Investigation of miRNAs controlling fruit and seed development. K78351

During the four-year period of our grant we attempted to better understand the role and molecular action of microRNAs (miRNAs) in plant developmental processes focusing mainly on fruit formation.

MicroRNAs (miRNAs) are small, 21–22 nucleotide RNA molecules encoded in the genomes of plants, animals. They are derived from single-stranded RNA precursors that can form stem–loop structures. MiRNAs are generated by sequential processing of genome-coded long single-stranded RNA precursors and are incorporated into the RNA induced silencing complexes (RISCs). ARGONAUTE1 (AGO1) is one of the most important AGO proteins in RISCs playing central role in miRNA mediated regulation of endogenous mRNAs and siRNA directed control of virus infections. MiRNAs regulate the expression of genes by binding to complementary sequences in specific mRNAs. This binding leads either to cleavage-induced degradation of the mRNA or suppression of its translation. Both processes eventually result in down-regulation of the product of the target gene. Importantly AGO1 mRNA itself is a subject of a miRNA mediated regulation via the activity of miR168. MiRNAs are indispensable for the control of wide variety of biological functions, including development, hormone responses, feed-back mechanisms, biotic and abiotic stresses.

In this work we investigated two major aspects of miRNA mediated regulation (1) the action and presence of miRNAs during fruit formation and (2) the regulation of the central AGO1 protein.

(1)

We investigated the role of miRNAs in seed and fruit development. Using our *in situ* hybridisation data base results together with the latest online databases and scientific literature we selected miRNAs present in *Arabidopsis thaliana* seed or fruit. We studied the role of these miRNAs (miR166, miR827, miR169, miR828, miR390, miR824, miR858, miR775, miR857, miR394, miR472) in fruit development by transgenic over-expression of miRNA precursor genes in transgenic plants. To our surprise only miR824 and miR857 over-expressing transgenic plants showed significant phenotipical changes during silique formation. Transgenic plants over-expressing miR824 produced abnormally small siliques, while miR857 over-expressing plants, produced siliques with fewer seeds than wild type plants. We analysed the expression of miR824 and miR857 using small RNA Northern blot and we found correlation between the severity of the phenotype and enhanced accumulation of the investigated miRNAs. Currently we are investigating the level of potential target mRNAs in the transgenic plants. These results were rather surprising since the investigated miRNAs were conservative miRNAs, they are present in different species, indicating their evolutionary fixation. Regarding the miR824 and miR857 transgenic plants

we are producing homozygous lines to be able to reliably investigate the phenotypes. However, we also would like to investigate the overexpressor lines, which show no altered phenotype, to reveal whether the down regulation of the particular host genes are neutral or the over expressed miRNA is disabled in some way to regulate of its host.

During our experiments we analyzed the spatial accumulation pattern of selected conservative miRNAs in *Arabidopsis thaliana* embryonic tissues with *in situ* hybridisation. MiRNA mediated regulation has been demonstrated to be essential for normal embryo development processes. Earlier reports suggested, that miRNAs function cell autonomously. However, other studies showed that miRNAs can function non-cell autonomously moving cell-to-cell trough plasmodesmata. Several reports demonstrated the presence of miRNAs in the phloem and phloem indicating the potential of miRNAs to travel long distances in plants. Grafting experiments showed that miR395 and miR399 are phloem mobile and can regulate their targets in long distance supporting the signalling function of some miRNAs. The ability of miRNAs to move cell-to-cell can produce a gradient-like expression pattern as the miRNA moves away from the place of its generation. This miRNA gradient may act similarly to animal morphogens mediating a dose-dependent regulation of developmental processes. Since the mobility of a particular miRNA in a given tissue can fundamentally determine its regulatory mode, the importance of the investigation the putative non-cell autonomous activity of miRNAs is evident. During our experiments we analyzed the spatial accumulation pattern of selected conservative miRNAs in *Arabidopsis thaliana* embryonic tissues with *in situ* hybridisation. The majority of the investigated miRNAs showed uniform accumulation across the embryo demonstrating their role at this developmental stage. In the case of miR167, however, we detected a gradient like expression profile which is usually considered to be the hallmark of the non-cell autonomous activity of miRNAs. Using a previously described GUS reporter assay we analyzed the spatially highly coordinated expression of the four precursor genes producing mature miR167. We found that one of precursors, *MIR167a,* also showed a gradient like expression pattern in the embryo. These data point to the necessity of careful investigation of gradient like expression patterns of miRNAs since this phenomenon can be generated not only by the non-cell autonomous action of miRNAs but also by the specific expression characteristic of miRNA precursor genes.

Publication associated with this part of the work:

Agyi, A and Havelda, Z (2013) Analysis of gradient-like expression of miR167 in Arabidopsis thaliana embryonic tissue, JOURNAL OF PLANT BIOLOGY 56 (5): 336-344.

Várallyay E. and Havelda Z. (2011) Detection of microRNAs in plants by in situ hybridisation. In MicroRNAs in Development. *Methods in Molecular Biology 732*, Humana Press, (Edited by Tamas Dalmay) pp. 9-23.

Havelda Z. (2010) *In situ* detection of miRNAs using LNA probes. In *Plant MicroRNAs*. *Methods in Molecular Biology 592*, Humana Press, (Edited by Pamela Green and Blake C. Meyers) pp. 127-136.

(2)

Our very important focus was the investigation of the regulation of AGO1 level, the master component of the RISC. We took the advantage that AGO1 is active in siRNA based antiviral defence system similarly to the miRNA pathway. The use of virus infected plants allowed the easy induction of AGO1 mRNA as we detected that virus infection is associated with AGO1 mRNA over accumulation previously. AGO1 level regulation is very well regulated by different mechanisms. As a feedback mechanisms, AGO1 homeostasis itself is controlled by coordinated action of miR168 and *AGO1*-derived siRNAs on *AGO1* mRNA. Moreover, additional components of the miRNA mediated feedback regulation of *AGO1* have been also described involving the AGO1 mediated post-transcriptional stabilization of miR168 and the co-regulated expression of *AGO1* and *MIR168* genes. These results demonstrate the existence of a complex refined feedback regulatory loop, which balances AGO1 and miR168 accumulation. Analyses of plant-virus interactions revealed that the induction of *AGO1* mRNA level is not accompanied by increased AGO1 protein accumulation. By studying virus infected *Nicotiana benthamiana* plants we show that the induction of *AGO1* mRNA level is a part of the host defence reaction while induction of miR168 level, which overlaps spatially with virus-occupied sectors, is mediated mainly by the viral p19 RNA silencing suppressor (VSR). The absence of p19 RNA silencing suppressor from the virus infection process brings about the elimination of miR168 induction and in parallel the enhanced accumulation of AGO1 protein. In transient expression study p19 was able to mediate the induction of miR168 and the down regulation of endogenous AGO1 level. Moreover, we also show that p19 is not able to efficiently bind miR168 in virus infected plants indicating that this activity is uncoupled from the small RNA binding capacity of p19.

Our results suggest that p19, in addition to its small RNA binding activity, can inhibit the translational capacity of *AGO1* mRNA by modulating the endogenous miR168 level to alleviate the anti-viral function of AGO1 protein. Our results strongly suggested that virus infection induced miR168 accumulation is responsible for AGO1 protein down regulation via the inhibition of the translational capacity of *AGO1* mRNA. Next we investigated wild type and mutant *A. thaliana* plants disabled in the activity of miR168 mediated control (4mAGO1) or compromised in the activity of ZWILLE/PINHEAD/AGO10 (zll-3) and AGO1 (ago1-25). To test whether virus infection associated repression of AGO1 protein accumulation is specific phenomenon we investigated the accumulation copper chaperone for superoxide dismutase (CCS1), which have been previously demonstrated to be under miR398 directed translational control. We found that CCS1 protein level did not change in virus infected wild type *A. thaliana* plants indicating that the observed down regulation of AGO1 protein is not a part of general enhancement of translational repression in the infected plants. We investigated the role of miR168 in this process by infecting 4mAGO1 mutant plants possessing *AGO1* gene

which contains four silent mutations in the miR168 target site rendering the mRNA to be resistant for miR168 mediated control. In these mutant plants the down regulation of AGO1 protein was inhibited in spite of the enhanced accumulation of miR168. These results demonstrated that miR168 is directly involved in the down regulation of AGO1 since elimination of correct target site from the mRNA resulted in less effective inhibition of AGO1 accumulation. Previously it has been shown that ZWILLE/PINHEAD/AGO10 is able to exert translational regulation. Analyses of virus infected zll-3 plants revealed that down regulation of AGO1 protein is inhibited in spite of the enhanced accumulation of miR168. In contrast, ago1-25 hypomorphic showed no efficient inhibition of AGO1 repression. Moreover, the enhanced accumulation of AGO1 is connected to the disabled activity of ZWILLE/PINHEAD/AGO10 in virus infected plants implicating the pivotal role of ZWILLE/PINHEAD/AGO10 in translational regulation of *AGO1* mRNA by miR168.
Since these results were very interesting and we were able to publish the data in a high profile journal we continued the experiments to analyse in depth the miR168 mediated AGO1 regulation.

In a further work we demonstrate that the ability of p19 to induce the accumulation of miR168 is not connected to its ability to bind virus specific siRNAs. We show that the presence of a siRNA binding deficient p19 VSR in the infection process is associated with the induction of miR168 level and the subsequent control the AGO1 accumulation. These data indicate that the control of AGO1 protein level is an important component of the efficient tombusvirus invasion. Moreover, our data suggest that siRNA binding and mir168 inducing capacities of p19 VSR evolved independently providing a multiple parallel counter defence activities to suppress RNA silencing successfully. Moreover, we also showed that infection of RNA viruses belonging to various genera is associated with the transcriptional induction of *MIR168a* precursor gene. In transient expression study we reveal that different unrelated VSRs are responsible for the enhanced accumulation of miR168. The induction of miR168 accumulation is an early function of VSRs and this activity is associated with the control of endogenous AGO1 protein level. During these experiments we applied gel infiltration technology to virus infected and mock plants, revealing the miR168 accumulates not only RISC bound forms but also in free from suggesting and new regulatory mechanism at the level of RISC loading.

Publication associated with this part of the work:

Várallyay E, Válóczi A, Agyi A, Burgyán J and Havelda Z (2010) Plant virus-mediated induction of miR168 is associated with repression of ARGONAUTE1 accumulation. The EMBO Journal , 20;29(20):3507-19.

Várallyay É, Oláh E, Havelda Z (2014) Independent parallel functions of p19 plant viral suppressor of RNA silencing required for effective suppressor activity. Nucleic Acids Res. 2014 Jan;42(1):599-608.

Várallyay E, Havelda Z. (2013) Unrelated viral suppressors of RNA silencing mediate the control of ARGONAUTE1 level. Mol Plant Pathol. 2013 Apr 11. doi: 10.1111/mpp.12029.

Burgyán J. and Havelda Z. (2011) Viral suppressors of RNA silencing. Trends Plant Sci. 16(5):265-72.

Conclusion and perspectives

During our work we tested the role of several miRNAs, supposed to be have role in fruit formation, in transgenic over expression approach. These miRNA were selected based on our miRNA in situ data base and public results. We learnt a lot about the cloning and testing miRNA precursors in transient systems. Hopefully, this knowledge will help us to better understand the structural requirement of miRNA precursors for producing biologically active miRNAs and will also help us to construct artificial miRNAs for reliable and precise experimental purposes. In these experiments we faced with the suppressing results the over production of important miRNA species did not induce usually dramatic phenotypic changes and if phenotypic alteration is present (seed loss etc,) this trait not always correlates with the level of the over expressed miRNA. We are producing homozygous lines for further investigation of this phenomenon. However, the investigation of AGO1 regulation was more lucrative resulting in several publications. These results prompted us to investigate AGO1 regulation in transgenic systems. We have produce mir168 over expressing lines and we are currently investigating the progeny of transgenic lines. As a new result we found that the flowering time is affected in these lines and also the AGO1 level control shows differences in different tissue types. This system seems very promising for understanding the regulation of the key AGO1 protein. Now we are utilizing different Arabidopsis mutants to better understand AGO1 regulation.

The experience and the technological knowledge (miRNA in situ hybridization in Arabidopsis and in Solenaceae species (pepper etc.), miRNA precursor cloning and testing, artificial miRNA production, transgenic systems etc.) we accumulated will help us to carry out ongoing projects. Hopefully, new publication will be produced in the near future based on our very recent results.

Gödöllő, 2014/03/28

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