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Investigation of genes participating in antiviral RNA silencing against Cymbidium ringspot virus and, the RNA silencing suppressor, protein p19

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1. Background and objectives

Plant viruses are among the most important pests of agriculture. The breeding of tolerant, resistant plant species and the use of virus free propagation material are the primary means of defense. To achieve this, it is crucial to understand the plant-virus interactions. The aim of molecular virology is to study this interaction on a molecular level. One important antiviral mechanism in plats is RNA silencing.

RNA silencing in plants are initiated by a DICER LIKE gene through cleaving an RNA to a small RNA (sRNA). These are methilated by HUA ENHANCER1 (HEN1), than loaded into an ARGONAUTE (AGO) protein which is the core of the RNA INDUCED SILENCING COMPLEX (RISC). Besides this there is an RNA DEPENDENT RNA POLYMERASE pathway which amplifies the silencing by producing secondary sRNAs. RNA silencing is conserved in most eukaryotes (Meister & Tuschl 2004).

SRNAS are the hallmarks of RNA silencing. There are two types of canonical sRNAs: micro RNAs (miRNA) and small interfering RNAs (siRNA). Amongst siRNA one can find the subgroups of viral siRNA (vsiRNA) and trans acting siRNA (tasiRNA). Also siRNAs can be divided into primary and secondary siRNAs. (Vaucheret et al. 2006). SRNAs are cleaved by a type III endoribonuclease, the DCL from double stranded RNAs (Fire et al. 1998) or highly structured RNAs (Molnár et al. 2005, Bartel 2004). The HEN1 dependent 3’ end 2’-O-methylation is responsible for the stability of sRNAs. Without methylation sRNAs are vulnerable to 3’-5’ exonucleases (Kamminga et al. 2010). SRNAs are loaded into a member of the ARGONAUTE protein family (Vaucheret 2008), to cleave or to cause translational inhibition on target RNAs (Rivas et al. 2005, Wilczynska & Bushell 2015). In plants novel sRNAs can be produced from cleaved RNAs. It is the RDR proteins role to synthesize the

complementer strand of cleaved RNAs for sRNA biogenesis (Schiebel et al. 1998).

Nicotiana benthamiana is a model organism for plant virology, which is due to the susceptibility for a wide range of viruses (Bally et al. 2015, Nakasugi et al. 2013).

Viruses are sub cellular infective nucleoproteins. They are obligated parasites incapable of autonomic metabolism. It is a general trait of viruses to infect host plants and cause disease. The Cymbidium ringspot virus (CymRSV) is a tombus virus (Hull *szerk.* 2014). It's fifth open reading frame encodes the viral suppressor of RNA silencing (VSR) called p19, which is in the center of our research (Grieco et al 1989). P19 is capable to inhibit RNA silencing by bounding sRNAs (Silhavy et al. 2002). The sRNA haveing the right size and being double stranded are the main criteria for p19 binding, and having 2 nt 3' overhang is not (Vargason et al. 2003). P19 inhibits the HEN1 mediated metylation and the AGO loading but not the DCL cleavage. (Lózsa et al. 2008, Lakatos et al. 2004; Lakatos et al. 2006).

The aim of our research was to find out:

1. Can p19 bind plant endogenous sRNAs in case of viral infection and thus have an impact on symptom formation?
2. How can the difference between CymRSV symptom and the phenotype of p19 transgenesis be explained?
3. What type of sRNAs does p19 prefer?
4. How can the difference between the vsiRNA size profile of CymRSV and that of Cym19stop be explained?
5. What is the role of DCL2 and DCL4 against CymRSV?
6. What is the effect of p19 sRNA binding on AGO loading?

2. Material and methods

We used the plazmid construction of CymRSV from Dalmay et al. (1993) and of Cym19stop from Szittya et al. (2002) for viral infection as described in the article.

For agroinfiltration the following constructs were used: GREEN FLURESCENT PROTEIN (GFP) from (Brigneti et al. 1998), a construction carrying its GF region as palindrome (GF-IR) (Csorba et al. 2007) and a construction expressing p19 (Burgyán et al. 1996). Details can be found in Kontra et al. (2016).

RNA extraction was preformed with the phenol-chlorophorm method as described in Kontra et al. (2016). Protein were extracted in Laemmli buffer as described in Kontra et al (2016).

For Northern blots samples were separated on a 15% acrylamide:bisacrylamide (19:1) 8,6 M urea 1 x TBE gel. The samples in the gel were electrobloted onto the membrane. Chemical crosslink (Pall & Hamilton 2008) was used to crosslink the RNAs to the membrane. For LNA containing DNA oligonucleotide probes the hybridization was carried out at 50 °C, for standard DNA probes 37 °C was used. For details please see Kontra et al. (2016).

For *in situ* hybridization the Várallyay & Havelda (2011) protocol was used.

Immunoprecipitation (IP) was carried out as described in Baumberger & Baulcombe (2005).

For in vitro RNase-A resistance assay we labeled synthetic RNA olignucleotides on their 5' end and made them double stranded with the 2 nt overhang. We added purified p19 (Várallyay et al. 2014) and incubated it in the

presenc of RNase-A. Samples were gel electrophorated and the radioactive signal was detected directly (Kontra et al. 2016).

Libraries were constructed with TruSeq technology. In case of sRNA seq reads were 50 bp long and were single end sequenced. In the p19-IP experiment 4-4 samples, in the AGO-ip experiments 9-9 samples were pooled together on a sequencing lane (100 M read). In case of RNA seq sequences were 100 bp and pair-end sequenced. 3 samples were sequenced on a lane (100 M reads) (Kontra et al. 2016)

For sRNA seq analysis UEA sRNA workbanch v3.0 (Stocks et al. 2012), PatMaN (Prüfer et al. 2008) and costume made scripts were used (https://github.com/kontral/Burgyan_Lab). The *N. benthamiana* v 1.0.1. (Bombarely et al. 2012) and CymRSV (Grieco et al. 1989) genomes were used for alignment. The quality control of the RNA seq libraries were done with the help of FastQC v0.10.1, cutadapt v1.9.2.dev0 (Martin 2011) and FastX_trimmer v0.0.13. By using RSEM v1.2.30 (Bo & Colin 2011) reads were aligned to the transcriptome database published by Nakasugi et al. (2014). The alignments were evaluated with the help of Trinity v2.2.0 (Grabherr et al. 2011) and edgeR (Mark et al. 2010).

3. Results

3.1. The role of p19 in symptom formation

At the beginning of my research it was a generally accepted dogma that sRNA binding viral suppressors of RNA silencing (VSR) inhibit the endogenous sRNA pathways thus are central players in symptom formation (Chapman et al. 2004, Jay et al. 2011., Schott et al. 2012). However, the phenotype of p19 transformant plant does not resemble the symptoms of CymRSV (Silhavy et al.

2002, Szittya et al. 2002). We raised the question what is the molecular explanation of this?

A p19 transgene construction without sequence similarity to the original viral p19 sequence, so that vsiRNA loaded RISCs would not target it, was required. One such construction was made by our collages in the Tavazza lab (Kontra et al. 2016). The transgenic construction was named „synthetic p19" (p19syn). We ascertained that p19syn maintained its VSR properties. To assure that p19 biogenesis only occurred form one source, in our experiments p19syn plants were infected by the p19 deficient Cym19stop. This also raised the opportunity to observe the sRNA binding properties of p19 when it is expressed *„in trans"* (non viral, transgenci expression) and *„in ciss"* (authentic expression from CymRSV).

We made p19 IP and validated it with Western blots and Northern blots. Previous results suggested that p19 bounds endogenous sRNAs (Chapman et al 2004, Schott et al. 2012). indeed p19 was able to bound vsiRNA and the miR159. Surprisingly however, the level of miR159 decreased drastically in the p19-IPs of virus infected samples, compared to their respective inputs.

We continued our investigation with the sequencing of the sRNAs. It showed that in both „in ciss" and „in trans" cases p19 almost exclusively bound vsiRNAs. We also assessed this on the miRNA level. In the case of uninfected p19syn plants an enrichment of miRNAs in the p19 IPs compared to their inputs can be observed, meaning that p19 can efficiently bind miRNAs.

However, if the virus is present and vsiRNA is formed the ratio of miRNA binding is drastically reduced. In uninfected p19syn plants p19 binds miRNAs efficiently enough to make their targets expression rise, therefore biologically relevant effect is expected. The same cannot be observed in case of Cym19stop infection.

3.2. The sRNA binding preference of p19

In a mock p19syn plant p19 binds endogenous sRNAs but in case of Cym19stop infection it essentially binds vsiRNA, meaning that miRNAs in the p19 are replaced by vsiRNA. One might presume that this is due to the overwhelming number of vsiRNA. This however, cannot fully explain the phenomenon. In virus infected samples the ratios of vsiRNA in p19-IPs are further increased compared to their inputs.

We have found that p19 do not show sequence specificity in general nor for the 5' starting nucleotide the likes of which AGO proteins have. However, in the p19-IP an the GC ratio increased compared to the input.

Based on our lab's results, it can be stated that the structural differences between miRNA and siRNA have significant impact on binding affinity (Kontra et al. 2016). This is further supported by the increase of negative stranded vsiRNA in the p19-IP. Negative stranded vsiRNAs originate from mismatch free double stranded RNAs.

3.3. The effect of p19 on the loading of AGO1 and AGO2

According to the currently accepted model, one function of p19 is to bind vsiRNA thus preventing their loading into an AGO. There are several member of the AGO gene family in plants. It is known that AGO1 and AGO2 plays important roles against RNA viruses. We immunoprecipitated these two AGOs and sequenced the loaded sRNAs. AGO1 primarily bound 21-22 nt long sRNAs with "U" as the 5' starting nucleotide, wheres AGO2 bound 21-22 nt long sRNAs with "A" as the first nucleotide. This is the first conformation of this in *Nicotiana bennthaminana*.

We anticipated that p19 will inhibit the sRNA loading of both AGO1 and AGO2. Surprisingly however, we only observed p19 specific change in the case of AGO1. In the AGO1 IP of CymRSV, but not Cym19stop, infected plants

significant amount of 5’ unsorted vsiRNA “background” can be found. We hypothesize that p19 effects between biogenesis and AGO loading. The 5’ end preference of AGO1 is abolished in case of vsiRNAs, but not in case of endogenous sRNAs, this coincides with p19 preference towards vsiRNA if the virus is present. The vsiRNA profile of CymRSV and Cym19stop in the AGO2 IP do not show any difference. We validated these results with northern blot (Kontra et al. 2016). AGO1 is essential in antiviral defense. On the other hand AGO2 is insufficient in itself for antiviral response, the plant dies.

3.4. The effect of p19 on sRNA size distribution

In CymRSV infected *N. benthamiana* 21 nt vsiRNA is the most abundant, but in Cym19stop infected there is more 22 nt vsiRNA. We vere curious, what is the molecular explanation of this?

To compare precisely the relative expression of DCL2 and DCL4 we conducted *in situ* hybridization. We did not find expressional difference in DCL2 nor DCL4 expression in CymRSV and Cym19stop infected and uninfected samples. To assess that whether they differ in enzymatic activity we sequenced sRNAs. However we did not find difference in the ratio of 21-22 nt ratio on a tissue level.

We investigated, whether p19 stabilizes the sRNAs it binds? For this we used the p19syn transgenic plants. MiR168 and miR159 exhibited elevated levels in the p19syn plants compared to the wild type. Both strands of the miR168 increased. The primary siRNAs of the miR7122 initiated TAS1 pathway and miR7122 itself was increased.

Given that p19 blocks methylation and thus induces 3’-5’ exonuclease digestion, we raised the question can p19 bound sRNAs get shorter due to a partial digestion on their overhanging 3’ ends, that are sticking out of the p19 homodimer. An enrichment of 20 nt vsiRNA can be detected in CymRSV if

compared to Cym19stop. There are no known DCL enzymes capable of cleaving 20 nt vsiRNA. We detected sRNAs with increased gel electrophoretic mobility, if the p19 was present in the sample, in northern blots as well. We used GFP siRNA inducing constructions to understand the shortening effect of p19. In this experiment we detected GFP siRNA in the 20 and 19 nt range besides the 21, 22 and 24 nt range. The shortening was more pronounced in the p19 IP. On the other hand we did not detected shortened sRNAs in case of miR168 which was evidently not bound by p19, nor in the range of 23 nt. As a proof of concept we made an *in vitro* experiment. In this experiment the ratio of sRNA shortening correlated with the concentration of p19. Also, we could not detect sRNAs shorter than 19 nt. The biogenesis of evolutionally conserved miRNAs is precise, thus giving us the opportunity to verify the shortenings direction and also it's ratio. We compared wild type and p19syn plants and the p19 IP from the p19syn plant. In the presence of p19 several miRNA got shorten on the 3' end, and this was more pronounced in the p19 IP.

3.5. The role of DCL2 and DCL4 in anti CymRSV defense

We investigated DCL2, DCL4 silenced and DCL2/4 double silenced plants to shed some light on their importance against CymRSV. In the presence of p19 nor DCL2 nor DCL4 silencing had any effect as expected. The viral RNA level of Cym19stop was much like the level of CymRSV in DCL2/4 double silenced plants. However the knockdown of DCL2 nor DCL4 in itself did not cause significant change. This manifested on the symptoms as well: the symptom of Cym19stop infected DCL2 and DCL4 silenced plant resembled that of the wild type, but the DCL2/4 did not, there was no recovery.

3.6. Novel scientific results

We contradicted the generally accepted dogma, that VSR-s general attribute that they are major players in viral symptom development directly due to their ability to inhibit RNA silencing. We proved that even though p19 is

capable to significantly inhibit endogenous sRNA pathways and change the plants appearance, this does not occur if the virus is present. In case of authentic virus infectin (p19 *in ciss*) or in ectopic p19 expression p19 deficient virus combination (p19 *in trans*) p19 binds vsiRNA almost exclusively. We proved that the main cause of this is the difference in the affinity in which p19 binds miRNA and siRNA, which is due to the structural difference between them. Besides this there is a high GC preference as well but this effect is less substantial.

P19 binding induces shortening of sRNAs on their 3' end. It is known that p19 inhibits 3' end methylation thus sRNAs are exposed to exonucleases. We proved, that the p19 saves the duplex part of the double stranded sRNA from the digestion, resulting in shortened sRNAs. *In vivo* 1 nt shortening occurs primarily. It is likely that this contributes to the shift towards 21 nt vsiRNA in CymRSV infected plants. Another contributor is p19's ability to stabilize sRNAs and its preference toward 21 nt siRNA rather 22 nt.

P19 inhibits the loading of vsiRNAs into AGO1, but not AGO2. We assume that the role of AGO1 is essential in the defense against CymRSV. The knockdown of both DCL2 and DCL4 abolished the recovery from Cym19stop infection. On the other hand the knockdown of either DCL2 or DCL4 did not cause significant difference compared to the wild type.

4. Conclusions and recommendations

Our research contributed to the understanding of the CymRSV – *N. benthamiana* interactions. We contradicted the generally accepted misconception that sRNA binding VSRs have direct effect on viral symptom formation by inhibiting the endogenous sRNA pathways. We proved that in case of virus infection p19 barley bind endogenous sRNAs at al, independently where the p19 was expressed from. Then what is the molecular mechanism

behind the symptom formation? Our extensive NGS datasets form a foundation in answering this question.

Based on uninfected p19syn plants we can assume that p19 prior the virus infection binds endogenous sRNAs. However, as the vsiRNAs appear they replace them in the p19. The affinity towards p19 us negatively affected by mismatches or bulges between the two strands of the sRNA. This is supported by the enrichment of negative stranded vsiRNA in the p19 IP, which originate from perfectly matched double stranded precursor. Our results, that p19 bind different structured sRNAs with different affinity could be important in the perfection of the quickly spreading p19 based cancer diagnostics (Mittal et al. 2017). Due to the differenc how miRNAs are enriched, raises another question: whether the position of mismatches and bulges have an effect on p19 affinity? Answering this question would take us further in understanding the molecular preferences of the binding of p19. Our observation, that p19 enriches GC ratio, is unfortunately does not give us much to speculate. At this point we can say that p19 prefers duplexes with higher GC content or these are more stable or both.

We have found the answer why is there a difference in the vsiRNA profiles of CymRSV and Cym19stop infections. By utilizing in situ hybridization and sRNA seq we investigated and ruled out that a possible difference in DCL expression, in tissues that CymRSV, but not Cym19stop replicates, causes this.

SRNAs are truncated on their 3’ end due to p19 binding. In the in vitro assay we detected both 1 and 2 nt shortened forms, in different ratio. p19 was able to defend the sRNA duplex and to some content the 1 nt overhanging end. In vivo we detected primarily 1 nt shortening both in cas of siRNA and miRNA. This is presumably due to lower RNase pressure. This contributes to the shift in

the ratio of 21:22 nt visRNA. It is highly likely that some of the originally 22 nt vsiRNA shortens to 21 nt.

The miRNA levels are significantly higher in p19syn plants compared to wild types. This suggests that the half-life of the sRNAs are increased. This is supported by the enrichment of miR168 in duplex form. It is known that p19 binds 21 nt sRNAs with higher affinity than those of 22 nt (Vargason et al. 2004). This is backed by our sequencing data also. By conserving the 21 nt vsiRNA p19 further contributes to tipping the ratio.

Based on the research conducted on DCL2 and DCL4 knockdown plants we can say that neither DCL has more importance in this host-patogen interaction. However, decreasing the expression of both had drastic result. Other DCLs were insufficient to counter the knockdown of DCL2 and DCL4. DCL2 and DCL4 acts redundantly.

The sorting and probably the functioning of AGO1 was compromised in CymRSV but not Cym19stop infected plants. Also, the function of AGO2 was not inhibited. Based on this we can say that the correct function of AGO1 is essential. The level of AGO2 did not change. Meaning that p19 did not inhibit the endogenous regulating function of AGO1. The explanation of this is that p19 do not bind endogenous sRNAs if the virus is present. We made an observation, that there are 20 nt vsiRNA in AGO2. These are sRNAs that had been bound by p19, than left it and were loaded into AGO2. Whether these are biologically active remains a question, and could be investigated further. Another interesting question is, what is the molecular mechanism of p19's ability to inhibiting the 5' nucleotide sorting of AGO1?

During our research we generated a huge amount of NGS data and developed scripts for their processing. A realistic goal would be to identify the full sRNA profile of *N. benthamiana*. The p19syn plants gives excellent

opportunity to identify the sRNA duplexes due to the stabilizing and conserving effect of p19 on the biologically irrelevant strand. From these detected sRNAs, based on our AGO-IP libraries, it would be easy to sort out functional sRNAs.

5. Publications

5.1.1. Scientific articles:

International, scientific articles with impact factor

Kontra Levente, Csorba Tibor, Tavazza Mario, Lucioli Alessandra, Tavazza Raffaela, Moxon Simon, Tisza Viktória, Medzihradszky Anna, Turina Massimo, Burgyán József: Distinct effects of p19 RNA silencing suppressor on small RNA mediated pathways in plants. Plos Pathogen. 2016 Oct 6;12(10):e1005935. doi: 10.1371/journal.ppat.1005935.

Csorba Tibor, Kontra Levente, Burgyán József: viral silencing suppressors: Tools forged to fine-tune host-pathogen coexistence. Virology. 2015 May;479-480:85-103. doi: 10.1016/j.virol.2015.02.028.

Domestic, scientific articles whit out impact factor

Kontra Levente, Burgyán József: Antivirális géncsendesítés és annak gátlása (Antiviral genesilencing and it's supression) Növényvédelem, 2015. 12. szám

5.1.2. Other scientific publications:

Conference proceedings

Kontra Levente, Burgyán József: The role of DCL2 and DCL4 in plant-virus interaction (A DCL2 és a DCL4 szerepe a növény-vírus interakcióban)**,** Hungarian Molecular Life Sciences 2015. március 27-29 Eger, Magyarország (ISBN 978-615-5270-15-4)

Conference abstracts

Kontra Levente, Csorba Tibor, Tavazza Mario, Lucioli Alessandra, Tavazza Rafael, Moxon Simon, Baksa Ivett, Tisza Viktória, Burgyán József: The effect of p19 RNA silencing suppressor on small RNAs in plants (A p19 RNS csendesítés szupresszor hatása kisRNS-ekre növényekben) International Conference on Genomics and Bioinformatics, 2016 május 7. Izmir, Törökország

Kontra Levente, Csorba Tibor, Tavazza Mario, Lucioli Alessandra, Tavazza Rafael, Moxon Simon, Tisza Viktória, Burgyán József: The effect of p19 RNA silencing suppressor on small RNAs in plants (A p19 RNS csendesítés szupresszor hatása kisRNS-ekre növényekben), Fiatal Biotechnológusok Országos Konferenciája II. 2016. március 21.-22. Gödöllő, Magyarország

Kontra Levente, Burgyán József: A p19 silencing szuppresszor hatása a kis RNS-ek méreteloszlására (the effec of the p19 silencing supressor ont he size distribution of sRNAs) Genetikai Műhelyek Magyarországon XIII. 2014. szeptember 12. Szeged, Magyarország

Kontra Levente, Burgyán József: The effect of the p19 silencing suppressor on antiviral siRNA biogenesis (A p19 RNS csendesítés szupresszor hatása az antivirális siRNS biogenezisére) Plants for the future, 2013. szeptember 30. – október 2. Cluj-Napoca, Románia

Kontra Levente, Szabó Emese Xóchitl, Burgyán József : A p19 Silencing szupresszor fehérje hatása a virális siRNS-ek biogenezisére (The effect of p19 silencing suppressor protein on viral siRNA biogenesis), MBK napok 2012. 12. 05. Gödöllő, Magyarország

Other scientific publications

Kontra Levente, Csorba Tibor: Hadakozó gazdanövények és paraziták – Géncsendesítéssel a vírusok ellen (The warfare between hostplants and parasites – Genesilencing against viruses) Természetbúvár 69: 2014 6/12

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