajamäki and Valkonen (2009)
Tritimo viruses	WSMV, ONMV	P1	Unknown	Young et al. (2012)
Poace virus	TriMV, SCSMV	P1	Unknown	Tatineni et al. (2012)
Ipomo virus	SPMMV	P1	AGO binding	Szabo et al. (2012) and Giner et al. (2010)
	CVYV	P1b	21 and 22 nt siRNA binding, protease activity, putativ Zn-finger	Valli et al. (2011) and Valli et al. (2008)
Tombus virus	CymRSV, CIRV, TBSV	P19	ds-sRNA binding, interfering with sRNA 3' methylation, mir168 upregulation mediated AGO1 downregulation, HR elicitor, Hin19 interaction, ALY interaction	Law et al. (2013), Rawlings et al. (2011), Cheng et al. (2009), Xia et al. (2009), Koukiekoloa et al. (2007), Merai et al. (2006), Lakatos et al. (2006), Omarov et al. (2006), Havelda et al. (2005), Dunoyer et al. (2004), Silhavy et al. (2002), Ye et al. (2003), Vargason et al. (2003), Lozsa et al. (2008), Yu et al. (2006), Chapman et al. (2004), Varallyay et al. (2010, 2014), Várallyay and Havelda (2013), Angel and Schoelz (2013), Hsieh et al. (2009), Park et al. (2004) and Uhrig et al. (2004)
	CNV	P20	Unknown	Hao et al. (2011)
Aureus virus	PoLV	P14	dsRNA binding	Merai et al. (2005, 2006)
Carmo virus	TCV	P38	AGO1 and 2 binding, DCL1 upregulation to antagonize DCL4 and DCL 3, dsRNA binding, blocking primary siRNA biogenesis by RAV2 interaction, TIP-interaction, DRB-HRT mediated HR elicitor, AGO1 downregulation via miR168 upregulation	Azevedo et al. (2010), Jin and Zhu (2010), Várallyay and Havelda (2013), Zhang J. et al. (2012), Merai et al. (2006), Endres et al. (2010), Donze et al. (2014), Choi et al. (2004), Ren et al. (2000), Zhu et al. (2013, 2014), Jeong et al. (2008) and Pérez-Cañamás and Hernández (2015)
	PFVB, HCRSV, PLPV	P37	siRNA binding	Martinez-Turino and Hernandez (2009), Meng et al. (2006) and Pérez-Cañamás and Hernández (2015)
	MNSV	P7B	Unknown, movement Protein	Genovés et al. (2011) and Genoves et al. (2006)
	MNSV	P42	Unknown	Genoves et al. (2006)
Diantho virus	RCNMV	replication	Unknown (DCL1 dependent), miRNA pathway interference	Takeda et al. (2005)
	RCNMV	MP	Unknown	Powers et al. (2008)
Clostero virus	BYV	P21	ds-sRNA binding, blocking HEN1 methyltransferase	Merai et al. (2006), Yu et al. (2006), Reed et al. (2003) and Chapman et al. (2004)
	CTV	P20	Unknown	Lu et al. (2004)

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Table 1 (*continued*)

Genus	Species	VSR	Function	Reference
	CTV	P23	Unknown (nucleolar localization)	Ruiz-Ruiz et al. (2013) and Lu et al. (2004)
	CTV	CP	Unknown	Lu et al. (2004)
Crini virus	SPCSV, SPVD	RNase3	Endonuclease activity	Cuellar et al. (2009) and Kreuze et al. (2002, 2005)
	SPCSV	P22	Unknown	Kreuze et al. (2005)
	CYSDV	P25	Unknown (down stream of siRNA biogenesis)	Kataya et al. (2009)
	ToCV	P22	Unknown (local silencing)	Canizares et al. (2008)
	ToCV	CP	Unknown, SAHH-interaction	Canizares et al. (2008, 2013)
	ToCV	Cpm	Unknown	Canizares et al. (2008)
Ampelo virus	GLRaV-3	P19,7	siRNA and miRNA pathway interference	Gouveia and Nolasco (2012) and Gouveia et al. (2012)
Polero virus	BWYV, PLRV, CYDV, BMYV, TuYV, MABYV, ScYLV, CLRDV, CABYV	P0	Destabilizing AGOs, HR elicitor	Hendelman et al. (2013), Fusaro et al. (2012), Derrien et al. (2012), Kozlowska-Makulska et al. (2010), Han et al. (2010), Csorba et al. (2010), Mangwende et al. (2009), Bortolamiol et al. (2007), Pazhouhandeh et al. (2006), Delfosse et al. (2014) and Wang et al. (2014a,b)
Enamo virus	PEMV-1	P0	Destabilizing AGO1	Fusaro et al. (2012)
Tymo virus	TYMV	P69	Unknown (inhibition of DNA methylation, miRNA and DCL1 upregulation)	Chen et al. (2004)
	CVB	P12	Unknown (ZF domain dependent but NLS independent)	Lukhovitskaya et al. (2014)
Potex virus	PVX, PlAMV, AV3, WclMV, TVX, PepMV	P25 (TGBp1)	AGO1 degradation, coaggregation with SGS3/RDR6, CAT1 interaction, X-Body organization	Chiu et al. (2010), Yan et al. (2012), Senshu et al. (2009), Okano et al. (2014), Mathioudakis et al. (2013) and Tilsner et al. (2012)
	PepMV	CP	Unknown (blocks systemic signaling)	Mathioudakis et al. (2014)
Carla virus	PVM	TGBp1	Unknown (no local suppression)	Senshu et al. (2011)
	PVM, SPCFV, PlAMV	CRP (NaBp)	Unknown (local and systemic silencing)	Deng et al. (2014), Senshu et al. (2011) and Okano et al. (2014)
Tricho virus	ACLSV	P50	Unknown (no local suppression)	Yaegashi et al. (2007, 2008)
Vitivirus	GVA	P10	siRNA binding	Zhou et al. (2006)
Citri virus	CLBV	MP	Unknown	Renovell et al. (2012)
Tobamo virus	TMV, YoMV(ORMV)	P126	Unknown, interference with HEN1-mediated methylation, accumulation of novel miRNA-like sRNAs	Wang et al. (2012), Vogler et al. (2007), Ding et al. (2004) and Hu et al. (2011)
	ToMV	P130	siRNA binding	Kubota et al. (2003)
	TMV	P122	siRNA and miRNA binding, AGO1 downregulation via miR168 upregulation	Csorba et al. (2007) and Várallyay and Havelda (2013)
Tenui virus	RHBV, RSV	NS3	Binding to RNA/RNA or RNA/DNA duplex, larger than 9 nt and long ssRNA	Hemmes et al. (2007), Shen et al. (2010) and Xiong et al. (2009)
	RSV	P2	Interaction with SGS3	Du et al. (2011)
Tobra virus	TRV	16K	Downstream of dsRNA biogenesis	Andika et al. (2012), Ghazala et al. (2008), Martínez-Priego et al. (2008) and Reavy et al. (2004)
	TRV	29K	Unknown	Deng et al. (2013)
	PepRSV	12K	Unknown	Jaubert et al. (2011)
Furo virus	SBWMV, CWMV	19K	Unknown, interaction with N-ext/CP	Sun et al. (2013a,b) and Te et al. (2005)
Peclu virus	PCV	P15	siRNA binding, miRNA pathway interference	Merai et al. (2006), Dunoyer et al. (2002, 2004)
Beny virus	BNYVV, BSBMV	P14	Unknown	Andika et al. (2012), Guilley et al. (2009), Chiba et al. (2013) and Kozlowska-Makulska et al. (2010)
	BdMoV	P13	Unknown	Andika et al. (2012) and Guilley et al. (2009)
	BNYVV	p31	Unknown, interaction with PR-10	Rahim et al. (2007) and Wu et al. (2014)
Hordei virus	BSMV, PSLV	γB	siRNA binding	Merai et al. (2006) and Yelina et al. (2002)
Sobemo virus	CfMV, RYMV	P1	Unknown (siRNA binding ruled out), dcl4 dep.21 nt siRNA delocalization, reduction of 24 nt siRNAs, Zn-finger like binding	Lacombe et al. (2010), Gillet et al. (2013), Sarmientoa et al. (2007) and Weinheimer et al. (2010)
	CfMV	CP	Unknown, nuclear localization	Olspert et al. (2010, 2014)
Nucleo rhabdo virus	RYSV	P6	Blocking RDR6-mediated secondary siRNA biogenesis	Guo et al. (2013)
Curto virus	BCTV	L2	Methylation interference by ADK inhibition	Buchmann et al. (2009), Raja et al. (2008), Yang et al. (2007), Wang et al. (2003, 2005) and Hao et al. (2003)
Begomo virus	ACMV	AC4	ss-sRNA binding	Chellappan et al. (2005)
	CaLCuV, MYMV, ACMV	AL2/ AC2	Methylation interference by ADK inhibition, tran activation of host genes, interference with host gene expression levels	

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Table 1 (*continued*)

Genus	Species	VSR	Function	Reference
				Buchmann et al. (2009), Raja et al. (2008), Yang et al. (2007), Wang et al. (2003, 2005), Hao et al. (2003), Trinks et al. (2005) and Soitamo et al. (2012)
	EACMCV	AV2	Unknown (downstream of siRNA biogenesis)	Chowda-Reddy et al. (2008)
	BSCTV, TYLCV-C, AYVV, ToLCJAV	C2	Methylation interference by SAMDC1 degradation	Zhang et al. (2011), Wezel et al. (2002), Sharma et al. (2010) and Kon et al. (2007)
	AYVV, CLCuMV,TYLCV, ToLCJV-A	V2	Interaction with SGS3, HR elicitor, interaction with PLCPs	Zhang J. et al. (2012), Glick et al. (2008), Sharma et al. (2010), Sharma and Ikegami (2010), Bar-Ziv et al. (2012), Zrachya et al. (2007) and Amin et al. (2011)
	AYVV	C4	Unknown	Sharma et al. (2010)
	ToLCJB, ToLCJAV, TYLCCNV, TYLCCNV-Y10, TbCSV-Y35, CLCuMV,	βC1	Unknown, rgs-CAM mediated RDR6 disruption, SAHH inhibition	Sharma et al. (2011), Kon et al. (2007), Cui et al. (2005), Einia et al. (2012), Li et al. (2014) and Yang et al. (2011), Amin et al. (2011)
	GDARSLA, GmusSLA	Alpha-Rep	Unknown	Nawaz-ul-Rehman et al. (2010)
Mastre virus	WDV	Rep	siRNA binding	Wang et al. (2014a,b) and Liu et al. (2014)
	WDV	RepA	Unknown	Liu et al. (2014)
Caulimo virus	CaMV	P6 (TAV)	DRB4 inactivation, interference with NPR1 (SA/JA crosstalk regulator), cell-to-cell movement, translational trans activation	Laird et al. (2013), Haas et al. (2008), Shivaprasad et al. (2008), Love et al. (2007, 2012) and Rodriguez et al. (2014)
	CaMV	Decoy RNA	Overloading DCL capacity	Blevins et al. (2011)

protein binds to and inhibit DRB4 activity strongly suggesting for an antiviral role of DRB4 upon DNA virus infections (discussed latter). DCL3 cooperates with DRB3 (and AGO4) in antiviral defense through genome methylation against DNA viruses *Cabbage leaf curl virus* (CaLCuV) and *Beet curly top virus* (BCTV) (Raja et al., 2014). HEN1 is also required for the antiviral defense through methylation and stabilization of vsiRNAs (Vogler et al., 2007). Indeed, *Hen1* mutants are more susceptible to *Cucumber mosaic virus* (CMV) and TCV virus infections (Boutet et al., 2003; Zhang J. et al., 2012).

Effector phase of antiviral silencing

In theory the initiation phase of silencing could be enough to limit virus replication and spread through processing of the viral RNA into vsiRNAs. Dicing *per se*, however, is not sufficient to restrict or limit the viral infection (Wang et al., 2011), suggesting that the DCLs' substrates may be the byproducts or aborted transcripts of the viral replication process. It has been shown that the effector step of silencing involving AGO-dependent activity is required to restrict virus replication and spread (Wang et al., 2011). Indeed, AGO proteins are essential in antiviral defense against both RNA and DNA viruses (Azevedo et al., 2010; Pantaleo et al., 2007; Qu et al., 2008; Raja et al., 2008, 2014; Carbonell et al., 2012; Harvey et al., 2011; Wang et al., 2011). sRNA loading into AGOs is governed mostly by their 5′ terminal nucleotides but length, thermodynamical properties of sRNA duplex ends and duplex structure are also important factors (Mi et al., 2008; Schuck et al., 2013; Schwarz et al., 2003; Zhang et al., 2014). *ago1* and *ago2* mutants are hypersusceptible to virus infections like CMV, *Turnip mosaic virus* (TuMV) or TCV (Carbonell et al., 2012; Harvey et al., 2011; Morel et al., 2002). AGO1 participate in removal of viral RNA through slicing activity (Carbonell et al., 2012), although translational repression activity was also found to play a role (Ghoshal and Sanfacon, 2014) (Fig. 1). It was shown that during RNA virus infections AGO1 homeostasis (Mallory et al., 2008) is disrupted and AGO1 protein levels are decreased probably through translational repression of AGO1 mRNA by miR168 activity (Varallyay et al., 2010). As AGO1 is the negative regulator of AGO2 through miR403 action in absence of AGO1 activity AGO2 levels are elevated (Azevedo et al., 2010; Harvey et al., 2011). AGO2 therefore emerges as a second layer in antiviral pathways. AGO2 was shown to be important in defense against CMV, TCV and *Potato virus X* (PVX) viruses in *A. thaliana* (Harvey et al., 2011; Jaubert et al., 2011) and against *Tomato bushy stunt virus* (TBSV) infection in *Nicotiana benthamiana* (Scholthof et al., 2011). In this scenario AGO1 is both a sensor of the infection that activates antiviral pathways such as AGO2 activity but also a direct effector of silencing. The phenotype of *ago1ago2* double mutant indicates that the two proteins act in a synergistic manner and have non-overlapping functions, as supported by their phylogenetic distance (Mallory and Vaucheret, 2010; Wang et al., 2011). Besides AGO1 and AGO2, AGO5 and AGO7 were also proposed to possess antiviral activities against RNA viruses (Qu et al., 2008; Takeda et al., 2008). AGO7 seems to work as a surrogate of AGO1 but with a preference for the less structured RNA targets (Qu et al., 2008; Takeda et al., 2008). The nuclear localized AGO4 has been shown to possess important antiviral functions against geminiviruses. *dcl3, drb3* and *ago4* mutants fail to hypermethylate the viral genome that is required for host recovery (Raja et al., 2014). Besides, AGO4 was proposed be important in transcriptional regulation of host transcriptional response during CMV virus infection (Hamera et al., 2012). The knowledge about RISC cofactors that cooperate with AGOs in plants is very limited. Heat shock protein 70 and 90 (HSP70, HSP90) have been found to be important players in AGO loading by using an *in vitro* cell-free system that recapitulates the loading process (Iki et al., 2010). Further understanding of RISC components, assembly and function may be helped by this and similar *in vitro* systems (Iki et al., 2010; Schuck et al., 2013).

Amplification of antiviral silencing

In many plant-virus combinations RDR activities contribute to the amplification of the antiviral response in order to achieve a robust defense response (Bologna and Voinnet, 2014; Wassenegger and Krczal, 2006) (Fig. 1). RDR polymerase activity is stimulated by the presence of aberrant RNAs lacking *bona fide* features like cap or polyA tail (Gazzani et al., 2004; Moreno et al., 2013). In some host-virus interactions (like during VSR-deficient CMV infection) sliced products of AGO1 or AGO2 were not required for secondary vsiRNA production (Wang et al., 2011) suggesting that the process may be different from that of ta-siRNA biogenesis. RDR-derived dsRNAs are

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processed by DCL4 and DCL2 into 21–22 nt long vsiRNAs, respectively. Both 21 and 22 nt long vsiRNA are effective in antiviral response as has been shown in case of many virus infections (CMV, *Oilseed rape mosaic virus* (ORMV), TCV, *Tobacco rattle virus* (TRV), CaLCuV, CaMV) (Xie et al., 2004; Bouché et al., 2006; Deleris et al., 2006; Blevins et al., 2006; Donaire et al., 2008). DCL2 activity becomes more pronounced under higher temperature (Zhang X. et al., 2012b). However, Wang et al. (2011) have found that the 21- but not 22-nucleotide long vsiRNAs guide efficient silencing through AGO1 and AGO2 effectors in CMV infection although AGO1 efficiently incorporates both of them. 22 nt long vsiRNAs contribute to secondary siRNA production, as was shown for 22 nt long miRNAs and ta-siRNAs or mediate systemic silencing (Garcia-Ruiz et al., 2010; Wang et al., 2011). It is assumed that the secondary vsiRNA are able to move on short and long distances within the plant to immunize distant tissues ahead of the viral infection, however this awaits experimental validation. The requirement of RDR6-activity for systemic movement of a silencing signal suggesting that RNA silencing amplification has antiviral roles in uninfected distant tissues. RDR1, RDR2 and RDR6/SDE1/SGS2 were all found to be crucial factors in secondary vsiRNA production during RNA virus (PVX, CMV, *Tobacco mosaic virus* (TMV), *Sugarcane mosaic virus* (SCMV), TuMV, TRV infections (Diaz-Pendon et al., 2007; Donaire et al., 2008; Garcia-Ruiz et al., 2010; Qu et al., 2008; Schwach et al., 2005). Host RDR involvement in secondary vsiRNA production could not be verified in certain virus-host interactions. Indeed, upon tombusvirus infections the overwhelming part of vsiRNA derive from the positive RNA strand of the virus genome suggesting that they are primary DCL products (Aregger et al., 2012; Blevins et al., 2011; Donaire et al., 2008; Molnar et al., 2005; Szittya et al., 2010). In case of DNA viruses, viral transcripts appear to be poor templates of RDRs. Majority of viral siRNAs accumulating during CaLCuV geminivirus infection were RDR1/2/6-independent primary siRNAs (Aregger et al., 2012).

RDR6 activity is facilitated by protein cofactors like Suppressor of GENE SILENCINIG 3 (SGS3) (Mourrain et al., 2000), SILENCING DEFECTIVE 5 (SDE5) (Hernandez-Pinzon et al., 2007) and SILENCING DEFECTIVE 3 (SDE3) (Dalmay et al., 2001). SGS3, a plant specific protein, was found associated with RISC complex and to be important in ta-siRNA biogenesis (Allen et al., 2005; Yoshikawa et al., 2005). It was proposed that SGS3 stabilize the RISC-cleavage product following slicing and enhance its conversion into dsRNA by RDR6 activity (Yoshikawa et al., 2013). Elimination of SGS3 not only abolishes ta-siRNA biogenesis but also leads to enhanced susceptibility to infection, at least in certain virus-host combinations. *sgs3* mutants have severe symptoms when challenged with CMV but do not show any difference in their viral symptoms during TuMV or *Turnip vein-clearing virus* (TVCV) infections (Adenot et al., 2006; Yoshikawa et al., 2013). SGS3 was shown to be required for CaLCuV virus induced VIGS of endogenous genes and further suggested that may be involved in the antiviral response against DNA viruses (Muangsan et al., 2004). This is supported by the fact that *Tomato yellow leaf curl virus* (TYLCV) encodes a VSR to compromise its activity (discussed below) (Glick et al., 2008; Fukunaga and Doudna, 2009). SDE5 is an RNA trafficking protein homologue of human mRNA export factor. It was proposed that SDE5 acts together with RDR6 in converting specific ssRNAs into dsRNA. *sde5* mutant plants are hypersusceptible to CMV but not TuMV infection (Hernandez-Pinzon et al., 2007). The amplification process is facilitated by the SDE3 an RNA-helicase like protein. SDE3 was shown to associate to AGOs through its GW motifs (Garcia et al., 2012). *sde3* mutant plants are affected in defense response against CMV or PVX but not TRV infections (Dalmay et al., 2001). SDE3 activity occurs downstream to RDR6 and requires AGO1 and AGO2 activities (Garcia et al., 2012). SDE3 was proposed therefore to facilitate the amplification process by unwinding a fraction of RDR6-sythetized dsRNA products using its helicase activity. Furthermore, SDE3 could increase the efficiency of AGO-targeting of ssRNA targets (Garcia et al., 2012).

Actions of viral suppressors of RNA silencing

Some viruses avoid RNA silencing by replicating within well-defined subcellular compartments/structures like ER spherules (Schwartz et al., 2002) or by replicating and moving fast enough to outrun the mobile silencing signal. However, the most common way to protect viral genome against RNA silencing-mediated inactivation is to encode proteins that act as suppressors of RNA silencing (viral suppressors of RNA silencing, VSRs) (Lakatos et al., 2006). In fact, the strongest support of RNA silencing having antiviral roles was the discovery of VSRs. In an early work Ding and coworkers identified the cucumoviral 2b protein as responsible for induction of a non-conventional virus synergistic disease. Although at that time it was thought that 2b facilitate hypervirulence through its movement protein function (Ding et al., 1996), latter it was demonstrated to be a potent VSR (Brigneti et al., 1998). Another hint regarding viral suppression of plant RNA-based defense was the synergistic interaction of potyviruses with other viruses that relayed on potyviral Hc-Pro protein activity (Pruss et al., 1997). Whilst Hc-Pro was shown to block PTGS at tissue level, CMV 2b was shown to prevent systemic silencing (Brigneti et al., 1998). Since these first observations more than a decade ago, numerous VSRs were discovered and characterized (Table 1). Available evidences suggest that most viruses encode at least one VSR that in most cases is essential for successful viral life cycle. Although plant virus' VSRs are more studied, silencing suppression has been documented on insect and fungus-infecting viruses as well (Bronkhorst and van Rij, 2014). The extraordinary diversity in sequence and structure of VSRs within and across kingdoms indicates that they have evolved independently. Diversity of VSRs implies diverse mechanistic activities, and indeed, VSRs were shown to block virtually all steps of RNA silencing (Fig. 1) such as dicing, effector assembly, targeting, amplification, transcriptional regulation of endogenous factors that control RNA silencing and its connections with protein-based immunity and hormone signaling.

Blocking initiation of antiviral response

One strategy used by VSRs is to hinder mounting of antiviral silencing by blocking the silencing initiation step. This can be achieved through multiple ways like dicer protein or co-factor activity inhibition, dsRNA/siRNA-sequestration or AGO protein destabilization prior of RISC assembly. A widespread suppressor strategy is the ds-siRNA sequestration that is used by several VSRs encoded by diverse virus genera (P19, Hc-Pro, P21, p15, p130/p126/p122, γB, NS3, Pns10, NSs etc.) (Csorba et al., 2007; Harries et al., 2008; Hemmes et al., 2007; Kubota et al., 2003; Lakatos et al., 2006; Merai et al., 2005, 2006; Silhavy et al., 2002) (Table 1 and Fig. 1). Probably the most characterized siRNA binder is the tombusviral p19 protein (Silhavy et al., 2002). Crystallographic studies have shown that p19 head-to-tail homodimer acts as a molecular caliper to size-select and sequester siRNA duplexes in a sequence-independent manner (Silhavy et al., 2002; Vargason et al., 2003). P19 siRNA sequestration prevents RISC assembly as shown by the heterologous *in vitro* Drosophila embryo extract system (Lakatos et al., 2006). Similarly p19 sequestration of miRNAs is efficient to prevent RISC-loading in p19-transgenic *A. thaliana* (Schott et al., 2012) and *N. benthamiana* plants (Kontra and Burgyan unpublished results). However, during authentic virus infections miRNA sequestration by p19 is not efficient (Lozsa et al., 2008), suggesting that, miRNA binding by P19 may depend on spatial and temporal co-expression of miRNA duplex and the virus encoded suppressor protein (Lozsa et al., 2008; Schott et al., 2012). A consequence of siRNA binding by VSRs is the block of HEN1-

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dependent methylation of sRNAs (Csorba et al., 2007; Lozsa et al., 2008; Vogler et al., 2007), however this also depends on the co-expression of sRNA and the suppressor (Lozsa et al., 2008). In addition to blocking silencing activation at cellular level, p19 interferes with the systemic spread of mobile silencing signal as well (Dunoyer et al., 2010; Molnar et al., 2010). This latter characteristic is an excellent indicator whether a VSR acts indeed as a *bona fide* siRNA sequester during authentic viral infection. Some VSRs bind dsRNAs in a size-independent manner: *Pothos latent aureusvirus* (PolV) P14, TCV p38 and CMV 2b (Deleris et al., 2006; Goto et al., 2007; Merai et al., 2005) have been all described to bind dsRNA and suggested therefore to block vsiRNA maturation. In turn nuclear localized P6 suppressor of CaMV diminish dicing efficiency through protein-protein interaction. The two importin-alpha dependent nuclear localization signals of P6 are mandatory for CaMV infectivity. P6 genetically and physically interacts with the nuclear DRB4, a cofactor required for DCL4-dependent vsiRNA processing (Haas et al., 2008). (TCV p38, CMV 2b and CaMV P6 are discussed latter). Similar strategy was described in insect infecting viruses: *Flock house virus*, *Nodamura virus* and *Wuhan nodavirus* encoded B2, *Drosophila C virus* (DCV) encoded canonical dsRBD, the VP3 proteins of *Drosophila X virus* or the dsDNA virus *Invertebrate iridescent virus 6* (IIV-6)-encoded 340R suppressors can block Dicer-2 activity and prevent RISC loading via size-independent dsRNA/siRNA-binding (Bronkhorst and van Rij, 2014).

A completely different strategy (to siRNA-binding) but with very similar outcome is used by *Sweet potato chlorotic stunt crinivirus* (SPCSV) suppressor RNase3. In this case the build-up of an efficient antiviral silencing complex is prevented by endonuclease activity of RNase3 that cleaves the 21−24 nt vsiRNAs into 14 bp products rendering them inactive (Cuellar et al., 2009; Kreuze et al., 2005). Insect infecting *Heliothis virescens ascovirus-3e* orf27 (RNase3 enzyme) suppressor works in analogous manner: it competes with Dcr-2 for dsRNAs and degrades dsRNAs and siRNAs (Hussain et al., 2010).

Arrest of functional RISC assembly through AGO interaction

The arrest in the assembly of a functional RISC can be carried out also through direct binding the protein component of minimal RISC, AGO protein. P0 protein (Mayo and Ziegler-Graff, 1996; Sadowy et al., 2001) the suppressor of *Poleroviruses* does not possess RNA binding activity (Csorba et al., 2010; Zhang et al., 2006), instead interacts with E3-ligase S-phase kinase regulated protein 1 (SKP1) through its F-box motif. P0 was shown to enhance the degradation of multiple AGOs (AGO1, 2, 4–6, 9) before holo-RISC assembly. Mutations in the F-box motif abolished P0 suppressor activity (Baumberger et al., 2007; Bortolamiol et al., 2007; Csorba et al., 2010; Derrien et al., 2012; Pazhouhandeh et al., 2006). These suggested a model where P0 destabilizes AGOs through the proteasome pathway. Instead, it was shown that P0-mediated AGO destabilization is not sensitive to proteasome inhibitor and that AGO degradation occurs through autophagy pathway (Baumberger et al., 2007; Bortolamiol et al., 2007; Csorba et al., 2010; Derrien et al., 2012; Pazhouhandeh et al., 2006). Massive accumulation of K48-linked ubiquitinated host proteins accumulated in the presence of P0, including the N-terminal cleavage product of AGO1. P0 therefore may have a role in promoting degradation of AGOs and other host proteins through ubiquitination-proteasome pathways (Csorba et al., 2010). *Tomato ringspot virus* (ToRSV) suppressor CP binds to AGO1 to suppress its translational inhibitory activity and to enhance AGO1 degradation through autophagy (Karran and Sanfacon, 2014). It was shown that PVX p25 physically interacts with AGOs (AGO1, 2, 3 4 but not AGO 5 and AGO9) to promote their destabilization in a proteasome-dependent manner. Consistently with these, plants treated with proteasome inhibitor were less susceptible to PVX (Chiu et al., 2010).

Inactivation of programmed antiviral RISC complex

VSRs may mimic cellular protein cofactors to inactivate programmed RISC. In *Sweet potato mild mottle ipomovirus* (SPMMV) the role of suppressor is played by P1 protein, a serine protease, in spite of the presence of HC-Pro (the suppressor of *Potyviruses*). P1 interacts directly with siRNA and/or miRNA-loaded AGO1 present in the high molecular weight holo-RISC but not minimal-RISC through GW/WG-motifs (AGO-hook). The conserved GW/WG-motif containing protein family (GW182 family) has been shown to bind to AGOs and to be required for diverse RISC function (Eulalio et al., 2009). Site-directed mutagenesis in the P1 protein proved that the conserved AGO-hook motifs (three GW/WG domains) located at the N-terminal part of P1 are absolutely necessary for both binding and suppression of AGO1 function (Giner et al., 2010). SPMMV P1 not just inhibits de novo RISC assembly but also block si/miRNA-loaded RISC activity. The role GW/WG-motif in silencing suppression was further evidenced by the restoration of naturally inactive P1 protein suppressor activity through the introduction of two additional GW/WG motifs (Szabo et al., 2012).

Coat protein of TCV also known as p38 may suppress silencing at multiple levels including vsiRNA generation and assembled RISC activity block. Since p38 possesses dsRNA-binding activity, and in its presence siRNAs are undetectable it was proposed that p38 acts to suppress Dicer's activity (Qu et al., 2003). Genetic evidences supported the role of p38 in inhibiting DCL4 but not DCL2 (Deleris et al., 2006). Although 22 nt vsiRNA were produced, they were inactive in the presence of p38 because Δp38 mutant TCV was not able to infect systemically the single *dcl, dcl2dcl3* or *dcl3dcl4* double mutant plants except *dcl2dcl4* mutant (Deleris et al., 2006). In a later study however, p38 suppressor impact on DCL4 was attributed to an indirect effect of AGO1-mediated DCL-homeostasis and has been shown that p38 blocks AGO1 but not AGO4 activity through its GW-motif binding. DCL2-derived 22 nt vsiRNA production, HEN1 and AGO2-activity pathway is crucial against TCV infection at elevated temperature (Zhang X. et al., 2012). Of note, p38 is also capable to bind and inactivate AGO2 (Zhang X. et al., 2012). P38 interacts with unloaded AGOs. Site-directed mutagenesis (GW-to-GA) in the p38 proved that GW motif is absolutely required for both binding and suppression of AGO1 function. GA mutation was enough to abolish TCV virulence that is restored in *ago1* hypomorph plants showing GW motif functionality during authentic virus infection (Azevedo et al., 2010). The coat protein (p37) of *Pelargonium line pattern virus* (PLPV) is a GW-containing protein that also functions as a VSR. It was shown that P37 mainly suppress silencing through siRNA sequestration. Mutations within its GW-motif concurrently affected p37 localization, its interaction with AGO1 and its sRNA-binding ability. Furthermore, binding assays have shown that also in case of TCV p38, the GW-mutation abolished p38 sRNA and long dsRNA-binding capacity (Pérez-Cañamás and Hernández, 2015). The overlapping multiple functions of p38 may lead to misinterpretation of experimental data. However, the data may suggest that these functions could cooperate: VSR interaction to AGO could enhance sRNA duplex sequestration in order to prevent RISC programming.

Silencing effector complex activity block can be achieved also through targeting holo-RISC's RNA component, the guide RNA. *African cassava mosaic virus* (ACMV) encoded AC4 was shown to bind to the ss-sRNAs but not dsRNA forms *in vitro*. Transgenic expression of AC4 correlated with decreased accumulation of miRNAs and upregulation of target mRNAs. AC4 acts downstream of the unwinding process: to bind mature miRNAs presumably loaded into AGO protein (Chellappan et al., 2005; Xiong et al., 2009; Zhou et al., 2006). *Rice stripe virus* suppressor NS3 is able to suppress and reverse GFP silencing and also prevent long distance spread of silencing signal. NS3 was found to bind to various RNA

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forms like ss-siRNA, ds-siRNA or long ssRNA but not long dsRNA (Chellappan et al., 2005; Xiong et al., 2009; Zhou et al., 2006). *Grapevine virus A* p10 suppressor was also suggested to act through RNA sequestration: recombinant p10 was able to bind to both ss- and ds-si/miRNA species (Chellappan et al., 2005; Xiong et al., 2009; Zhou et al., 2006).

VSR activities downstream of RISC and RITS

VSRs may inactivate host defense downstream to RISC or RITS. Several DNA viruses encode VSRs that have been described to alter DNA/histone methylation, the effector step of TGS. *Tomato golden mosaic virus* (TGMV) suppressor AL2 and *Beet curly top virus* (BCTV) suppressor L2 inhibit adenosine kinase (ADK) activity that plays crucial role in adenosine and methyl-cycle maintenance or cytokinin regulation. In the presence of AL2 and L2 global reduction in cytosine methylation was observed that leads to inactivation and reversal of antiviral silencing (Buchmann et al., 2009; Wang et al., 2003, 2005). *In vitro* methylated TGMV cannot replicate in protoplasts suggesting that viral genome methylation is a bona fide defense against geminiviruses that is worth to be suppressed (Bisaro, 2006). Similarly, *Tomato yellow leaf curl China virus* (TYLCCNV) another *Begomovirus* encodes ßC1 that interacts and inhibit activity of S-adenosyl-homocystein-hydrolase (SAHH) that is involved in methyl-cycle and therefore indirectly affects TGS (Yang et al., 2011).

Modulation of AGO1 homeostasis

Similarly to the aforementioned examples of DNA virus encoded VSRs, RNA virus-encoded VSRs were also described to modulate host gene expression on transcriptional level to their benefit. During tombusviral infection AGO1 transcription is induced as part of the host antiviral arsenal. AGO1 homeostasis in plants depends on the miR168-guided AGO1 mRNA cleavage and translational inhibition (Rhoades et al., 2002). To counteract AGO1-based defense, the virus promotes miR168 transcriptional induction that results in miR168-guided AGO1 down-regulation. The miR168 accumulation spatially correlates with the virus localization and depends on its p19 VSR (Varallyay et al., 2010) (Fig. 1). Similarly to p19 all VSRs, which are very heterogeneous in protein sequence but bind vsiRNA, promote miR168 transcriptional induction and AGO1 down-regulation suggesting that VSR-siRNA complexes are effectors and recognized by the plant surveillance system (Várallyay and Havelda, 2013).

Plant RDR-based activity suppression

Host RDRs (RDR1, 2 and 6) contribute to amplification of RNA silencing and spread of a systemic signal by synthesis of vsiRNAs (Schwach et al., 2005). Interestingly, plant RDR1 itself was suggested to have adverse functions. RDR1 is an antagonist of RDR6-mediated sense-PTGS silencing therefore behaves as an endogenous silencing suppressor (Ying et al., 2010). Suppression of RDR activities may constitute a target point for VSRs since it dampens cell-autonomous silencing amplification and systemic signal movement in distant tissues to facilitate the virus replication and spread. It was shown that V2 protein of *Tomato yellow leaf curl virus* (TYLCV) directly interacts with SGS3, the cofactor of RDR6, to block silencing amplification (Glick et al., 2008). Another *in vitro* study has shown that V2 competes with SGS3 for dsRNA having 5′overhang ends that may be an RDR6/SGS3 intermediate in vsiRNA amplification (Fukunaga and Doudna, 2009; Kumakura et al., 2009). This may suggest that a 5′-overhanged intermediate may be the RDR6-complex template. Similar structures may be present on viral RNAs. Similarly, potexviral TRIPLE GENE BOX PROTEIN1 (TGBp1) was also shown to inhibit RDR6/SGS3-dependent dsRNA synthesis (Okano et al., 2014). βC1 suppressor of *Tomato yellows leaf curl China virus* (TYLCCNV) DNA satellite interacts with the endogenous suppressor of silencing calmodulin-like protein (rgsCAM) in *N. benthamiana* to repress RDR6 expression and secondary siRNA production (Li et al., 2014). *Sugarcane mosaic virus (SCMV)* encoded HC-Pro and TAV 2b were shown to downregulate RDR6 mRNA in *N. benthamiana* transient assay (Zhang et al., 2008). Pns10 suppressor of *Rice dwarf phytoreovirus* (RDV) downregulate RDR6 to enhance viral invasion of shoot apices (Ren et al., 2010). RDR6-based activity suppression therefore is a widely used strategy that effectively blocks antiviral silencing but has only a limited impact on endogenous silencing pathways (discussed latter).

Targeting multiple steps of antiviral pathways

VSRs may act at multiple points to suppress silencing (Fig. 1). There is substantial evidence that the cucumoviral 2b protein can interfere with silencing at many steps. 2b prevents the spread of long-range silencing signal (Guo and Ding, 2002). N-terminal domain of 2b contains a dsRNA-binding domain that exhibits high affinity for short and long dsRNAs (Duan et al., 2012). CM95R strain of CMV and the related *Tomato aspermy virus* (TAV) 2b were also shown to bind siRNAs and ds-miRNAs *in vivo* and *in vitro* (Chen et al., 2008; Gonzalez et al., 2012; Goto et al., 2007). 2b (Fny and SD strains) was found to interact with AGO through the PAZ- and partly PIWI domains and blocks AGO1/RISC slicer activity (Duan et al., 2012; Zhang et al., 2006). FnyCMV 2b transgenic expression phenocopies *ago1* mutant plants (Duan et al., 2012; Zhang et al., 2006). Additionally, CMV 2b encoded by the SD strain alters RdDM pathway as well. 2b facilitates cytosine methylation through the transport of siRNAs into the nucleus (Kanazawa et al., 2011). 2b interacts both with AGO4-related siRNAs and with AGO4 protein through PAZ and PIWI domains. Interaction of 2b with AGO4 reduces AGO4 access to endogenous target loci and consequently modulates endogenous transcription to create a favorable cellular niche for CMV proliferation (Duan et al., 2012; Gonzalez et al., 2010, 2012; Hamera et al., 2012).

VSR interactions with host factors

There are emerging evidences that besides the “canonical” block of RNA silencing (through ds-, si-, mi-ssRNA-binding *e.g.* p19, RNase3 etc. or manipulating silencing-related protein activities via direct/ indirect interactions *e.g.* P0, V2, P1 etc.) some suppressors may target endogenous regulators of the silencing to modulate host defense. Potyviral helper-component protease (HC-Pro) is a multifunctional protein involved in many aspects of virus infection (Anandalakshmi et al., 1998; Carrington et al., 1989; Guo et al., 2011; Kasschau et al., 1997; Lakatos et al., 2006; Mallory et al., 2001). *Tobacco etch virus* (TEV) HC-Pro suppresses silencing through vsiRNA-sequestration (Lakatos et al., 2006) and interferes with vsiRNA methylation (Lozsa et al., 2008). *Zucchini yellows mosaic virus* (ZYMV) Hc-Pro interacts with HEN1 directly in *in vitro* assays (Jamous et al., 2011). TEV HC-Pro was found to interact with rgsCAM in a yeast two-hybrid system. RgsCAM itself is a host suppressor of RNA silencing (Li et al., 2014; Anandalakshmi et al., 2000; Endres et al., 2010). Interestingly, geminivirus AL2 protein induces expression and interacts with rgsCAM. Overexpression of rgsCAM leads to increased virus susceptibility (Yong Chung et al., 2014). Contradictory, in another study it was shown that rgsCAM counteracts HC-Pro through binding to its positively charged dsRNA-binding surface. RgsCAM binding prevents HC-Pro ds-siRNA binding activity and promotes its degradation through autophagy pathway (Nakahara et al., 2012). Multiple host interactors may further modulate activities of HC-Pro. It was proposed that RAV2, a transcription factor is required for suppression of

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silencing mediated by TuMV HC-Pro. RAV2 downstream targets include *FIERY1* an endogenous silencing suppressor in *Arabidopsis* (Gy et al., 2007) and *CML38,* the likely homologue of rgsCAM in *Arabidopsis* (Anandalakshmi et al., 2000; Endres et al., 2010). Genome expression analysis reveals that RNA silencing related genes were unaltered in HC-Pro transgenic lines, instead RAV2 was required for HC-Pro-mediated induction of stress and defense-related genes (Endres et al., 2010). The unrelated *Carmoviral* p38 had a very similar effect on RAV2-mediated changes. These findings may suggest that RAV2 is a cross-talk point between pathogen stress-defense and silencing pathways that is used by VSRs to manipulate host reactions. *Papaya ringspot virus* (PRSV) HC-Pro interacts with papaya calreticulin to modulate host defense to virus infection through calcium signaling (Shen et al., 2010a; Shen et al., 2010b). HC-Pro of *Potato virus A* (PVA), *Potato virus Y* (PVY) and TEV interacts also with microtubule-associated protein (HIP2) through its highly variable region (HVR). HIP2 depletion reduces virus accumulation, whereas mutations affecting HC-Pro HVR domain induces necrosis and ethylene- and jasmonic acid-mediated systemic induction of host pathogen-related defense genes (Haikonen et al., 2013a,b).

Another example of how the endogenous factors may be used to modulate silencing efficiency is provided by *Red clover necrotic mosaic virus* (RCNMV). RCNMV recruits DCL enzymes into its replication complex and therefore deprives them from the silencing machinery. *dcl1* mutant plants show reduced susceptibility to RCNMV (Takeda et al., 2005). A similar strategy is used by CaMV pararetrovirus: massive amounts of vsiRNAs derive from the 35S leader sequence produced by all four DCLs. These do not restrict viral replication but instead may serve as decoy RNAs to divert the effectors of the silencing machinery from important viral features like promoter and coding sequences (Blevins et al., 2011) (Fig. 1).

Driving factors in VSRs' evolution

The high diversity in structure and function, the various position of their gene-code within the viral genome, the alternative expressional strategies like transcriptional read-through, leaky ribosomal scanning, proteolytic maturation and being often encoded by out-of-frame ORFs within conserved viral genes suggests that VSRs are of recent evolutionary origin (Ding and Voinnet, 2007). Therefore, in most cases, the suppressor function of VRSs may have evolved after the ancient role as replicase, coat protein, movement protein, protease, transcriptional regulator etc. or co-evolved with these to combine within the suppressor role and other essential roles important for viral life cycle. The different VSRs can inhibit all steps of the antiviral RNA silencing pathway, including cell-autonomous and non-cell autonomous aspects of it. Using mathematical modeling of dynamics of suppression has been shown that the different strategies employed result in slightly different outcomes regarding suppression of antiviral silencing. Suppressors targeting effector step are more potent at single cell level whereas siRNA binding is more effective at tissue level (Groenenboom and Hogeweg, 2012). Besides this however, an important driving factor in the suppressors' evolution was probably the availability of ancient/original viral protein activities that could be selected from with a minimum number of changes to acquire an additional suppressor features. Many of viral proteins have RNA binding capacity (like replicase, coat protein, movement protein). This may explain why an overwhelming number of suppressors act through RNA binding. Silencing functions could have become established in the cases when the tradeoff between its positive effect on viral life cycle and negative effects on host were worth it. The suppressors being too weak or too strong were out-selected through evolution.

The control of pathogen impact on host

Antiviral and endogenous silencing pathways share common elements. The ability of viruses to block antiviral silencing may have an impact on endogenous silencing pathway that results in alteration in short RNAs expression profile/activity and changes in gene expression both in a direct and in an indirect manner. vsiRNA-binding VSRs can bind endogenous si- and miRNAs that could result in alteration of their downstream targets as was shown previously (Chapman et al., 2004; Kasschau et al., 2003; Lozsa et al., 2008). In case of miRNAs that target RNA silencing target components an unpredicted number of genes will be altered indirectly (*e.g.* miR162-mediated DCL1 negative feed-back loop, DCL1-dependent suppression of DCL3 and DCL4, miR168 and AGO1 mRNA-derived siRNA control of AGO1, miR403 control of AGO2) (Allen et al., 2005; Mallory and Vaucheret, 2009; Qu et al., 2008; Rajagopalan et al., 2006; Vaucheret et al., 2006; Xie et al., 2005). The situation is similar in case of AGO-targeting VSRs (P0, P1, P38) (Azevedo et al., 2010; Baumberger et al., 2007; Derrien et al., 2012; Giner et al., 2010). An elegant demonstration of this was described for TCV p38. AGO1 quenching by p38 had a profound impact on DCLs' homeostasis uncovering the strong interconnection of the silencing components into a functional network (Azevedo et al., 2010). VSRs' presence therefore may have a big impact and lead to an altered developmental program of host organism and symptom development. In support of VSRs as contributors to the viral symptoms, VSR-transgenic lines were created and analyzed. In many cases the VSR-expressing transgenic plants display phenotypes similar to viral infections (Dunoyer et al., 2004; Jay et al., 2011; Kasschau et al., 2003; Lewsey et al., 2007; Zhang et al., 2006). However, transgenic expression of VSR does not recapitulate the expression pattern in time and space of an authentic viral infection, therefore conclusions need to be drawn very carefully.

Limitation of VSRs' suppressor strength

Some of the VSRs differentially impact the antiviral and the endogenous pathways. For example vsiRNA binding *Tombusviral* p19 protein binds more efficiently to free ds-siRNA forms. It was shown previously that p19 blocks very efficiently HEN1-dependent methylation of vsiRNA but not miR159 (Lozsa et al., 2008). Furthermore, vsiRNA but not miR159 are bound into p19-dimer:siRNA nucleoprotein complex whereas miR159 incorporates efficiently to AGO/RISC complex (Varallyay et al., 2010). The miR168, a particular miRNA that has a weak AGO-loading rate (Mallory and Vaucheret, 2009), was also available for *in vivo* p19-binding (Varallyay et al., 2010). All these suggests that p19 affects more potently vsiRNA- but at less extent miRNA-pathway (Lozsa, Kontra and Burgyán, unpublished.). These differences may rise probably from the differences in the DCL1-dependent and DCL4/2-dependent si/miRNA-maturation pathways and distinct sub-cellular localization of silencing components. Conversely to p19, the TEV HC-Pro significantly affected 3′-methylation of both si- and miRNAs. miRNA 3′-methylation may take place in the nucleus where HC-Pro could access them (Lozsa et al., 2008). Similarly, TMV suppressor localizes to the nucleus as well (dos Reis Figueira et al., 2002). This may explain the more efficient sequestration of ds-miRNAs as assessed by the miRNA-star strand accumulation (Csorba et al., 2007). Therefore modulating sub-cellular localization of VSR will result in differential impact on antiviral and endogenous pathways respectively and therefore may constitute a regulatory point (Papp et al., 2003).

P6 is mostly present in the cytoplasm consistent with its transactivator function, but a small fraction of it is imported into the nucleus (where it blocks DRB4). It was shown that cytoplasmic-nuclear shuttling is prerequisite for VSR function and successful virus infection

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(Haas et al., 2008). Although P6 contains a dsRNA-binding domain, this is not required for its suppressor function. In fact, perhaps surprisingly, the dsRNA-binding domain has an indirect negative effect on P6 suppressor function, presumably because nucleoplasmic localization of P6 is decreased by the dsRNA-binding domain interaction with ribosomal proteins L13 and L18 in the nucleolus and consequently P6 localizes more effectively to the nucleoplasm. Sub-cellular localization of P6 therefore alters its impact on host silencing. Similarly, enhanced nuclear targeting of CMV Fny strain 2b by addition of a nuclear localization signal compromises its suppressor activity but in the same time enhances 2b-mediated pathogenicity and CMV virulence, making the virus necrotic and accentuating the disease symptoms in *Arabidopsis*. Enhanced virulence was unrelated to effects of nuclear localized 2b on vsi- or miRNA-regulated target functions (Du et al., 2014). Consequently, CMV Q strain 2b mild suppressor activity could be explained by its preferential accumulation in the nucleus in contrary to cytoplasmic Fny 2b.

The suppression of antiviral pathways could additionally also protect the host endogenous targets from the vsiRNA-"off-target" effects. Recently it was shown that in the absence of 2b suppressor of CMV virus-activated siRNAs (vasiRNAs) are produced from endogenous transcripts in a DCL4- and RDR1-dependent manner. AGO2 is essential for the silencing activity of vasiRNAs (Cao et al., 2014).

Another VSR strength-limitation strategy is exemplified by the *Poleroviruses*. *Poleroviral* P0 protein expression is restricted by its suboptimal translation initiation codon, even though the use of such a weak suppressor-strategy results in phloem restriction of the virus. Attempts to restore optimal P0 translation lead to secondary mutations indirectly affecting P0 (Pfeffer et al., 2002). This shows that P0's impact on endogenous pathways is so devastating that is unfavorable for the virus.

Suppressor strength of HC-Pro was tested on a collection of point mutants in a GFP co-agroinfiltration assay and parallel the virulence of mutant alleles encoding TEV viruses *in vivo* (Torres-Barcelo et al., 2008). Both hyposuppressor and hypersuppressor HC-Pro alleles were found suggesting that the wild type protein has intermediate suppressor activity strength. Interestingly, while viruses carrying hyposuppressor alleles induced milder symptoms and accumulated to a lower level, hypersuppressor mutant viruses produced symptoms and accumulated to levels characteristic to wild type virus (Torres-Barcelo et al., 2008). This suggests that the suppressor strength could have been optimized by natural selection.

Since antiviral pathways strongly relay on amplification and systemic spread of silencing, those suppressors that target amplification of silencing (*e.g.* V2 or TGBp1 inhibition of SGS3) will have a definite impact to fight off antiviral pathway and promote virus spread (Fukunaga and Doudna, 2009; Glick et al., 2008; Kumakura et al., 2009; Okano et al., 2014). In certain cases however, in order to keep a tight control and spare the host, virus' systemic spread is limited by enhancing systemic silencing signal by the pathogen itself. *Rice yellow mottle virus* (RYMV) P1 was described originally as the movement protein of the virus. Recently it was shown that P1 suppresses local transgenic silencing and inhibits DCL4-dependent endogenous siRNA pathways in *Nicotiana* plants and its expression caused similar developmental defects in rice as *dcl4* mutation. In the same time P1 facilitated the short- and long distance spread of silencing signal in *Nicotiana* plants. P1 therefore may have a dual role, both to suppress and to elicit silencing of host (Lacombe et al., 2010). Although the precise mechanism of RYMV P1 activities are not known and need to be confirmed during authentic virus infections, it seems that P1 plays a fundamental role for an efficient infection process where the virus preserves the integrity of the host. Similarly to RYMV movement protein P1, the TMV MP (30kDa) has been shown to support the spread of antiviral silencing signal (Vogler et al., 2008) and therefore counter-balance the activity of p130 the suppressor of TMV to attenuate pathogen impact (Kubota et al., 2003).

Decreasing rather than eliminating specific antiviral silencing activities may allow setting a balance between blocking antiviral pathway and host transcriptome control through silencing. CaMV P6 protein was described as a symptom and host range determinant, a translational transactivator and a silencing suppressor (Bonneville et al., 1989; Haas et al., 2008; Love et al., 2007). P6 suppressor blocks DRB4 function that is required for DCL4 function therefore only diminishes (but not blocks) DCL4 activity. This maybe sufficient enough to dampen antiviral silencing but in the same time allow host gene control by the 21 nt 35S-derived vsiRNA. It was shown previously that several CaMV vsiRNAs exhibit near-complete complementarity to many endogenous targets (Moissiard and Voinnet, 2006). Besides, DRB4 targeting may allow accumulation of 24 nt vsiRNA that are required for heterochromatin silencing of CaMV minichromosomes (Al-Kaff et al., 1998).

VSRs as links between RNA-based and protein-based immunity

Alteration of silencing pathways (an RNA-based immunity) in the presence of the VSR and/or viral infection triggers the protein-based immunity in host as part of the counter-counter defense response. Resistance (R) genes present in the plant genome convey disease resistance against pathogens by producing R proteins and their action are main part of the protein-based immunity arsenal. Conserved miRNA family controls a plethora of R genes (Li et al., 2012; Shivaprasad et al., 2012; Zhai et al., 2011). It is assumed that R genes are silenced in the absence of the pathogen in order to minimize the cost for the plants and prevent autoimmunity reactions (Tian et al., 2003). It was found that the NBS-LRR genes (the main class of R proteins with nucleotide binding site (NBS) and leucine-rich repeat (LRR) motifs are silenced in a siRNA-regulated cascade similarly to ta-siRNA biogenesis scheme: RDR6-dependent secondary siRNAs are produced following the original 22 nt miRNA-mediated cleavage on a R-gene transcript. The secondary siRNA may target other R-genes. When tomato plants were infected with viruses (TCV, CMV, TRV) (or bacteria) accumulation of miR482 was reduced. In the absence of miR482 activity the resistance R gene targets are released and consequently R gene products accumulate to enhance immunity of the plants (Shivaprasad et al., 2012). In tobacco the R gene (TIR-NB-LRR immune receptor *N*) that confers resistance against TMV stays under nta-miR6019 and nta-miR6020 regulation. It was also shown that miRNA repression of *N* attenuates resistance to TMV (Li et al., 2012). Eight miRNA families were identified from *Solanaceae* (tobacco, tomato and potato) that may target R genes (Li et al., 2012). In summary miRNA-regulated R genes participate in a non-race-specific immunity mechanism where the miRNAs are the sensors of the infection. It is supposed that release of R-gene based defense may be the cause of the inhibitory action of pathogen-encoded suppressors of silencing (VSRs and bacterial suppressor proteins, BSR) on miRNA activity during infection, however this assumption needs to be experimentally tested in the future. It was shown, however, that in specific *Nicotiana* species the presence of p19 activates extreme resistance (ER) to protect tissues against TBSV and siRNA binding of p19 was necessary for ER (Sansregret et al., 2013). TAV 2b suppressor was found to elicit hypersensitive reaction (HR) when expressed in tobacco from the unrelated TMV genome (Li et al., 1999). Whether the R proteins are the monitors of the VSR presence or activity remains to be established.

Connecting antiviral silencing to hormone signaling

Several studies have shown that antiviral silencing might be connected to signal transduction pathways responsible for induction of salicylic acid (SA)-mediated resistance (Alamillo et al., 2006; Ji and Ding, 2001). SA is a plant hormone that is involved in local and systemic antiviral defense responses including systemic acquired resistance (SAR). SA induces expression of key antiviral silencing

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factor RDR1 (Liao et al., 2013; Xie et al., 2001). In turn, RDR1 affects many jasmonic acid (JA)-regulated genes (Pandey et al., 2008). JA was implicated as a defense-related hormone (Lewsey et al., 2010). VSRs seem to interfere with hormone signaling-based responses, although the precise mechanisms are elusive. It was shown that CaMV P6 suppressor inhibits SA-induced gene expression through NPR1 (Laird et al., 2013; Love et al., 2012). Upon CMV but not CMVΔ2b suppressor mutant strain infection the SA levels accumulated (Lewsey et al., 2010; Zhou et al., 2014). 2b was shown to have a dual action: it inhibited expression of few SA-regulated genes but in the same time enhanced the effect of SA on others. Remarkably, 2b changes the expression of 90% of jasmonic acid (JA)-regulated genes (Lewsey et al., 2010). 2b protein is known to affect RDR1 activity (Diaz-Pendon et al., 2007) that may explain partly the 2b effect on JA-signaling. HC-Pro was also shown to interfere with JA-regulated transcript expression (Endres et al., 2010). TCV p38 has been shown to interact with a NAC transcription factor called TIP in *Arabidopsis*. It was suggested that p38 interaction with TIP alters defense signaling to favor enhanced TCV invasion (Donze et al., 2014). VSRs therefore emerge as regulators of hormone-based signaling to create favorable conditions for the virus. Although at the moment the complex interplay between the RNA silencing and SA-mediated defense is elusive VSRs might be important coordinators of this crosstalk during infection.

VSRs as tools

Unraveling molecular basis of silencing itself

Due to their versatility and availability with a wide range of actions covering theoretically every aspect of RNA silencing, VSRs can be used to unravel the molecular mechanisms of both antiviral and endogenous RNA silencing pathways. Transgenic expression of cytoplasmic and nuclear p19 variants has brought evidence for nuclear DCL activity (Papp et al., 2003). Employing B2 and the cytoplasmic and nuclear variants of p19 it was suggested that a portion of cytoplasmically located endo-siRNAs are translocated back into nucleus to induce chromatin modifications. B2 or the nuclear p19 but not cytoplasmic p19 suppressed silencing of heterochromatic gene markers and altered H3K9 methylation and distribution of histone modifying complexes in flies (Fagegaltier et al., 2009). p19 size-specific dsRNA binding capacity was crucial to define the 21 nt ds-siRNAs but not long dsRNA precursors as mobile silencing signals (Dunoyer et al., 2010). The differential effects of transgenically expressed VSRs in *Arabidopsis* on miRNA and siRNA pathways have shown that these pathways are only partially overlapping (Dunoyer et al., 2004). Employing TCV p38 it was shown that DCLs work in a hierarchical manner and that there is a complex autoregulation of silencing pathways based on DCLs- and AGOs-activity feedback loops (Deleris et al., 2006; Mallory and Vaucheret, 2009). As TCV p38 could prevent siRNA but not miRNA loading, it was suggested that AGO1 is located in two distinct cellular pools (Dunoyer et al., 2010). The use of HC-Pro has led to discovery of endogenous regulators of silencing like rgsCAM following a yeast-two hybrid assay (Anandalakshmi et al., 2000). The study of poleroviral P0 and potexviral p25 has unraveled the degradation pathways responsible for AGO protein turnover both in a nonviral and viral context (Chiu et al., 2010; Derrien et al., 2012). By using P0, a VSR that destabilizes exclusively the unloaded AGO protein, the half-life of AGO/RISC complex was estimated to be in the range of 2–3 days (Csorba et al., 2010). Besides the study of VSRs helped to start understanding the complex multilateral connections of RNA- and protein based immunity and hormone regulation during host-pathogen interactions.

VSRs employed in molecular research

Apart of these, VSRs can be also employed as molecular biology tools in research. P19 was used to probe the si- and miRNA content of cellular milieu following immunoprecipitations. The use of P19 provided sensitive detection without the need for amplification (Qavi et al., 2010). A surface plasmon resonance (SPR)-based miRNA sensing method was developed where the RNA probes are immobilized on gold and p19 recognize and binds the miRNA: probe duplexes. This allowed detection of miRNA at nanomolar range. Coupled to immunoassay p19 aided miRNA detection at femtomole sensitivity (Nasheri et al., 2011). Similarly P19 was used in protein-facilitated affinity capillary electrophoresis for miRNA detection in blood serum (Berezovski and Khan, 2013). These high-sensitivity methods are very powerful especially because there is no amplification bias in contrary to the PCR-based techniques.

VSRs as biotechnological and medical tools

VSRs also emerge as important and useful tools for biotechnology and medicine. RNA silencing impairs or limits the use of transgenic plants in numerous biotechnological applications. VSRs can be employed efficiently to limit transgene silencing and attain consistently high-level expression of diverse products like vaccines and pharmaceuticals, high-nutritive foods, high-value products etc. (Kanagarajan et al., 2012; Naim et al., 2012).

Conclusions and perspectives

VSRs regulate the multiple layers of the complex defense, counter-defense and counter-counter-defense arm race between host and pathogen. Although the study of VSRs was at the frontline of investigations for more than ten years, many aspects of VSR' molecular behaviors are still elusive. It is becoming more obvious now that VSRs are not just simply blockers of RNA silencing but serve as central hub regulators to dynamically integrate connections between antiviral silencing, protein-based immunity, hormone signaling, RNA metabolism and subcellular organization (Pumplin and Voinnet, 2013). As most suppressor proteins have parallel functions, the silencing function and the non-silencing activities (*e.g.* coat protein, movement protein, replicase, protease etc.) need to be synchronized in order to fulfill these multiple tasks and achieve "optimal" infection. In most cases, how the silencing function and the other functions of VSRs are selected and how the interconnections between the alternative functions are maintained is not known. We are just at the beginning to appreciate the complex regulatory network involving VSR multiple activities and there are still several unanswered questions. More detailed information is needed about viral replication, subcellular localization of viral proteins including VSRs and their interaction with host factors. Very little is known about not just "antiviral" – but the "endogenous" – RISC complex as well. The relatively recently developed *in vitro* system may help to better understand the RISC components and activities. In exploring RISC complex VSRs such as P0, p38 or P1 may be powerful tools in the future research. It was suggested that some VSRs facilitate viral long-distance movement by suppressing the spread of antiviral silencing (*e.g.* p19 sequesters the signal, 2b or p38 blocks the production of the signal). Understanding antiviral systemic signaling is a very important but still unknown aspect of host defense. The assumption that vsiRNA move throughout the plant to convey immunity still awaits direct experimental validation. Several studies have shown that antiviral acquired resistance can be passed transgenerationally to offspring through siRNAs and RdDM by the use of virus-induced TGS (VITGS) (Kanazawa et al., 2011; Kon and Yoshikawa, 2014; Martín-Hernández and Baulcombe, 2008; Otagaki et al., 2011). How VSR activities could modulate/interfere with

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resistance "priming" in subsequent generations is not known. VITGS could provide a useful technology for plant breeding via epigenetic modifications that needs to be explored in the future.

In spite of its paramount economical importance viral symptom development is a poorly understood aspect of virus infection process. How does VSR presence/activities relate to viral symptom development? Due to their effect on accumulation and function of endogenous small RNAs it is supposed that VSRs could be major contributors to the development of viral symptoms. It was shown however that VSRs might be dispensable for disease induction (Diaz-Pendon et al., 2007), therefore, answering this question need more investigation. Most of the knowledge on VSRs and antiviral silencing comes from studies in model organisms like *Arabidopsis* that may miss important aspects of the authentic virus infection and host defense responses that occur in nature. The recent development of genetic tools such CRISPR/Cas9 technology will allow us to generate specific mutation in several other plant species including important virus hosts (*e.g. N. benthamiana*) and economically significant crops to extend our knowledge on RNA silencing.

The use of natural host-virus pairs would be an important track to follow in the future in order to be able to understand and ultimately control viruses that cause huge economical losses on crop plant worldwide.

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