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REVIEW

Biochemical Mechanisms of Suppression of RNA Interference by Plant Viruses

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Abstract—RNA interference (RNAi) plays an important biological role in regulation of gene expression of eukaryotes. In addition, RNAi was shown to be an adaptive protective molecular immune mechanism against viral diseases. Antiviral RNAi initiates from generation of short interfering RNAs used in the subsequent recognition and degradation of the viral RNA molecules. As a response to protective reaction of plants, most of the viruses encode specific proteins able to counteract RNAi. This process is known as RNAi suppression. Viral suppressors act on various stages of RNAi and have biochemical properties that enable viruses to effectively counteract the protective system of plants. Modern molecular and biochemical investigations of a number of viral suppressors have significantly expanded our understanding of the complexity of the nature of RNAi suppression as well as mechanisms of interaction between viruses and plants.

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Previously known as post-transcriptional gene silencing (PTGS) in plants, RNA interference (RNAi) is a process that plays a key role in regulation of gene expression. In higher plants, RNAi is a natural molecular constituent of tolerance leading to selective recognition of viruses and their subsequent degradation.

The initial starting mechanism of RNAi is synthesis of long double-stranded RNA (dsRNA) [1]. The next functional step of RNAi is the action of the Dicer enzymes (Dicer-like DCL) (members of the RNase III group) catalyzing formation of 20-30-nucleotide-long short interfering RNAs (siRNAs) or microRNAs (miRNAs) with 2-nucleotide-long sticky 3′-ends [2, 3]. These small RNA molecules can be generated as a result of enzymatic hydrolysis of long replicative forms of viral RNA as well as transgenes and transposons [4, 5]. During viral infection of plants, siRNAs can be generated directly from the viral genome, but there are data obtained indicating participation of RNA-dependent RNA polymerases (RDRP) in amplification of the key RNA molecules [3-6].

The latest plant studies have shown that enzymatic methylation of siRNAs also plays an important functional role in providing stability of these molecules from oligouridylation and subsequent degradation [7]. The siRNAs are methylated on the 3′-ends, and this enzymatic modification is catalyzed by methyltransferase (HEN1) [8, 9].

In the next step of RNAi, double-stranded siRNAs unwind and one of the strands incorporates into multicomponent effector complex (RNA-induced silencing complex (RISC)) and functions as "searching template" for recognition complementary nucleotide sequences of specific transcripts with subsequent enzymatic hydrolysis and translational repression [3]. Complementary pairing between siRNA nucleotides and the RNA provides effective and high-specificity location of the target of interest. In work [10], it is shown that siRNAs and the proteins of the Argonaut (AGO) family are universal RISC components. AGO proteins are characterized by the presence of specific conservative domains called PAZ and PIWI [11]. Structural investigations revealed that the PAZ domain directly interacts with siRNAs [12]. Moreover, the PAZ domain has been established to interact with the 3′-ends of siRNAs. The PIWI domain in the AGO proteins is a key catalytic center as it possesses endonuclease activity [10, 13].

As a response to RNAi, viruses have developed specific strategies for counteraction to the molecular mechanism of immune resistance of plants. The most effective and active countermeasure against RNAi is viral suppression of the molecular immunity. For instance, most viruses encode specific suppressor proteins able to effectively block RNAi. Expression of the suppressors by viruses for

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counteraction to the protective system of plants reasonably suggests that, initially, the function of RNAi in plants was to counteract the viral pathogens [14].

Many viral proteins currently known as suppressors were initially determined as pathogenicity or virulence factors since their expression determines to a large extent formation and severity of the viral disease symptoms [15]. Expression of these proteins is usually not an obligatory factor for viral replication, but the viral suppressors are necessary for successful accumulation and distribution during the infection [16]. A large number of viral proteins possessing suppressor activity have been found. However, descriptions of their biochemical mechanisms have appeared rather recently in the literature. The latest molecular, biochemical, and structural studies of various viral suppressors have enabled examination of RNAi suppression mechanisms in detail. A common property of all the viral suppressors is ability to counteract the RNAi protective system in its different stages. This counteraction is a striking example of complex and intense "evolutionary struggle" between viruses and plants [17]. In addition, co-evolution between the viral suppressors and the RNAi mechanism of plants indicates the extremely complex nature of adaptation of viruses to the protective system of plants.

The goal of the present review is to generalize the current data about molecular and biochemical mechanisms of RNAi suppression by several plant viruses.

Potyvirus HC-Pro. Viruses of the Potyviridae family encode the HC-Pro suppressor (helper component proteinase), which is a classical example of a viral multifunctional protein responsible for successful systemic distribution of viruses of this family in an infected organism. Numerous biological processes in which the HC-Pro functionally participates are viral replication, systemic and intercellular transport, and protein cleavage of the viral polyprotein [18-20]. However, the most important biological function of HC-Pro is its participation in RNAi suppression. The first settled but still indirect proof that HC-Pro participates in RNAi suppression was an observation that in transgenic plants expressing the 5′-end segment of the tobacco etch virus (TEV) genome encoding the P1/HC-Pro sequence, the disease symptoms intensified during infecting with other viruses [21]. Subsequent independent studies have shown that the protein is the core factor in RNAi suppression in infected plants [15, 22, 23]. Further mutational analysis of the virus revealed that the central region of HC-Pro is necessary for suppressor activity, while its N-terminal part is not crucial for this function [24]. An observation that HC-Pro interacts with the rgsCaM protein, which is an endogenous RNAi suppressor in plants [25], is quite interesting.

Further, it was suggested that the mechanism of action of HC-Pro consists of the inhibition of DCL, since transgenic expression of the viral protein in plants is connected with accumulation of long unwound dsRNAs [26, 27]. It was also shown that HC-Pro expression leads to defects in growth and differentiation of *Arabidopsis* plants, presumably due to inhibition of miRNA-associated hydrolysis of RNA of transcription factors [28]. Thus, functional connection between molecular factors involved in growth and differentiation processes on one hand and antivirus RNAi on the other hand was first established. Moreover, the reason for the appearance of the disease symptoms in infected plants as a result of viral suppressor expression was first suggested [29].

Biochemical investigations of HC-Pro have revealed that its ability to form dimers and multimers is critical for its function as RNAi suppressor [30]. In addition, the suppressor function of HC-Pro can be also connected with decrease in stability of siRNA, since transgenic protein expression leads to significantly reduced 5′-end modification of the viral 21-nucleotide-long siRNAs [31]. Moreover, HC-Pro was revealed to impede functional methylation of mi/siRNA [32] and binding of ds siRNA [33]. Recent studies have revealed a functional role of the FRNK region within the HC-Pro structure for binding siRNA and shown that this function is correlated with selective binding of miRNAs and the severity of the viral infection symptoms [34].

Tombusvirus P19. Earlier genetic studies of the P19 protein encoded by the viral genome of the Tombusviridae family showed the participation of the protein in the processes of reproduction, transport, RNA packaging, and vector transmission of the virus [35]. Protein P19 was subsequently found to be a significant pathogenic factor required for the progress of infection symptoms [36]. For instance, P19 of the tomato bushy stunt virus (TBSV) does not significantly affect the initial stages of the infection in *Nicotiana benthamiana* plants, but it is necessary for systemic invasion in other organisms such as pepper (*Capsicum annum*) and spinach (*Spinacia oleracea*) [37, 38]. Participation of P19 in RNAi suppression was first demonstrated on transgenic plants expressing green fluorescent protein (GFP) and infected by potato virus X (PVX), which was used as the expression vector of P19 [39]. Further investigations demonstrated a crucial role of TBSV P19 in protection of the viral RNA during systemic infection in *N. benthamiana* plants [40, 41]. Moreover, the biological activity of the protein depended on its quantity, i.e. successful infection, severity of symptoms, as well as stability of the viral RNA require quite high level of P19 expression [41, 42].

Structure determination is likely to provide the clearest explanation of protein function. X-Ray crystallographic investigations, carried out by two independent research teams, have indicated that there is a complex between P19 dimers and ds siRNA molecules [43, 44]. These structural studies have suggested the first explanation of the possible molecular mechanism of the viral suppressor during RNAi suppression. Moreover, direct physical interaction between P19 and the viral siRNAs was

also found *in planta*, i.e. in infected plants [45, 46]. These studies have revealed a correlation between the ability of P19 to effectively bind siRNA and the severity of the viral infection symptoms in several plants [47].

So the function of P19 as a viral suppressor is that, during infection, it binds abundantly circulating viral siRNAs making them inaccessible for programming of RISC, the activity of which is directed toward cleavage of the viral RNA. As a result, viral RNA molecules accumulate in the infected organism. The proof supporting this model is the fact that infection of *N. benthamiana* with mutant TBSV defective in P19 is associated with the presence of the RISC complex containing viral siRNAs and possessing specific ribonuclease activity in plants [48, 49]. P19 has also been demonstrated to prevent protective methylation of miRNAs [32]. Thus, there is a reason to assume that the ability of P19 to bind siRNA can prevent activity of the HEN1 enzyme responsible for siRNA methylation, which has been demonstrated for several other viral suppressors.

Cucumovirus 2b. Like HC-Pro, the 2b protein encoded by Cucumovirus family viruses is one of the first discovered RNAi suppressors. In experiments with the *GFP* transgene, it was demonstrated that 2b expression counteracts RNAi [15]. Investigations with suspended tobacco cells and whole plants showed the presence of arginine-rich nuclear localization signal (NLS) in the 2b structure, which is responsible for localization of the protein in the nucleus. Mutations in the NLS lead to decrease in suppressor activity of the protein, indicating the necessity of nuclear localization of 2b for realization of the suppressor function [50]. Further investigations demonstrated that 2b blocks the distribution of the RNAi cellular signal and inhibits DNA methylation in the nucleus [51]. It is of interest that 2b also participates in inhibition of viral tolerance of plants through salicylic acid, but how this process relates to the suppressor function of the protein remains unclear [52].

Recent studies have revealed that 2b expression significantly decreases accumulation of 21-, 22-, and 24-nucleotide-long types of siRNA, the generation of which is catalyzed by DCL4, DCL2, and DCL3, respectively [53]. Moreover, the absence of infectiveness of the virus defective in 2b is compensated in plants having double mutations *dcl2* and *dcl4* defective in synthesis of 21- and 22-nucleotide-long siRNAs [53].

RNAi suppression by the 2b protein is also associated with binding of siRNA molecules. The 2b protein of a mutant variant of CMV having one amino acid substitution in 2b and weak plant infection was shown to be significantly defective in ability to bind siRNA [54]. This fact might indicate that the ability of the viral suppressor to strongly bind siRNA is a critical pathogenic factor of the virus.

Recent studies have demonstrated that, in contrast to most known viral suppressors, 2b directly interacts with the catalytic center of the RISC AGO1 *in vitro* and *in vivo* [55]. Moreover, it was found that the 2b–AGO1 interaction leads to specific inhibition of enzymatic hydrolysis by the RNA nuclease complex.

The ability of 2b to directly interact with the RISC and lower its activity is a striking example of complexity of cooperative evolution and adaptation of plants and viruses.

Polerovirus P0. The beet western yellow virus (BWYV), a representative of the Poleroviridae family, encodes the P0 protein, which is a powerful RNAi suppressor. It was first found in experiments with agro-infiltration of the viral protein into leaves of GFP transgenic *N. benthamiana* plants [56]. Further studies did not reveal RNA binding ability of P0, but the protein was found to interact with a homolog of S-phase kinase-related protein 1 (SKP1), which is a component of the SCF family of ubiquitin E3 ligase [57]. This interaction involves the so-called F-box domain of the viral protein. Point mutations in the F-box impede interaction with the SKP1 homolog and, at the same time, decrease the pathogenicity of the virus [57]. So silencing of the SKP1 homolog gene expression in *N. benthamiana* plants leads to tolerance of the plant to the viral infection.

It was later found that P0 expression in transformed *Arabidopsis* plants leads to various abnormalities in plant development and high level of several miRNA-targeted transcripts, indicating that P0 acts at the level of the RISC [58]. It is quite interesting that P0 expression leads to degradation of AGO1 protein *in planta* and P0 directly interacts with AGO1 [58]. Parallel biochemical investigations of the mechanism of RNAi suppression by P0 demonstrated that the F-box protein interacts with PAZ domain in AGO1. F-Box proteins are components of the E3 ubiquitin ligase complexes, which mark the protein for proteasomal degradation [59], and interaction with SKP1 mentioned earlier is likely to be functionally critical. Notably, this interaction is completed by proteolytic degradation of AGO1. However, exactly what leads to AGO1 degradation remains unclear since the process is not sensitive to a specific inhibitor of proteasomal activity [59].

Thus, the ability of P0 to lead to AGO1 degradation is an additional example of the complexity of viral adaptation to the protective mechanism of RNAi.

Tobamovirus replicase. Tobacco mosaic virus (TMV) activates protective RNAi in plants since the viral infection is followed by generation of viral siRNA [60, 61]. The TMV genome encodes a 126-kDa protein responsible for replication and transport of the virus and, as was found later, is involved in RNAi suppression [62]. In addition, the TMV-related tomato mosaic virus (ToMV) encodes a 130-kDa replicase RNAi suppressor protein [63]. Moreover, a single amino acid substitution in the ToMV replicase leads to lack of symptoms during the viral infection.

Biochemical examination of the interaction between the TMV replicase and RNA molecules indicates that the protein can bind the siRNA molecules [61]. Like TMV, another 122-kDa replicase protein of the virus strain (cr-TMV) infecting Cruciferae family plants also can bind 21-nucleotide-long siRNAs and the paired miRNA thereby preventing their incorporation into the RISC [64]. Moreover, it was shown that the binding ability of siRNA does not prevent activity of already programmed RISC, indicating irreversibility of the programming mechanism of the nuclease complex.

Recent studies indicate that TMV infection impedes siRNA methylation by HEN1 methyltransferase [65, 66]. Furthermore, this effect and formation of the diseases symptoms were directly related to expression of the 126-kDa replicase protein [66]. However, whether the suppressor directly affects the HEN1 activity or it participates in demethylation of already premethylated siRNA molecules remains unclear. Of interest and quite contradictory, expression of the 122-kDa replicase protein of cr-TMV is coupled with increased accumulation of siRNA molecules despite its negative effect on HEN1 methyltransferase, the activity of which is necessary for maintenance of stability of siRNA molecules [64].

Closterovirus P21. Earlier studies with the beet yellow virus (BYV) demonstrated that the 21-kDa P21 protein suppresses RNA-induced GFP expression silencing [67]. RNAi suppression was also revealed for the P21 homolog encoded by the other representatives of the Closteroviridae family. Furthermore, in infected plants BYV P21 is found in cells as a soluble cytoplasmic protein as well as in the form of insoluble protein bodies in the cell periphery. Another P21 homolog of the *Citrus tristeza* virus suppresses RNAi on intracellular and intercellular levels [68]. Biochemical examinations demonstrated that the P21 interacts with paired miRNAs and siRNA *in vivo* [29]. The ability of P21 to selectively bind paired siRNAs was confirmed in other studies [33]. Notably, like P19, P21 does not affect the RISC activity but impedes miRNA methylation [32].

Structural studies revealed the interaction of α-helical monomers of P21 protein in which the N- and the C-terminal regions contact with adjacent monomers through symmetrical head-to-head and tail-to-tail interactions [69]. The protein forms octameric rings with a central hollow ~90 Å in diameter and positively charged inner surface of the ring, which is likely to play a role in binding siRNA molecules. Furthermore, it was shown that, in contrast to selective interaction of tombusvirus P19 with the 21-nucleotide-long siRNA molecules, BYV P21 forms a binding surface responsible for electrostatic interaction with the 21-nucleotide-long siRNAs as well as with longer ss- and dsRNAs *in vitro*. It must be suggested that the ability of P21 to bind long dsRNAs involves additional suppressor activity of the protein, possibly at the level of siRNA synthesis.

In conclusion, P21 is a viral RNAi suppressor, which, like the examples discussed earlier, impedes the RISC programming essential for nuclease degradation of viral RNA.

Capsid protein of turnip crinkle virus (TCV). The p38 capsid protein (CP/p38) of turnip crinkle virus (TCV) is another example of a viral protein exhibiting many biological functions. Along with its structural role in the virus formation, this protein was shown to be responsible for systemic distribution and intercellular transport of the virus [70, 71]. Moreover, the TCV CP functions as a critical factor of disease symptom formation during infection as well as affecting modulation of the symptoms in the presence of satellite viral RNA [72].

The first sign of possible participation of TCV CP in RNAi suppression was the observation that this protein makes up the disadvantage of the TBSV P19-defective mutant [40]. Further experiments including the method of *Agrobacterium* infiltration demonstrated that TCV CP is a very strong RNAi suppressor [73, 74]. Moreover, TCV CP impeded accumulation of viral siRNAs [73].

Further investigations demonstrated that, in contrast to P19 and HC-Pro, TCV CP binds dsRNAs independently of the size of the molecule, i.e. the protein also binds long dsRNAs [75]. This means that the CP–dsRNA interaction can impede the accessibility of the substrate (dsRNAs) to the DCL nuclease, which decreases siRNA accumulation. Indeed, recent studies established the crucial role of p38 in inhibition of the DCL4 responsible for production of the 21-nucleotide-long viral siRNAs [2]. Of interest, p38 does not impede activity of the DCL2 enzyme essential for synthesis of the 22-nucleotide-long siRNAs. Moreover, the results of this work demonstrated that the p38 suppressor activity does not depend on the virion-forming function of the protein.

Small cysteine-rich proteins. Cysteine-rich proteins encoded by viruses of the Hordeivirus, Tobravirus, Pecluvirus, Furovirus, and Carlavirus families do not show significant relationship, but they are structurally similar, play a critical role in viral infections, and function as pathogenic viral factors [76-79].

Tobravirus 16K. The ability of tobacco rattle virus (TRV), a representative of the Tobravirus family, to suppress RNAi was first discovered on inoculating GFP-expressing transgenic plants with the virus [39] with following identification of a 16-kDa cysteine-rich protein (16K) as a suppressor [77]. In the infection process, this protein plays the key role for effective accumulation of TRV. Furthermore, mutational inactivation of the *16K* gene was compensated by CMV 2b coexpression, which indicated functional similarity of the proteins in the RNAi suppression mechanism. Moreover, it was found that the 16K protein is able to particularly suppress RNAi in the *Drosophila* cells [80]. Subsequent transformation experiments on *N. benthamiana* plants containing transgenic GFP using *Agrobacterium* have shown that, for 16K

activity, expression of the full protein sequence is required [81]. Expression of 16K leads to a slight decrease in the level of GFP siRNA accumulation, this emphasizing a probable role of the protein in suppression of initial stages of RNAi in infected plants [81]. Very recent data indicate that TRV-16K blocks RNAi before dsRNA formation, since suppressor activity of the protein was smoothed by increasing the dsRNA dose [82].

Hordeivirus γb. The barley stripe mosaic virus (BSMV) encodes a 17-kDa cysteine-rich γb protein not critical for replication and transport of the virus but significantly affecting the pathogenic process [83]. The first indirect sign of possible participation of γb in RNAi suppression was obtained in experiments using a TRV mutant not expressing p16. It was demonstrated that the absence of the protein could be compensated by BSMV γb expression [77]. Similarly, a BSMV mutant lacking γb expression was unable to be transported systemically in the plant, but this functional defect was not observed in transgenic plants expressing the potyvirus HC-Pro suppressor, at the same time indicating a critical role of γb in systemic transport and its participation in RNAi suppression [84].

It was later demonstrated that the C-terminal of γb forms a helical structure and participates in protein–protein interactions as well as being critical in RNAi suppression [85]. Of interest, poa semilatent virus (PSV) encoding γb is localized in the cytoplasm and peroxisomes [86].

Biochemical examinations have revealed that BSMV γb interacts with ssRNAs through three Zn-binding sites localized on the N-terminal region of the protein [78, 87]. Furthermore, RNA-binding ability of the γb protein is significantly stimulated in the presence of zinc ions [88].

Despite the need for further biochemical data, these results suggest that the interaction of the viral protein with RNA is the key function of γb in RNAi suppression.

Modern molecular and biochemical studies of several viral proteins have significantly widened our understanding about viral strategies of RNAi suppression. Viral suppressors possess a wide spectrum of biochemical properties essential for the struggle with RNAi at different stages of this protective system.

For a more complete study of the mechanism of molecular interactions between the protective system of an organism and their viruses, further detailed molecular, biochemical, and structural investigations of viral suppressors are required. Eventually, these data can be used for development of effective strategies of creating plants tolerant to the viral pathogens.

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