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Antiviral Defense in Plants

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ABSTRACT

RNA silencing is a conserved pathway and it may result in gene expression blockage in eukaryotic organisms. RNA silencing is also part of a highly adaptable immune system response against viruses in plants and animals. It is generally thought that virus-induced RNA silencing is that double-stranded replicative intermediates of RNA viruses, and/or double-stranded RNA produced from the viral RNA by host RNA-dependent RNA polymerases are recognized by Dicer-like proteins for the production of viral siRNAs (vsiRNAs). However, recent studies show that vsiRNAs originated predominantly from highly structured single-stranded viral RNAs is a general characteristic for RNA viruses, dsDNA virus as well as for sub-viral pathogens, e.g. viroid. Increasing lines of evidence has also shown that the plant antiviral response involves hierarchical action of DCLs. To counteract antiviral silencing, many viral genomes encode suppressor proteins to combat the defense pathway. The most common strategy for viral suppressors to inhibit RNA silencing is via binding to siRNAs. Some viral suppressor can also bind to long dsRNA and maybe compete with DCLs to access viral RNA substrates, or inhibit the activity of specific DCLs in the production of vsiRNAs. This review will give an update on the current view of these researches on antiviral silencing and defense in plants.

Keywords: DCL, RNA silencing, viral siRNAs, viral suppressor
Abbreviations: AGO, argonaute; **DCAP**, DCL-Associated protein; **DCL**, Dicer-like enzyme; **DRB**, double-strand RNA binding protein; **PTGS**, posttranscriptional gene silencing; **RDR**, RNA dependent RNA polymerase; **RISC**, RNA-induced silencing complex; **VIGS**, virus-induced gene silencing; **vsiRNAs**, viral small interference RNAs

CONTENTS

INTRODUCTION

RNA silencing (RNA interference, RNAi) is a conserved pathway and it may result in gene expression blockage in eukaryotic organisms in both the cytoplasm and the nucleus, which is involved in regulating sequence-specific gene expression, transposon control, chromatin modification, virus resistance and development (Baulcombe 2004; Meister and Tuschl 2004; Baulcombe 2005). Small RNAs, the unifying feature of RNA silencing, involved in these RNAi-mediated processes include microRNAs (miRNAs) and other small inference RNAs (siRNAs), while the key host protein components in these processes include RNaseIII-type enzymes called Dicers, RNA-dependent RNA polymerases and Argonaute protein-containing effector complexes called RNA-induced silencing complexes (RISCs). In Arabidopsis, there are four Dicer-like enzymes (DCLs), six RNA-dependent RNA polymerases (RDRs) and ten Argonautes (AGOs). In plant, the miRNA pathway generates 21-24 nt miRNAs from single-stranded hairpin RNA precursor by DCL1, regulating the sequence-specific gene expression, especially in some crucial developmental processes (Jones-Rhoades *et al*. 2006). miRNAs also control endogenous TAS-derived 21-nt *trans*-acting siRNAs (ta-siRNAs), which guide the target mRNA cleavage and function as regulators of gene expression as plant miRNAs do (Peragine *et al*. 2004; Vazquez *et al*. 2004; Allen *et al*. 2005; Yoshikawa *et al*. 2005). DCL4 is required for the 21-nt ta-siRNAs synthesis. RNA SUPPRESSOR OF GENE SILENCING3 (SGS3) and RDR6 synthesize another strand of the miRNA-dependent, AGO1-mediated single-stranded TAS primary cleavage products to form double-stranded RNA (dsRNA) for DCL4-mediated cleavage (Peragine *et al*. 2004; Vazquez *et al*. 2004; Allen *et al*. 2005). Heterochromatin-related siRNAs, a kind of 24-nt endogenous siRNAs, produced by DCL3, have important functions in DNA methylation and chromatin remodeling (Xie *et al*. 2004). RDR2, proposed to contribute to dsRNA conversion from heterochromatin-related transcripts (Xie *et al*. 2004), and RNA Pol IVa complex, proposed to function as the RDR in a self-amplifying loop (Kanno *et al*. 2005), are required for formation of these 24-nt siRNAs. Recently, a new class of endogenous 24-nt nat-siRNAs due to DCL2-mediated cleavage for natural antisense overlapping transcripts has been identified (Borsani *et*

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al. 2005). Taken together, the production of these siRNAs is always triggered by dsRNAs, which is processed into 21-24nt RNA duplex by Dicer and its homologues. One strand of small RNA duplex is then incorporated into RISC and leads to sequence-specific RNA degradation, translation inhibition or DNA and/or histone modification.

Viral infection would trigger a set of RNA silencing reactions, which is named as virus-induced gene silencing (VIGS). Different classes of viral siRNAs (vsiRNAs) derived from various viruses are detected in corresponding virus-infected host (Blevins *et al*. 2006; Deleris *et al*. 2006; Moissiard and Voinnet 2006). DCL2 is the first identified DCL which contributes to viral siRNAs biogenesis and functions in antiviral defense (Xie *et al*. 2004), and recently, emerging evidences indicate that all four DCLs in Arabidopsis have hierarchical contribution to viral siRNAs biogenesis (Voinnet 2005; Blevins *et al*. 2006; Deleris *et al*. 2006; Moissiard and Voinnet 2006). Meanwhile, some RDRs, such as RDR6 and RDR1, are proposed to amplify VIGS signal and function in systemic virus silencing (Xie and Guo 2006). The Arabidopsis *ago1* mutants and AGO2-deficiency flies were shown to be hypersensitive to virus infection, respectively (Morel *et al*. 2002; van Rij *et al*. 2006). Therefore, besides its endogenous functions, RNA silencing is also part of a highly adaptable immune system response against viruses in plants and animals. Correspondingly, viruses have also evolved suppression mechanisms to counteract host RNA silencing system, and various virus-encoded proteins have been identified as RNA silencing suppressors to interfere with host silencing machinery. Induction, amplification and suppression of RNA silencing due to virus invasion have been well reviewed (Voinnet 2005; Xie and Guo 2006). This review will give an update on how vsiRNAs originate, how plant virus and plant RNA silencing antiviral system, especially DCLs, fight against each other.

TRIGGERS FOR RNA-MEDIATED PLANT ANTIVIRAL DEFENSE

In plant and animal, several possible primary sources of RNA silencing as elicited by viral and sub-viral pathogens have been well summarized (Voinnet 2005). Most plant viruses belong to positive single-stranded RNA virus. Depended on viral RDR, dsRNAs intermediates are generated. Moreover, viral normal and aberrant RNAs could also be transformed into dsRNAs by host RDRs, for instance, RDR6 (Voinnet 2005). This makes the general thought that these dsRNA are probably processed into viral siRNAs, which trigger primary VIGS. Alternatively, vsiRNAs also could originate from highly structured region of viral single-stranded RNAs. Recent studies suggest that viral siRNAs originate predominantly from highly structured single-stranded viral RNAs (Silhavy *et al*. 2002; Landry and Perreault 2005; Molnar *et al*. 2005). On the assumption that viral dsRNAs intermediates are the only or main triggers for DCLs processing, the content of viral negative-strand-derived siRNAs should be closely equal to the one of viral positive-strand-derived siRNAs, and the length of target RNAs should probably determine the efficiency of the targeting step. However, the study of defective interfering RNAs (DI RNAs) of *Cymbidium ringspot tombusvirus* (CymRSV) has shown that is not really the case (Havelda *et al*. 1997; Silhavy *et al*. 2002). They show that the large DI RNAs containing a highly base paired structure could be efficiently targeted by RNA silencing machinery, and the target activity depends on specific sequence/structures rather than the length of target molecules (Silhavy *et al*. 2002). A highly base-paired structure in the DI RNAs is found to be sufficient to trigger viral RNA silencing and the mutation that increased the stability of this structure conferred higher accumulation of vsiRNAs (Havelda *et al*. 1997; Silhavy *et al*. 2002). Analysis of the population of the viral siRNAs in several RNA virus-infected plants also shows that the vsiRNAs are derived mainly from plus-stranded RNA, especially from the region thought to fold into base paired structures (Silhavy *et al*. 2002; Molnar *et al*. 2005). An *in vitro* dicer cleavage assay also indicates that the pre-miRNA-like hairpin from peach latent mosaic viroid (PLMVd) is sufficient to trigger Dicer cleavage (Landry and Perreault 2005). Potato spindle tuber viroid (PSTVd)-derived siRNAs have recently been cloned and identified to be derived from the secondary structure of viroid RNAs (Itaya *et al*. 2007). Moreover, in a case about dsDNA virus caulimovirus, the siRNA northern blot assays on *Cauliflower mosaic virus* (CaMV)-infected plants showed that the ratio between 21-nt and 24-nt siRNAs in infected *dcl4* mutants differed from different CaMV regions detected (Blevins *et al*. 2006). The differences might be due to the structure of viral RNA intermediates and the structure characteristics determine the specificity of DCLs. Recently, the 35S leader of CaMV genome, which likely forms fold-back structure, was found to be a major source of CaMV-derived siRNAs in CaMV-infected plants (Moissiard and Voinnet 2006). Therefore, it's obvious that highly structured viral single-stranded RNAs could be accessible for DCLs and might be the main triggers for plant antivial defense activity, at least for primary VIGS reaction.

The primary VIGS initiation is RDR6-independent (Schwach *et al*. 2005), and this was further supported by the study on CaMV 35S leader-derived vsiRNAs, whose accumulation level is not reduced in *rdr6* mutant (Schwach *et al*. 2005; Moissiard and Voinnet 2006). However, RDR6 in Arabidopsis, functions as the amplifier of VIGS signals so that it's important for secondary VIGS reaction and systemic virus silencing in antiviral defense (Voinnet 2005; Xie and Guo 2006). Long dsRNAs amplified by host RDRs will be targeted by DCLs for cleavage and confer dominant content of secondary vsiRNAs, one should detect more dispersed population of the vsiRNAs from viral genome. However, it was not really the case in studies on some viruses (Silhavy *et al*. 2002; Molnar *et al*. 2005; Moissiard and Voinnet 2006). The possible explanation is that RDRs have specificity for viral RNAs (Schwach *et al*. 2005), and RDR6 activity may be more dependent on the quality rather the mere concentration of template (Xie and Guo 2006). The extensive fold-back structure may be such template that is not suitable for RDR6 to synthesize new viral dsRNAs, resulting in a little or no *de novo* amplification of viral siRNAs. Such highly structured single-stranded viral RNAs are, therefore, dominant to trigger DCLs antiviral activities.

Whether the secondary structures formed by single-stranded viral RNAs resemble the miRNA precursor hairpins that are recognized by DCL1, which DCL will mainly target highly structured viral RNAs and how DCLs cleave the respective secondary structure of viral RNAs remain to be further investigated. Therefore, more detailed analysis of population of vsiRNAs in virus-infected wild type plants and different *dcl* mutants, combined with structure biological analysis of RNA and Dicer proteins, will help us find the possible substrate of DCLs and the recognition specificity of different DCLs with antiviral activities.

DCLS ACTIVITIES IN PLANT ANTIVIRAL RESPONSE

DCLs function as the key enzymes of diverse RNA silencing pathway. As mentioned above, plant such as Arabidopsis have evolved more diverse sRNAs and RNA silencing pathway (Vaucheret 2006; Vazquez 2006). Coordinated or redundant functions of DCLs in different endogenous sRNAs pathways and transgene-induced silencing pathway (Brodersen and Voinnet 2006; Vaucheret 2006; Vazquez 2006) suggest the complex action of DCLs in the plant antiviral response. In numerous studies made with RNA virus infection of Arabidopsis *dcl* single mutant, neither viral RNA accumulation nor the extent and/or consistency of VIGS was altered in any of the single *dcl* mutants in comparison with WT-infected plants (Blevins *et al*. 2006; Deleris *et al*. 2006), which suggest redundancy among DCLs in

mediating antiviral silencing. Recent studies made with all possible combination of Arabidopsis *dcl* double and triple mutant strengthen the conclusion that plant antiviral response involves hierarchical action of DCLs (Blevins *et al*. 2006; Deleris *et al*. 2006; Fusaro *et al*. 2006). Deleris *et al*. (2006) have shown that the respective 21-nt and 22-nt siRNAs products of DCL4 and DCL2 guide an antiviral RISC to promote defense against *Tobacco rattle virus* (TRV) and p38-deficient *Turnip crinkle virus* (TCV), and DCL2 provides redundant vsiRNA-processing functions when DCL4 is genetically removed or suppressed (Deleris *et al*. 2006). Meanwhile, they have also shown that DCL3 and DCL1-derived vsiRNAs were not associated with defense against TRV. The similar siRNA/*dcl* profiles were also obtained in recent studies for some other RNA viruses including *Cucumber mosaic virus* (CMV), CMV+Satellite RNA, *Turnip mosaic virus* (TuMV) and *Oliseed rape mosaic virus* (ORMV), their results confirm such a hierachical action of DCL4 and DCL2, but further show that DCL3 can also limit viral RNA accumulation (Blevins *et al*. 2006; Fusaro *et al*. 2006). However, DCL3 might just function in cleaving the transient viral dsRNA replicative intermediates to reduce directly its steady-state level (Fusaro *et al*. 2006). In other words, DCL3 might with compete the substrates DCL4 and/or DCL2 and interfere with DCL4 and DCL2-directed antiviral response. Taking all together, it is undoubted that DCL4 plays a key role in antiviral response and RNA virus-VIGS.

In the other side, different from RNA viruses that replicate in cytoplasm, plant DNA virus, such as pararetrovirus and geminivirus, replicate in the nucleus, without coding for their own RDRs, generate dsDNA intermediates and form circular minichromosomes (Pilartz and Jeske 1992) in the nucleus, and. therefore, actions of DCLs in plant antiviral response to DNA virus might be different. Recently, Blevins *et al*. (2006) described the siRNA/*dcl* profiles for DNA viruses of the geminivirus *Cabbage leaf curl virus* (CaLCuV) and the pararetrovirus CaMV. Wild-type (wt) plants infected with these two viruses accumulated similar patterns of 21, 22 and 24-nt siRNAs of both sense and antisense polarities, and the DCL3-dependent 24-nt siRNAs is the most abundant (Blevins *et al*. 2006; Moissiard and Voinnet 2006). In these DNA viruses-infected *dcl3* mutants, 24-nt viral siRNAs are eliminated, however, there is no alternation in viral DNA levels or VIGS (Blevins *et al*. 2006). This suggests that it is probable that two RNA silencing pathways contribute to plant antiviral response to DNA viruses, and the DCL4 and DCL2-dependent siRNAs compensate the functions of DCL3-dependent siRNAs. DCL2-dependent 22-nt siRNAs accumulation increase in *dcl3*, *dcl4* and *dcl3 dcl4* double mutant, suggesting that DCL2 not only can substitute DCL4, as in production of endogenous siRNAs (Gasciolli *et al*. 2005; Xie *et al*. 2005; Yoshikawa *et al*. 2005), but also can substitute DCL3 in procession of DNA vsiRNAs (Blevins *et al*. 2006). DCL1, thought to process hairpin RNAs in miRNA pathway, appears to process hairpin RNAs and generate 21-nt siRNAs in triple *dcl* mutant lacking DCL2, DCL3, DCL4 transformed with hairpin RNA-mediated silencing constructs (Fusaro *et al*. 2006). Similarly, DCL1 appears to generate 21-nt vsiRNAs in the triple *dcl* mutant infected with DNA viruses (Blevins *et al*. 2006; Moissiard and Voinnet 2006), to a limited extent, in which, the production of miR173 seems to be decreased at the same time (Blevins *et al*. 2006). A supposed explanation is that DCL1 takes over the role of other DCLs' while other DCLs lose their function so that its energy has to be dispersed from processing miRNAs. While analysis of CaMV 35S leader-derived vsiRNAs in *dcl1* mutants suggested that DCL1 might be not directly involved in but facilitate the processing of vsiRNAs by the three other DCLs (Moissiard and Voinnet 2006).

Taken together, the four DCLs show hierarchical antiviral activities in plant. DCL4, DCL2 and DCL3 confer cleavage activity to produce 21-, 22- and 24-nt vsiRNAs, respectively, and DCL1 also can produce 21-nt vsiRNAs, albeit inefficiently; DCL4 may play a key role in posttranscriptional gene silencing (PTGS) antiviral pathway against both RNA virus and DNA virus. However, the actions of DCL1, DCL2 and DCL3 in antiviral response seem to be different between RNA and DNA virus infection, resulting probably from the difference of the replication strategies and subcellullar localization between RNA and DNA viruses. Supporting this idea, DCL3-dependent 24-nt siRNAs, which are related to DNA methylation and heterochromatin modification, are the most abundant in DNA virus infected plants but have no obvious effects in defensing against RNA virus. It is probable that 24-nt vsiRNAs play a key role in targeting the dsDNA intermediates and/or minichromosomes of DNA viruses, while have no obvious targets and becomes the by-products of silencing machinery in anti-RNA-virus response. The nuclear localization of DCL1, DCL3 and DCL4 (Papp *et al*. 2003; Xie *et al*. 2004; Hiraguri *et al*. 2005) seems not to be consistent with their actions on processing of RNA and DNA virus siRNAs. However, relocalization of DCLs during infection is possible. In fact, previous study has reported that DCL2 has a nucleocytoplasmic distribution, and the DCL2-GFP fusion protein has predominant accumulation in the nucleus of *N. benthamiana* cells in the transient assay system (Xie *et al*. 2004).

Moreover, some other factors might be also important for DCLs' cleavage properties. For instance, given that the interaction with some associated proteins (name them as Dicer-Associated Proteins, DCAPs) is required for DCLs cleavage, the cleavage properties of DCLs might be modulated by the properties of DCAPs: the subcellular localization of DCAPs might determinate the subcellular localization of functional DCLs; the affinity of Dicer- DCAP interaction might modulate the accessibility and specificity of Dicer to different substrates; even that, the Dicer- DCAP interaction might determinate the size of siRNAs products, considering that DCL2 can produce both 22-nt vsiRNAs and 24-nt nat-siRNAs (Borsani *et al*. 2005). Based on a well-developed biochemical system using Arabidopsis protein extract for Dicer activity assay, dsRNA substrates could be cleaved into 21- and 24-nt siRNAs by Arabidopsis total protein extract or purified DCL1/DCL3 complexes (Qi *et al*. 2005). Meanwhile, size-exclusion chromatography has indicated that DCL1 and DCL3 reside in >660 kDa complex conferring 21-nt siRNA-generating activity and ~440 kDa complex conferring 24-nt siRNA-generating activity, respectively (Qi *et al*. 2005). These observations indicate that some different factors associate with DCL1 and DCL3, respectively, conferring different siRNA products from the same dsRNA substrates. Furthermore, considering that the molecular weight of DCL1 and DCL4 is ~213.6 kDa and ~188.3 kDa, respectively, it also raises a question whether DCL4, which is thought to be the preferred Dicer for 21-nt siRNAs production from dsRNA substrates (Brodersen and Voinnet 2006), also resides in the >660 kDa complex and confers 21-nt siRNA-generating activity rather than DCL1. DRB family proteins might be essential for Dicer function, some cases of Dicer-DRB interaction in *Drosophila*, *Caenorhabditis elegans* and Arabidopsis were shown to be required for miRNA processing (Vazquez 2006). Specificity and redundancy in DCL-DRB interaction and specificity of subcellular localization of DRBs have been recently reported (Hiraguri *et al*. 2005). Therefore, DRB proteins might be one of the candidates of DCAPs. The patterns of vsiRNAs in infected *hyl1-2*, mutant of a member of Arabidopsis DRB protein HYL1, are shown no change with wild type plant, consistent with the strongly interaction between HYL1 and DCL1 (about 50-fold stronger than those between HYL1 and the other three DCLs) (Hiraguri *et al*. 2005), suggesting HYL1 might be not essential for vsiRNAs biogenesis. Recently, DRB4 was found to interact with DCL4 *in vivo* and function in the ta-siRNA pathway (Nakazawa *et al*. 2007). Responses of other DRB mutants in viral infection should be of interest in future works. Meanwhile, DCL-AGO interaction could not be excluded in this issue.

VIRAL SUPPRESSORS WITH RNA-BINDING ACTIVITY

To counteract plant host RNA silencing, many plant viruses have encoded proteins that suppress different steps of the RNA silencing machinery (Silhavy and Burgyan 2004; Voinnet 2005; Bisaro 2006). Elucidating the mechanism of viral suppression of RNA silencing is significative for us to learn the mechanism of host RNA silencing machinery. Till now, more than 30 viral suppressors have been identified in plant viruses (Silhavy and Burgyan 2004; Cao *et al*. 2005; Dunoyer *et al*. 2005; Bisaro 2006; Zrachya *et al*. 2007). Recently, based on multiple *in vivo* and *in vitro* approaches, increasing evidences suggest that many of viral suppressors are RNA-binding proteins, either size-selective or size-independent, though they evolve independently and show low sequence and/or structure homology. For instance, results of *in vivo* and *in vitro* assays show that viral suppressors of *Tobacco etch virus* (TEV) HC-Pro, Tombusvirus p19 and Closterovirus p21 have size-selective ds-siRNA binding activity (Lakatos *et al*. 2004; Lakatos *et al*. 2006; Merai *et al*. 2006). Through direct competition target cleavage assays and RISC formation direct competition assays using the *Drosophila* embryo extracts, Lakatos et al also showed that TEV HC-Pro, p19 and p21 uniformly inhibited the siRNA-triggered RISC assembly through sequestering siRNA, and none of them inhibit preassembled RISC activity *in vitro* or *in vivo* (Lakatos *et al*. 2006). P19-defective tombusvirus infected plants recovered from infection resulting from viral RNA degradation induced by vsiRNA-containing RISC antiviral activity has recently been confirmed by biochemical assays (Omarov *et al*. 2007; Pantaleo *et al*. 2007). The presence of an active p19 in wild-type tombusvirus inhibited ssRNA-specific rebonuclease activity (Omarov *et al*. 2007). Moreover, these suppressors are also shown to bind miRNA/miRNA* intermediates *in vivo*, as a consequence, inhibiting miRNA pathway. These are strengthened by the 3D structure of p19 (Vargason *et al*. 2003; Ye *et al*. 2003) and the octameric ring structure of p21 (Ye and Patel 2005). However, in transgenic plant system, HC-pro is shown to prevents the accumulation of siRNA (Mallory *et al*. 2002) and not to interact directly with miRNA/miRNA*, but do interfere with the incorporation of siRNAs into RISC (Chapman *et al*. 2004). The diverse results might be due to different experiment conditions in these independent studies, and might also reflect multiple suppression functions for HC-pro and only dominative functions display in a certain experiment system. For other size-selective ds-siRNAs binding suppressors, like *Peanut clump virus* p15 and *Barley yellow mosaic virus* γB, the presence of 3′ overhangs of ds-siRNAs is required for their binding activities (Merai *et al*. 2006). The 3′ overhangs ds-siRNAs also increase the TEV HC-pro binding efficiency. Nevertheless, siRNA duplex sequestration seems to be the most common strategy for viral suppressors to inhibit RNA silencing (Lakatos *et al*. 2006; Merai *et al*. 2006; Uhrig 2006; Wang *et al*. 2006).

Other viral suppressors such as *Turnip crinkle virus* (TCV) p38, *Pothos latent virus* (PoLV) p14, are shown to have size-independent ds-siRNA and long dsRNA binding activity (Thomas *et al*. 2003; Merai *et al*. 2005, 2006). Sequestration of wider range of RNA targets by p38 and p14 suggests that some viral suppressors might target multiple silencing steps, probably not only inhibiting the maintenance/amplification step, but also the other, such as the silencing-triggering step. Sequestering long dsRNAs may prevent certain DCL/DCLs cleavage, and/or prevent RDRs to yield more dsRNA substrates for DCLs. In addition, several viral suppressors are shown to bind ssRNA. A geminivirus-encoded suppressor AC4 from *African cassava mosaic virus* Cameroon Strain (ACMV), was reported to bind single-stranded form of miRNAs and siRNAs but not dsRNAs in *in vitro* binding assays (Chellappan *et al*. 2005), suggesting that ACMV-AC4 interferes with RISC loading at the downstream step of small RNA biogenesis and ds-sRNA unwinding. Therefore, long dsRNA, ds-sRNA and/or ssRNA in the silencing pathway are possible targets for most viral suppressors.

VIRAL SUPPRESSORS INTERACTING WITH HOST SILENCING COMPONENTS

Increasing line of evidences has shown that, besides targeting RNA components, some viral suppressors also interact with plant host protein component of silencing machinery. The plant protein rgs-CaM, which was found to interact with HC-Pro in a two hybrid system, is identified as the first endogenous suppressor of PTGS (Anandalakshmi *et al*. 2000). Not only by sequestering ds-sRNAs, HC-Pro was shown to inhibit silencing by activating rgs-CaM, suggesting that HC-Pro may activate an endogenous mechanism that negatively regulates RNA silencing (Anandalakshmi *et al*. 2000). Studies on TuMV suggest HC-Pro may inhibit multiple steps downstream from DCL1-processing and maturation of miRNAs, probably suppresses the assembly and activity of RISC (Kasschau *et al*. 2003). In a RISC formation direct competition assay with HC-Pro, it is striking that the addition of *Drosophila* embryo extract significantly increased the affinity of HC-Pro to siRNA and led to form a new complex containing labeled siRNAs, which did not happen without adding *Drosophila* embryo extract (Lakatos *et al*. 2006). It suggests that one or more cellular factor may interact with HC-Pro and increase the affinity of HC-Pro to siRNA and provides a clue for researchers to find the endogenous HC-Pro-interacting factor/factors.

DCL4 has been identified as a key component of non-cell autonomous RNA silencing in a genetic screen using the SUC-SUL system, which suggests cell-to-cell signaling requires DCL4-dependent 21-nt siRNAs (Dunoyer *et al*. 2005). Virus-derived 21-nt siRNAs are also shown to be DCL4-depentdent (Blevins *et al*. 2006; Deleris *et al*. 2006; Fusaro *et al*. 2006). DCL4-dependent 21-nt siRNAs accumulate in suppressor p38-deficient TCV-GFPΔp38 infected plants but not in TCV-GFP infected plants, suggesting that the production of DCL4-dependent 21-nt is inhibited by the p38 (Dunoyer *et al*. 2005). Moreover, TCV-GFPΔp38 has movement defects in wild type infected plant, which can be rescued in p38 transgenic plants and *dcl2dcl4* double mutant (Deleris *et al*. 2006). All of these evidences suggest that DCL4 plays an important antiviral role in producing 21-nt cell-to-cell silencing signals and restricting viral systemic infection, while its activity could be suppressed by TCV p38. According to the RNA binding activity of p38, the suppression of DCL4 is probably via competing with DCL4 to interact with the substrate RNAs. Alternatively, p38 may direct or indirect interact with DCL4 to inhibit its activity in the production of 21-nt siRNAs.

AGO1-containing RISC plays an important role in endogenous RNA silencing pathway. It is shown that AGO1 associates with endogenous miRNAs and ta-siRNAs but not virus-derived siRNA (Baumberger and Baulcombe 2005). However, recently, Zhang *et al*. (2006) showed that AGO1 also associated with CMV-derived siRNAs. The 2b protein encoded by CMV was shown to inhibit the activity of long range silencing signals (Guo and Ding 2002). *In vivo* and *in vitro* assays showed that CMV 2b suppressor directly interacted with AGO1, and the interaction occurred primarily on one surface of the PAZ-containing module and part of the PIWI-box of AGO1(Zhang *et al*. 2006). RISC reconstitution assay also showed that 2b specifically blocks the AGO1 cleavage activity resulting in accumulation of passenger siRNA of tasiRNA and star strand of miRNA, as a consequence, attenuating miRNA-mediated RNA silencing and increasing of accumulation of miRNA target mRNA (Zhang *et al*. 2006). Moreover, CMV-infected plant and 2b transgenic plant show the similar developmental abnormality with *ago1* mutant (Zhang *et al*. 2006). All these results suggest that CMV 2b interacts with AGO1 and AGO1-containing RISC is also important for VIGS. As PAZ-containing module is sufficient for the interaction with CMV 2b, it

could be anticipated that DCLs, which also contain PAZ domain, probably could interact with CMV 2b as well. Hopefully, more endogenous protein components of RNA silencing interacting with viral proteins would be identified in the near future.

OTHER VIRAL ACTIONS AGAINST RNA SILENCING

Besides the direct targeting silencing pathway, escape of antiviral silencing is also an effect counter-defense by viral suppressors to protect viral RNA inside the plant cells (Xie and Guo 2006). Additionally, actions of some other viruses to combat RNA silencing are also notable, which might provide us more clues to investigate the plant host antiviral response. A recent study show that a potent silencing suppressor Polerovirus P0 (Pfeffer *et al*. 2002), requires an F-box-like motif for its suppressor function (Pazhouhandeh *et al*. 2006). This suggests that P0 might act as an F-box protein that targets an essential component of the host RNA silencing defense pathway; or P0 might function as an antagonist of a cellular F-box protein, which normally degrades a negative regulator of the silencing pathway (Pazhouhandeh *et al*. 2006). The ubiquitination activity and the target of P0 will be important for the demonstration of this supposition, and will open a door for studying the correlation between the ubiquitination pathway and the RNA silencing pathway in plant. Interestingly, *Red clover necrotic mosaic virus* (RCNMV), a positive single-stranded RNA virus, suppresses sense-transgene-mediated RNAi by using multiple viral components required for viral RNA replication (Takeda *et al*. 2002). The requirement of DCL1 or its homologues for RCNMV infection and leading to inhibit miRNA biogenesis and host RNA interference (Takeda *et al*. 2002) suggest that DCL1, as one of components of host silencing machinery, becomes not a safeguard anymore but an accessory of viral invasion. Several different protein components encoded by different geminivirus genome, have been identified as the viral suppressor. Although their silencing mechanisms have not been fully understood, it provides us new sight of silencing suppression. The transcription activator protein AC2 is proposed to suppress RNA silencing by controlling the expression of host silencing effector genes (Trinks *et al*. 2005), while Tomato golden mosaic virus AC2 interacts and inactivates adenosine kinase (ADK), a cellular enzyme shown to be required for supporting RNA silencing. Another identified suppressor protein, the βC1 protein of *Tomato yellow leaf curl China virus*-Y10 (TYLCCV) is shown to bind ssDNA and dsDNA without sequence specificity and its nuclear localization is required for silencing suppression activity (Cui *et al*. 2005a). Developmental defects in its transgenic plant suggest that it target at the silencing step overlapping the miRNA pathway (Cui *et al*. 2005b).

SUMMARY

Increasing experimental evidences indicate that not only dsRNA replicative intermediates and dsRNA generated by host RDRs, but also single-stranded highly structured region of RNA genome triggers DCLs antiviral activity. DCLs function as the sensors for viral RNAs and cleave them into respective siRNAs in different sizes, leading to further actions of plant RNA silencing machinery against viruses. It becomes clearer that DCLs act cooperative and hierarchical. Moreover, DCLs combine with other silencing components, such as RDRs, DRBs, AGOs or even themselves, seem to form an even complex network in antiviral silencing pathway.

On the other hand, viruses have evolved diverse strategies to suppress host RNA silencing. Most common stratetegy is via targeting the viral RNAs, such as long dsRNAs, ds-sRNAs and ssRNA, dependent on their RNA binding activity, to compete with DCLs, RDRs or AGOs for substrates. Targeting the protein component, such as DCLs and AGOs, is another efficient counteraction to plant antiviral response. Some of viral suppressors may also inhibit multiple steps of RNA silencing pathway. Moreover, some new suppression mechanisms are also proposed. The divers suppression mechanisms also reflect that the complexity of antiviral defense in plant. In fact, the RNA-silencing and other defense pathways can act together to limit virus infection. This is supported by the discovery that both CMV 2b and TEV P1/HC-pro can interfere with SA-defense pathway, and SA can act as an enhancer of the RNA-silencing antiviral defense in plant (Ji and Ding 2001; Alamillo *et al*. 2006). The discovery of multiple functions of viral suppressors will help us to better understand the plant complex network on antiviral defense.

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