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Effect of *Tobacco etch virus* and *Carnation italian ringspot virus* on methylation of endogenous and virus derived small RNAs

Ph.D. thesis

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INTRODUCTION

RNA silencing is an evolutionarily conserved sequence-specific gene-inactivation system induced by double stranded RNAs.

The process is guided by the so called smallRNAs diced from longer double stranded RNAs (dsRNA). In plants small RNAs are stabilized by 3' methylation against degradation. This step is essential for smallRNA function and in *Arabidopsis thaliana* is made by a nuclear methyltransferase HEN1 (Yu *et al.*, 2005).

In plants RNA silencing functions as an effective antiviral system also. Small RNAs are diced from dsRNA regions of the virus (siRNAs) and incorporated into protein-complexes to guide cleavage of virus-RNA. To ensure success of infection, viruses have evolved silencing suppressor proteins what counteract antiviral silencing (Ding and Voinnet, 2007). Majority of these proteins bind double stranded smallRNAs, thus sequester virus derived siRNAs and inhibit assembly of antiviral complexes (Lakatos *et al.*, 2006).

Moreover RNA silencing has diverse role in endogenous gene regulation. Most important class of smallRNAs involved in this process are microRNAs (miRNA), derived from dsRNA region of MIR gene transcripts (Voinnet, 2009).

Antiviral and endogenous pathways are overlap. Hence, while silencing suppressors inhibit antiviral processes they interfere with plant gene expression too. This provide a tool for us to investigate plant gene regulation with the use of suppressor proteins.

In transgenic plants it has been described that smallRNA-binding suppressors partially inhibit miRNA methylation (Yu *et al.*, 2006). Two suppressors, HC-Pro and p19 had very similar effect on most miRNAs. p19 (Vargason *et al.*, 2003) is the suppressor of Tombusviruses while HC-Pro is the suppressor of many Potyviruses (Ruiz-Ferrer *et al.*, 2005). The two virus groups possess different genome organization and utilize different replication strategies (Hull, 2002). Both virus groups are cytoplasmic and the subcytoplasmic distribution of their proteins and molecular processes are strictly restricted and regulated (Wei and Wang, 2008) (Burgyán *et al.*, 1996). This raised the question whether these viruses are able to inhibit smallRNA methylation during natural virus infection and how could their suppressors interfere with theoretically nuclear processes.

We investigated the effect of *Carnation italian ringspot virus* (CIRV) coding p19 and *Tobacco etch virus* (TEV) coding HC-Pro on smallRNA methylation in *Nicotiana benthamiana*. We also examined the effect of P0 suppressor of *Beet western yellows virus* (BWYV) on silencing-complex stability.

AIMS OF THE STUDY

p19 and HC-Pro suppressors inhibit the crucial smallRNA methylation in transgenic plants which is made by the nuclear HEN1 protein. (+) stranded RNA viruses replicate cytoplasmically, inhibition of nuclear processes seemed ambiguous. Therefore we wanted to answer the following questions:

1) Are virus derived siRNAs methylated, if yes, are they able to enter the nucleus?
2) Are suppressors able to inhibit endogenous smallRNA methylation during virus infection?
3) If suppressors could inhibit smallRNA methylation do they transported into the nucleus?
4) Is there a difference between endogenous and virus derived smallRNA methylation?

METHODS

Northern-blot

Nucleic acid extracts were resolved by 12% denaturing polyacrilamide gel-electrophoresis and blotted onto nitrocellulose membranes. Filters were probed with specific radioactive oligonucleotides and in vitro transcripts. The radioactive signals were exposed to a Storage Phophor Screen of the manufacturer Amersham, detected with Storm 840 scanner and quantified with ImageQuant software.

Western-blot

Protein extracts were resolved by 10% SDS-polyacrilamide gel-electrophoresis and blotted onto polyvinyl-difluoride membrane. HC-Pro (containing His-tag) were probed with anti-His-antibody, p19 was probed with anti-p19 serum.

Cell-fractionation

Cell-fractionations were performed with Sigma Cell Lytic Plant Nuclei Isolation/Extraction Kit according to the manufacturer's instructions for semi-pure nuclei isolation protocol. Homogenates were prepared from plants to obtain whole cells. Then cells were lysed with Tween-20. Lysates were layered on a 2,3 M suchrose-cushion and nuclei and cytoplasmic fraction was separated with centrifugation.

β-elimination

3’ methylation of smallRNAs was probed with β-elimination. The reaction requires free hydroxil-groups on the ribose-ring. In the first step oxidation is carried out with periodate, then β-elimination is performed on pH10. Incubation in alcalic conditions brokes the last nucleoside thus the RNA get shorter. If RNA is methylated it resist the reaction and no truncation occurs.

Agroinfiltration

Transient expression of genes were made by *Agrobacterium tumefaciens* infiltration.

Co-immunoprecipitation

Suppressor-bound RNAs were isolated with co-immunoprecipitation. Suppressors were immobilised with specific antibodies on Sepharose-Protein-A beads. After elution of bound suppressors, RNA and protein was extracted from eluates and used for Northern- and Western-blots.

Fast protein liquid chromatography (FPLC)

To examine P0 induced silencing-complex destabilization, we used FPLC technique. Agroinfiltrated and control plants were lysed 2 or 4 days postinfiltration, extracts were chromatographed on Superdex 200 column (Pharmacia) to obtain size-dependent separation of protein-complexes. Then RNA and protein was extracted from the fractions and analysed by Northern- and Western-blot.

RESULTS AND DISCUSSION

1) HC-Pro and p19 inhibit smallRNA methylation in transgenic plants to a similar extent. In contrast with previous results we proved in our work that during virus infection CIRV has only slight effect on smallRNA methylation compared to TEV. Therefore we presented that suppressors act in a different way *in natura* than in transgenic systems.
2) We proved that both CIRV p19 and TEV HC-Pro able to bind methylated and non-methylated smallRNAs. This means that none of those suppressors recognize 3’ methyalation.

3) We assess that TEV inhibit methylation of viral siRNAs completely and partially inhibit methylation of certain miRNAs while has no effect on other miRNAs. HC-Pro was able to inhibit methylation only of bound smallRNAs. We hypothesize that HC-Pro compete with the methyltransferase for smallRNAs.
4) In contrast to TEV, CIRV infection had no effect on miRNA methylation and weekly inhibited viral siRNA methylation. p19 preferentially binds siRNAs and only slightly binds miRNAs. Therefore p19 compete with the methyltransferase only for siRNAs.
5) Double-stranded RNA binding activity was found to be responsible for inhibition of methylation. Mutant suppressors with disrupted binding-site were not able to influence methylation.
6) Suppressor proteins of CIRV and TEV and viral siRNAs localized in the cytoplasm. We found no evidence for their transport into the nucleus.
7) We proved that in *Nicotiana benthamiana*-ban methylation of CIRV derived siRNAs occurs entirely in the cytoplasm, while certain miRNAs are methylated at least partly in the cytoplasm. Thus we found indirect evidence for existence of a cytoplasmically active methyltransferase in *Nicotiana benthamiana*.
8) The two suppressor inhibited methylation to a similar extent during transient transgene-expression, but not in virus infection. We concluded that suppressors could only inhibit methylation if they co-localize with the expressing smallRNA. As small RNA duplex is the only substrate for these suppressors, physical accessibility is the requirement for inhibition of methylation. The accessibility is determinded by compartmentalization of the suppressor and the smallRNA duplex.
9) During examination of P0 suppressor of BWYV we found that half-life of certain miRNA containing high-molecular weight complexes is around 2-3 days. These complexes might correspond to the effector-complexes of post-transcriptional silencing and presumably contain the same or similar proteins with antiviral complexes.

CONCLUSIONS

We revealed that silencing suppressors has different effect in transgenic and in infected systems. We found indirect evidence for a cytoplasmic methyltransferase that methylates viral siRNAs and certain miRNAs. Our results show that plant viruses and their

silencing suppressor proteins are suitable to explore endogenous plant pathways. Furthermore we emphasize that informations concluded from transgenic systems could be misleading and it is necessary to confirm them in natural systems.

LIST OF PUBLICATIONS

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