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Crucifer infecting TMV replicase protein p122 is an RNA silencing suppressor

Ph. D. thesis

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Introduction

RNA silencing is a general term for a particular collection of phenomena in which short RNA molecules trigger repression of homologous sequences. It is a highly conserved pathway, found in a large variety of eukaryotic organisms, and its main characteristic is the use of small RNA molecules of 21–25 nucleotides (Hamilton et al., 1999) that confere high specificity to the target sequence. The triggers of RNA silencing are the double-stranded RNA (dsRNA) molecules or hairpin RNAs, which are recognized as abberant in the cell by RNase-like III type nucleases, the Dicers, and cleaved into small interfering RNAs (siRNA) or microRNAs (miRNAs) with specific two-nucleotide 3‘ overhangs. The siRNAs and miRNAs are 3’end methylated as a protective step against degradation by HEN1 methyltrasferase. The siRNAs/miRNAs following unwinding are incorporated into a multiprotein effector complexes, called RNA-induced silencing complexes (RISC). RISCs target homologous RNAs and exert silencing on post-transcriptional level, either by inducing cleavage or by blocking translation (Bartel 2004, Brodensen et al., 2008). RNA-dependent RNA polymerase (RdRP) plays a role in amplifing the RNAi response by generating more double-stranded RNA from single-stranded targets that can then enter and continue to stimulate the RNA silencing pathway. This positive-feedback system is crucial to amplify the siRNA signal transmitted from cell to cell and to mount a systemic form of silencing (see the model on page 7.).

The biochemical machinery of RNA silencing support several processes. Among these are transposone silencing due to sequence specific DNA methylation and chromatin condensation, developmental gene regulation and stress responses by micro RNA (miRNA) and endogenous siRNA pathway and antiviral defence.

Early studies in plants pointed to a role for silencing pathway as a defense against viruses. The first indications that RNA-mediated responses play an important antiviral role came from observations that transgenic expression of viral sequences protected plants from homologous viruses by conferring sequence-specific degradation of viral RNAs (Linbdo et al., 1992). Later it was shown that sequence-specific RNA degradation was a natural plant antiviral response (Ratcliff et al., 1997). Viral proteins were identified that suppress RNA-mediated defence (Kasschau & Carrington 1998), indicating that pathogens have evolved efficient counter defensive strategies. This was first shown to be the case for plant viruses, later animal-infecting viruses were also found to encode suppressors of RNAi (Silhavy & Burgyán, 2004; Baulcombe et al., 2004; Li & Ding, 2005).

As an experimental system *Tobacco mosaic virus* (TMV) has earned his reputation as a workhorse for many areas of plant biology. As limited data were available about the silencing suppressor strategy of tobamoviruses, our objective was to find and characterize the mode of action of TMV silencing suppressor.

For our study we choose crucifere-infecting TMV (cr-TMV) which successfully infect *Arabidopsis thaliana* plants. The virus genome code for 4 proteins: 122 kDa and 178 kDa, the small and the large

subunit of replicase complex, 29 kDa movement protein and 18 kDa coat protein. Earlier findings suggested that the small replicase subunit of TMV viruses could be responsible for silencing suppression. It has been demonstrated that the p126 protein of TMV OM strain (corresponding to the p122 of cr-TMV) forms a heterodimer replicase complex with p183 and two or more host proteins. The ratio between p126 and p183 is 1:1, although they are expressed in a 10:1 ratio during infection (Watanabe et al., 1999). The biological function of this excess amount of p126 was not determined. A substitution mutant of TMV-L strain in which the amber stop codon of p126 was replaced by tyrosine codon thus expressing only the p183 readthrough product was shown to replicate *in vivo* in the absence of the p126 protein. The growth rate of this mutant virus was about one-tenth of the rate of wild type (Ishikawa et al., 1986). All these results imply that the small replicase subunit besides being involved in the replication complex actions, has other functions as well. Indeed the other analogous protein p130 of *Tomato mosaic virus* (ToMV) has been shown to have silencing suppressor function (Kubota et al., 2003), however, the molecular bases of the suppression remained to be determined.

Materials and methods

Virus inoculation

Agroinfiltration assay

Northern blot analysis

Western blot analysis

Gel mobility shift assay

Gel filtration chromatography

Native gel electrophoresis

The *Drosophila* heterologous system *in vitro* RNA silencing

β-elimination assay

Results

1. The cr-TMV replicase subunit protein p122 is a potent silencing suppressor protein *in vivo*

Green fluorescence protein (GFP) induces strong transgene silencing, if is ectopically expressed in *N. benthamiana* plant leaves. The RNA silencing response is even stronger if GFP inverted repeat (GFP-IR) is simultaneously coexpressed, since this molecule bear a double-stranded region which is direct substrate of Dicer. When p122 protein was coexpressed with GFP or GFP + GFP-IR the RNA silencing was blocked, high level of GFP mRNA and protein was detected. p122 inhibited the accumulation of siRNAs when the RNA silencing was triggered by GFP, however, p122 did not inhibit the processing of GFP-IR (dsRNA) into siRNAs, but interfered with the silencing machinery

downstream of siRNA generation *in vivo*. Large amount of 21nt long viral-siRNA accumulated in the virus-infected, suggesting that p122 does not compromise the generation of viral siRNAs *in planta*. p122 was able to inhibit the accumulation of secondary siRNAs. These results suggest that p122 act downstream to Dicer and unstream to RdRP-mediated siRNA amplification.

2. RISC formation is impaired *in vitro* in the presence of p122 protein

RNA silencing complex formation can be followed in *Drosophila* embryio lysate by addition of labelled siRNAs. If the labelled siRNAs and p122 protein-expressing native plant extract was added in the same time, silencing complexes were unable to build up, if p122 native plant extract addition to the reaction mix was delayed 10 min., p122 was not able anymore to interfere with complex formation. So, p122 was able to inhibit the RISC-assembly while did not compromise the preassembled RISC

3. p122 inhibits siRNA-directed RNA cleavage of RISC complexes in *Drosophila* embryo extracts in a dose-dependent manner but cannot interfere with loaded RISC activity

We tested the ability of p122 to block RISC activity: if *Drosophila* embryo extracts are mixed with siRNA and target RNA, RISC complex is able to cleave the homologous RNA, and the 5‘-cleavage product can be observed upon gel electrophoresis. p122 was able to inhibit the RISC target cleavage when added to the reaction together with siRNAs, but did not affect the slicer activity, when its addition to the reaction was delayed. These results implyes that p122 is able to hinder RISC formation and activity, but has no effect on preassembled RISC activity.

4. p122 does not inhibit siRNA- or miRNA- preloaded RISC activity *in planta*

We tested p122 effect on plant RISC activity *in vivo.* In the plants are present high amount of preloaded miRNA- and viral siRNA- (in the virus infected plants) containing RISC complexes. GFP-sensor construct were co-expressed in plant leaves with p122 and monitored their RNA level. The GFP-sensors bear in the 3’UTR region miRNA- and siRNA- target sequences, which are silenced by the RISC machinery. Similarly to the *Drosophila* heterologous system, p122 could not interfere with siRNA- and miRNA- pre-programmed RISC cleavage *in planta.*

5. p122 binds double-stranded siRNAs in size-specific manner

Based on previous findings the most simple explanation would be that p122 sequesters the siRNAs and/or miRNAs. We extensively tested the p122 RNA affinities for different RNA species. p122 did not show any single stranded RNA binding activity irrespectively of the length of RNA, bound to 21nt RNA duplexes with 2nt 3’overhang with the highest affinity. The size and the 3‘overhangs of the siRNA are important: the binding affinity was slightly reduced when 19 nt blunt dsRNAs or 21nt blunt dsRNAs were used. The affinity to 24 nt siRNA species was much lower, while p122 did not

bind the other RNAs tested: 19nt dsRNA with 3' 2nt overhang 26nt dsRNA with 2nt 3'overhang, 49nt dsRNA. Since TMV siRNAs are 21 nt long, these results suggest a specific adaptation of the virus to counteract the antiviral silencing machinery.

6. P122 protein is the only suppressor of cr-TMV

As p122 and p178 ORFs are partially overlapping translation products of the virus, it would be expected that also p178 has RNA silencing suppressor activity. To test this assumption we checked the virus infected plant extract (p122 and p178 both present) siRNA binding activity and compared it to the p122-expressing plant extract activity. The mobility of the protein-siRNA complexes was the same in the two samples. To strenghten our results we made a p122-mutant virus: 64-128- times diluted crTMV-infected extract was able to bind siRNAs, in contrast the cr-TMV-Δp122 (p122 mutant virus) infected plant extract did not show any siRNA binding activity. This result suggests that the p178 readthrough product of p122 doesn't have siRNA binding activity and the p122 protein is solely responsible for anti-viral silencing upon cr-TMV infection. We measured the size of the siRNA-protein complex in the virus infected sample: 108,4 kDa. This is the p122 monomer-siRNA complex size (siRNA is 7kDa), as p178-siRNA or p122-p178-siRNA complex would elute at much higher molecular weight. This finding exclude any involvement of p178 in the siRNA binding and therefore silencing suppression.

7. p122 interferes with miRNA pathway: promotes miRNA accumulation by sequestration of miRNA duplexes

Severe mottling and serrated edges are visible on cr-TMV systemically-infected plant leaves, similar to those plants in which the miRNA pathways were compromised by mutation (Palatnik et al., 2003) or silencing suppressor proteins were expressed (Dunoyer et al., 2004). The most simple explanation would be that p122 can bind also miRNAs as their structure is very similar to siRNAs. We tested the p122 protein's affinity to miRNA/miRNA* duplexes. The binding affinity of p122 to the three miRNA/miRNA* (miR171a, miR171b, miR171c) duplexes are in the same range, (relative disotiation constant, K_r=1.1, 1.6 and 1.9, respectively) and only slightly reduced as compared to the 171 siRNA perfect duplex. (K_r=1). Upon cr-TMV infection both mature and star strands of all tested endogenous miRNAs (miR160c, miR162a, miR171c, miR168a, miR172) accumulated to a higher level compared to the mock-infected control plants, suggesting that p122 binds miRNA duplex intermediates also *in vivo*. Supporting this we observed that the miRNAs were inactive as the Argonaute1 (AGO1) mRNA (miR168 negatively regulates AGO1 mRNA) expression was upregulated in cr-TMV infected plants.

8. p122 interferes with siRNA and miRNA 3‘-terminal methylation

During their maturation miRNAs and siRNAs are methylated by HEN1 methyltransferase. In virus infected *A. thaliana* leaf RNA extract the tested miRNAs showed different level of methylation as compared to the miRNAs derived from mock-inoculated plants. All miRNAs tested were fully methylated in mock-inoculated plants, while miRNAs - both mature and star strands - derived from cr-TMV-infected plants were partially non methylated. We can rule out the existence of other unidentified methylase, which may operates in the cytoplasm since none of the miRNAs and siRNAs were methylated in the in *hen1-1* (HEN1 null mutant) plants. This could not be observed untill now, since in the absence of methylation the miRNAs are quickly degraded, in this case p122 protects them. The stabilization of partially methylated miRNA duplexes suggest that HEN1 is active both in the nucleus and in the cytoplasm as p122 is active only in the cytoplasm. The ability of virus to interfere with miRNA methylation may suggest that miRNAs exported from the nucleus are in both methylated and non-methylated forms.

9. p122 interferes with RNA silencing by sequestration of siRNAs, which is a very potent and widely used viral suppressor strategy.

Discussion

Many silencing suppressor proteins are evolutionary unrelated, and different molecular mechanisms were proposed for their activity.

To establish a more detailed model for the molecular basis of RNA silencing suppression by viral suppressor proteins, we used different approaches for their characterization. This included *in vitro* and *in vivo* approaches to explore the molecular mechanisms by which p122 (Csorba et al., 2007), p19, p21 and HC-Pro (Lakatos et al., 2007) interfere with RNA silencing machinery. We presented evidence that all these silencing suppressors are dsRNA-binding proteins that interact physically with siRNA duplexes *in vivo* as well as *in vitro*, inhibit siRNA-directed target RNA cleavage in the *Drosophila in vitro* RNA silencing system and the siRNA-initiated RISC assembly pathway by preventing RNA silencing initiator complex formation through siRNA sequestration (Lakatos et al., 2006, Csorba et al., 2007).

It is tempting to speculate that the siRNA binding is one of the most effective strategy to inhibit silencing. As most of the viruses can infect more than one plant the RNA-binding is more reliable strategy than the targeting of silencing proteins, which can widely vary from one species to the other, althought there are also counter-examples like 2b (Zhang et al., 2006) or p0 (Baumberger et al., 2007, Bortolamiol et al., 2007) silencing suppressors. Many dsRNA-binding suppressors are evolutionarily unrelated suggesting

that dsRNA-binding silencing suppressors have evolved independently many times in convergent evolution, resulting in remarkably similar dsRNA-binding characteristics (Merai et al., 2006). After the production of siRNAs in the silencing pathway this molecules are promptly sequestered and blocked to be incorporated by one hand into the effector complex and by the other hand to take part in the amplification processes mediated by the plant RDRP proteins.

In the same time the virus replication strategy evolved not to affect as much as possible the endogenous pathways. Because the miRNA duplex are very similar to the siRNAs, the suppressors bind them, blocking the loading of new RISC complexes, by this contributing to viral symptom development. Very important to remark that this strategy do not affect already programmed RISC complexes, which provides a delayed impact on miRNA pathway.

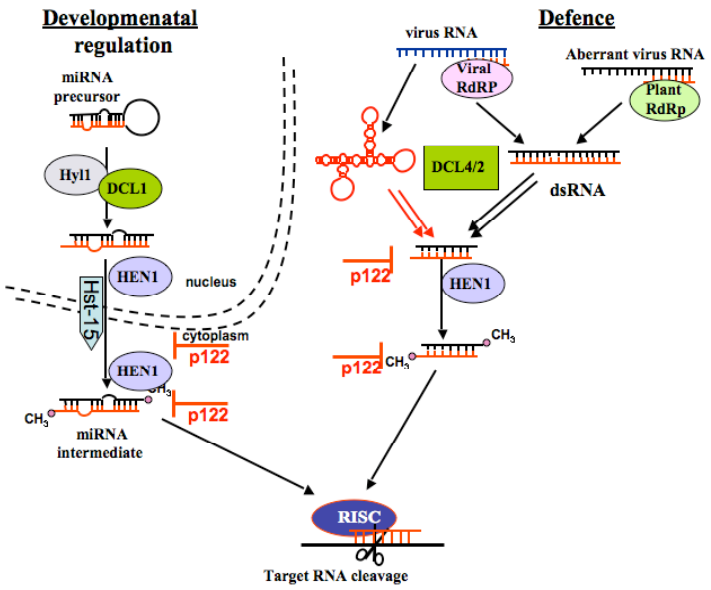

Figure 21: The model of silencing suppression mechanism of p122 protein.

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