Suppressors of RNA silencing encoded by geminiviruses and associated DNA satellites

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Abstract In plants, RNA silencing provides a major line of defence against viruses. This antiviral immunity involves production of virus-derived small interfering RNAs (vsiRNAs) and results in specific silencing of viruses by vsiRNAs-guided effector complexes. As a counter-attack against RNA silencing, many plant viruses encode suppressors of RNA silencing called viral suppressors of RNA silencing (VSRs), which interfere with the silencing pathway by various mechanisms. This review describes various methods that are being used to characterize viral proteins for suppressor function, VSRs found in geminiviruses and associated DNA satellites and their mechanisms of action.

Keywords Geminivirus · RNA silencing · Viral suppressors of RNA silencing (VSRs)

Introduction

Geminiviruses have circular, single-stranded DNA genome (ssDNA), the size of which can range from ~ 2.5 to 3.0 kb [29]. The family Geminiviridae has been classified recently to comprise nine genera: *Capulavirus*, *Becurtovirus*, *Begomovirus*, *Curtovirus*, *Eragrovirus*, *Grablovirus*, *Mastrevirus*, *Topocuvirus* and *Turncurtovirus* [74]. They are transmitted by insects and infect a wide range of plant species including some of the major food and fibre crops. The genomic DNA is encapsidated within twinned

(geminate), incomplete, non-enveloped, icosahedral particles of size ~ 18–30 nm. Geminiviruses can either be bipartite (two DNA molecules, DNA-A and DNA-B (only in genus *Begomovirus*) or monopartite (one DNA molecule analogous to DNA-A) [30]. Monopartite viruses encode all the proteins essential for successful infection, whereas, in bipartite viruses proteins required for viral DNA encapsidation and replication and a few other functions related to gene expression and RNA silencing suppression are encoded by DNA-A, the functions related to viral movement are encoded by DNA-B. The genes or Open Reading Frames (ORFs) are named according to the coding strand (V-sense or C-sense) and the genomic component.

Plant defence against virus attack: RNA silencing

RNA silencing is a generic term given to diverse yet related pathways, mediated by ~ 21–30 nucleotide double-stranded RNA molecules [small (s) RNAs], leading to regulation of the gene expression in a sequence-specific manner at the transcriptional or translational level. In plants, RNA silencing pathway is a major line of defence against viruses. Upon viral infection, virus-derived siRNAs (vsiRNAs) are formed from dsRNA originating from viral sequences, which then target the viral genome by various mechanisms [22].

Important steps in RNA silencing pathway

The first step in RNA silencing pathway is the formation of partially or perfectly dsRNAs. The latter can arise from various ways, for e.g. from self-complementary fold-back structure [12], imperfect duplexes in the folded regions of genome [41]; convergent, aberrant or RNA induced

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silencing complex (RISC)-mediated cleaved transcripts, stem-loop structure [46]; conversion of cleaved *TAS* transcripts [72]; overlapping region of a pair of natural antisense transcripts [6, 77]; transposons or repetitive genomic regions [40, 55, 73]; or gene/pseudogene duplexes [68]. The second step involves the formation of shorter dsRNAs (siRNAs) from the long dsRNA, by the ‘dicing’ activity of an RNaseIII-like enzyme called Dicer-like (DCL) in plants. In the case of viral infection, the siRNAs that are formed during this step are called ‘primary siRNAs’ as they are derived from the target region. Next, the duplex siRNAs are loaded onto a multi protein RISC or RNA-induced transcriptional silencing complex (RITS) [63], containing a protein from the Argonaute family (AGO). One of the strands from duplex siRNA, called passenger strand, is degraded. The strand, called guide strand, binds to complementary region in the homologous mRNA and initiates either post-transcriptional gene silencing (PTGS), leading to degradation of target mRNA or its translational arrest or transcriptional gene silencing (TGS), involving epigenetic changes to the target DNA [18, 22] (Fig. 1).

Key components of RNA silencing pathway

The key components in RNA silencing pathway include DCL ribonucleases and associated dsRNA binding (DRB) proteins, RNA-dependent RNA polymerases (RDR), and Argonaute (AGO) proteins. The widely studied model plant *Arabidopsis thaliana* has four DCLs (DCL1 till DCL4), five DRBs (HYL1/DRB1 till DRB5), six RDRs (RDR1 till RDR6) and ten AGO proteins (AGO1 till AGO10) [4, 5, 22, 34, 38, 46]. The functions of the various components of RNAi pathway mentioned above have been recently reviewed [19, 75].

Virus counter-defence against RNA silencing: viral suppressors

As has already been discussed in the preceding section of this review, RNA silencing serves as a major defence mechanism that plants employ to protect themselves against viral infections. Therefore, as a counter-defensive strategy many plant viruses encode proteins, known as viral silencing suppressors (VSRs) that interfere with RNA silencing pathway at one or the other step. VSRs are also found in viruses infecting insects, fish, mammals etc. Most of the plant viruses encode at least one VSR. In addition, several studies showed that mutations giving rise to non-functional VSRs led to increased resistance against viral infection in the host plant. These points emphasize the importance of RNA silencing as a major line of defence against viruses. Apart from acting as suppressors, most of

the VSRs are involved in functions that hold importance in viral life cycle like replication, movement, encapsidation, transmission etc. Since the discovery of first viral suppressor, the potyviral P1/Hc-Pro, numerous other VSRs have been discovered [2, 19, 45]. The following sub-sections will describe various methods that have been used to characterize viral proteins for suppressor function, some of the VSRs and their mechanism of action.

Assays for the identification of VSRs which suppress PTGS

Transient expression assays

This method involves the use of transgenic *Nicotiana benthamiana* plants (16c) constitutively expressing the reporter gene GFP [51]. This assay employs the technique of co-infiltration of two *A. tumefaciens* strains to the GFP-expressing transgenic plant; one carrying a construct having the GFP gene (inducer of silencing) and the other carrying the candidate viral suppressor gene [37], both the inducer and the candidate suppressor genes under strong constitutive promoters. In the assay for local silencing both the agrobacterium suspensions are co-infiltrated into a leaf and the infiltrated patch is observed for GFP expression over a period of time. The infiltrated patch initially appears green (under ultraviolet illumination) as it expresses high levels of GFP, but turns red in 3–5 days because the inducer triggers silencing of the transgenically-expressed GFP; the red colour resulting due to chlorophyll autofluorescence. If the candidate gene possesses a suppressor activity, the silencing is reversed and the GFP expression does not decrease and the patch retains its green colour.

In the assay for systemic silencing, both the agrobacterium constructs are infiltrated in the leaves of 16c plants and newly-emerging leaves are observed for changes in GFP expression after about 3 weeks. In this particular case, the GFP-inducer construct triggers GFP silencing in the infiltrated leaf and the silencing signal travels systemically, resulting in the emerging leaves appear red. However, if the candidate gene possesses a suppressor activity, such that it prevents the generation and/or spread of the silencing signal, the emerging leaves will appear green.

To assay whether the VSR suppresses the movement of silencing signal, a grafting experiment is generally undertaken. A root-stock, where the silencing of a transgenic reporter gene is initiated by infiltration of the same gene (inducer, as described above), is then used to graft a scion on it, in which the same reporter gene is expressed transgenically. The silencing signal initiates in the root-stock, spreads through the graft to the scion and silences the reporter gene in it. The same effect is seen if the scion is separated from the root-stock by an “intergraft” (middle

Fig. 1 The main steps in RNA silencing pathway where geminiviral suppressors have been shown to act. The dsRNA arising from various sources is cleaved into ~ 21–30 nt small RNA duplexes (siRNA/miRNA). One of the strands in this duplex, called the guide strand is incorporated in the RISC complex containing an AGO protein. This

siRNA then guides the AGO protein to the target sequence and mediate the gene silencing by various mechanisms. The RDRs work to amplify/maintain the gene silencing. The red arrow depicts the inhibition of the step shown in the figure (see text for more details) (colour figure online)

insert), taken from a wild-type plant, through which the silencing signal can pass and silence the reporter gene in the scion. However, if the intergraft is taken from a plant expressing the VSR capable of blocking the movement of the silencing signal, the reporter gene expressed in the scion is no longer silenced [28, 39].

Stable expression assays

This assay can be used when any of the above assays confirm the suppressor activity of a protein. It exploits two stable transgenic lines: one expressing the candidate suppressor and a line silenced for any well-characterized

reporter gene. These lines are then crossed and progeny is tested for the reporter gene expression. The reporter expression will be restored if the putative suppressor eliminates the silencing [2].

Assays for VSRs which suppress TGS

For assaying TGS suppressors, *N. benthamiana* 16-TGS lines are used. These plants carry a reporter gene (usually GFP) under the control of a cauliflower mosaic virus (CaMV) 35S promoter, which is transcriptionally silenced (by TGS) after infection with a tobacco rattle virus-based vector, which in turn, carries a portion of the CaMV 35S promoter, the inducer of silencing. The silenced promoter cannot drive the expression of GFP and hence the plant appears red under UV illumination. In the presence of TGS suppressor, the promoter becomes active and starts expressing GFP, resulting in the plant showing green fluorescence under UV illumination [11, 64].

Mechanism of action of geminiviral VSRs

VSRs can have diverse mode of action and can interfere with almost any step of silencing pathway. A single VSR can have multiples modes of action as will be evident in following section. Table 1 lists some of the important VSRs and their modes of action.

V2 protein

The V2 ORF (AV2 in bipartite begomoviruses) codes for pre-coat protein. V2, along with CP, is required for efficient accumulation of viral DNA forms and therefore, facilitates successful infection in the host [7, 47, 67]. It is also required for efficient viral movement [43, 50]. V2 can suppress both PTGS and TGS. *Tomato yellow leaf curl virus* (TYLCV) V2 protein interacts with the host SGS3 (SUPPRESSOR OF GENE SILENCING 3) which assists RDR6 in the production of dsRNA. The interaction between V2 and SGS3 affects the amplification step of silencing [26]. V2 protein encoded by *Tomato yellow leaf curl China virus* possibly sequesters the siRNAs generated against the virus and hinders the silencing pathway [76]. In a similar way it has been speculated that *Cotton leaf curl Multan virus* (CLCuMV) V2 also sequesters long dsRNA and prevents its dicer-mediated cleavage [1]. The TYLCV V2 also manifests its function as a suppressor by interfering with the innate immunity of the plant by binding to CYP1 protein, a member of the family of papain-like cysteine proteases [3]. V2 has been shown to elicit a hypersensitive response (HR) and acts as a pathogenicity determinant in *Tomato leaf curl Java virus-A* [53]. In

addition to blocking PTGS, V2 can also act as TGS suppressor as has been demonstrated in TYLCV [64].

Replication-associated protein (AC1, C1)

The replication-associated protein (Rep), encoded by C1 or AC1 ORF, plays an indispensable role in viral replication [24]. Rep has been reported to be involved in all the major steps of rolling-circle replication (RCR). Apart from its long recognized role in replication, a recent study implicated Rep as a TGS suppressor, whereby, it was shown to reduce the expression of plant DNA methyl transferases such as METHYLTRANSFERASE 1 (MET1) and CHROMOMETHYLASE 3 (CMT3) [49]. The Rep protein of *Wheat dwarf virus* (WDV) has been shown to interfere with silencing by binding to 21 and 24 nt siRNA duplexes and ss-siRNA [66].

Transcriptional activator protein (AC2, C2)

The C2 protein [also known as AC2, AL2, L2 or transcriptional activator protein (TrAP)] functions to activate viral gene expression. The C2 has also been shown to act both as PTGS and TGS suppressor. The C2 protein functions as a PTGS suppressor in *Tomato yellow leaf curl virus-China* [69, 70], *East African cassava mosaic Cameroon virus* [62], *Mungbean yellow mosaic virus-Vigna* (MYMV-Vig) [61] and CLCuMV [1]. It has been hypothesized that MYMV-Vig AC2 can alter the expression levels of the host genes involved in gene silencing to bring about its suppression activity. In addition, C2 inhibits the components of methylation machinery such as SUCROSE NONFERMENTING1 (SNF1) Kinase [31], adenosine kinase [11, 65] and certain histone methyltransferases [13]. Therefore, AC2 acts as TGS suppressor. C2 encoded by *Beet severe curly top virus* also suppresses TGS but with a different mode of action. It attenuates the 26S proteasomal degradation of S-adenosyl-methionine decarboxylase 1, consequently interfering with the DNA-methylation based gene silencing [78].

AC4/C4 protein

The C4 ORF lies entirely within the C1 ORF, however, in a different reading frame. AC4 (bipartite begomoviruses)/C4 (monopartite begomoviruses) ORF is among the least conserved begomoviral ORFs and has been shown to be associated with diverse array of functions, some of them being symptom determination, viral DNA accumulation and virus movement [33, 48, 50]. AC4/C4 has also been proved to act as RNA silencing suppressor in both bipartite and monopartite begomoviruses. The AC4 encoded by ACMV specifically binds to the single stranded form of

Table 1 A List of some of the important viral suppressors of RNA silencing encoded by geminiviruses and associated DNA satellites, and their modes of action

VSR	Genus	Species	Mode of action	Reference
AV2	Begomovirus	EACMCV	Unknown	[17]
V2	Begomovirus	TYLCV-Is	Acts at a step downstream of dicer-mediated cleavage of dsRNA	[79]
		ToLCJV-A	HR elicitor	[53]
		AYVV	Unknown	[54]
		TYLCCNV	Sequestration of siRNA molecules	[76]
		CLCuMV	Sequestration of long dsRNA	[1]
		TYLCV	Interaction with SGS3; Binding to CYP1 protein and hindering innate immunity of plant; TGS suppressor	[3, 26, 64]
Rep	Begomovirus	TYLCSV; TYLCV-Mld, ACMV, TGMV	Decrease the expression of MET1 and CMT3	[49]
	Mastrevirus	WDV	siRNA binding	[66]
TrAP	Begomovirus	TYLCV-C	Unknown	[69, 70]
		EACMCV	Unknown	[62]
		ICMV	Unknown	[62]
		MYMV-Vig	Alteration of host gene expression levels	[61]
		BYVMV	Unknown	[27]
		AYVV	Unknown	[54]
		CLCuMV	Unknown	[1]
		BSCTV	Inhibits proteasomal degradation of SAMDC1	[78]
		TGMV, CaLCuV	Genome-wide reduction in cytosine methylation; inhibit histone methyl transferases	[11, 13, 65]
		ACMV	Alteration in host gene expression	[57]
AC4/C4	Curtovirus	BCTV	Inhibit SNF1 Kinase and ADK	[31]
	Begomovirus	ACMV	Binds to ss-sRNA	[16]
		EACMCV	Unknown (might interfere with spread of silencing)	[25]
		BYVMV	Unknown	[27]
		ToLCV	Unknown (might interact and inhibit activity of shaggy-like kinase and, in turn, modify brassinosteroid signalling)	[23]
		AYVV	Unknown	[54]
		MYMV	Binds to sRNA	[60]
		CLCuMV	Binds to long dsRNA	[1]
β C1 protein	Betasatellite	TYLCCNB	Inhibit a methyl cycle enzyme, SAHH	[20, 71]
		BYVMB	Unknown	[27]
		ToLCJB	Unknown	[35]
		CLCuMB	Unknown	[1]
		TYLCCNB	Rgs-CaM mediated suppression of RDR6 expression	[36]

EACMCV: *East African cassava mosaic Cameroon virus*; TYLCV-Is: *Tomato yellow leaf curl virus- Israel*; ToLCJV-A: *Tomato leaf curl Java virus-A*; AYVV: *Ageratum yellow vein virus*; TYLCCNV: *Tomato yellow leaf curl China virus*; CLCuMV: *Cotton leaf curl Multan virus*; TYLCSV: *Tomato yellow leaf curl Sardinia virus*; TYLCV-Mld: TYLCV, mild strain; ACMV: *African cassava mosaic virus*; TGMV: *Tomato golden mosaic virus*; WDV: *Wheat dwarf virus*; TYLCV-C: *Tomato yellow leaf curl virus-China*; ICMV: *Indian cassava mosaic virus*; MYMV-Vig: *Mungbean yellow mosaic virus-Vigna*; BYVMV: *Bhendi yellow vein mosaic virus*; BSCTV: *Beet severe curly top virus*; CaLCuV: *Cabbage leaf curl virus*; BCTV: *Beet curly top virus*; ToLCV: *Tomato leaf curl virus*; TYLCCNB: *Tomato yellow leaf curl China betasatellite*; BYVMB: *Bhendi yellow vein mosaic betasatellite*; ToLCJB: *Tomato leaf curl Java betasatellite*; CLCuMB: *Cotton leaf curl Multan betasatellite*

sRNA [16]. The MYMV-AC4 binds to sRNAs and inhibits cleavage of target mRNA [60]. The CLCuMV-C4, on the other hand, binds both long and short RNAs, with a preference for the dsRNA form [1].

β C1 protein

Betasatellites are circular ssDNAs of ~ 1.35 kb found to be associated with monopartite begomoviruses

[14, 15, 21, 32, 52, 58]. However, more recently it has been reported that Cotton leaf curl Multan betasatellite (CLCuMB) contributed to the development of typical leaf curl symptoms when inoculated with DNA-A of a bipartite begomovirus *Tomato leaf curl New Delhi virus* (ToLCNDV) [56]. DNA β sequence can be demarcated into four different regions namely (1) a conserved stem-loop structure with conserved nonanucleotide sequence, (2) a region of high sequence similarity conserved termed as 'satellite conserved region' (SCR), (3) an adenine (A) rich region approximately 370–420 nucleotides (nts) upstream of the SCR, (4) a single gene encoded by the complementary strand, β C1 [8, 10, 59]. The β C1 proteins from several betasatellites have been shown to act as RNA silencing suppressors. Some of the examples include *Ageratum yellow vein virus*-Indonesia, Bhendi yellow vein mosaic betasatellite (BYVMB), Tomato leaf curl Java betasatellite (ToLCJB), CLCuMB [1, 27, 35, 54]. The β C1 gene of Tomato yellow leaf curl China betasatellite (TYLCCNB) has the ability to suppress TGS by interacting with a methyl cycle enzyme, S-adenosyl homocysteine hydrolase, which is required for methylation [20, 71]. The TYLCCNB encoded β C1 has been reported to enhance the expression of *N. benthamiana* calmodulin-like protein Nbrgs-CaM. The enhanced expression of Nbrgs-CaM hindered the production of secondary siRNAs, likely through inhibiting the expression of RNA-dependent RNA polymerases 6 (RDR6) [36].

Alphasatellites

Alphasatellites are found to be associated with both old world monopartite begomoviruses and new world bipartite begomoviruses [9, 44]. Similar to betasatellites, they possess circular, ssDNA genome of approximately half the size of their helper begomoviruses. They contain a single gene which encodes replication-associated protein (Alpha-Rep). The Alpha-Rep proteins from two alphasatellites, *Gossypium darwinii* symptomless alphasatellite and *Gossypium mustelinum* symptomless alphasatellite have been reported to act PTGS suppressors [42].

Concluding remarks

Geminiviruses are the largest family of plant viruses and are becoming increasingly important as pathogens infecting crop plants in the tropical and sub-tropical regions of the World. A better understanding of the mechanisms of action of geminiviral VSRs can give researchers new insights on the management of geminiviruses, as a means of improving the production of food and fibre crops in many parts of the World. This can also lead to a better

understanding of the plant defence mechanisms, especially the small RNA-based defence.

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