Friedrich-Schiller-Universität Jena

Biologisch-Pharmazeutische Fakultät

Max-Planck-Institut für Chemische Ökologie

Abteilung für Molekulare Ökologie

Prof. Ian Thomas Baldwin

Arbeitsgruppe Dr. Pandit

seit 1558

MicroRNAs as "direct defense" molecules

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Marcus Horn

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1st Examiner

Prof. Dr. Ian Thomas Baldwin

2nd Examiner

Dr. Sagar Subash Pandit

Presented By

Marcus Horn,

Dr. Hans-Loch Straße 3,

99867 Gotha, Germany.

University ID: 56241

Prepared in

Max-Planck-Institute for Chemical Ecology,

Department of Molecular Ecology

Hans-Knöll Straße 8,

07745 Jena, Germany.

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The thesis was delivered at due date to the Friedrich-Schiller-University Jena, Department for Biochemistry.

It was written by my own and the data have not been published somewhere else, yet. Quotations of all used references are denoted and described in detail.

I am very grateful to Prof. Ian T Baldwin who provided me with the opportunity to work in his Department of Molecular Ecology and especially to Dr. Thomas Hahn for his valuable support. My special recognition and my thanks go, of course, also to all the people in the Department of Molecular Ecology who supported me throughout the overall process.

Abstract

In this diploma thesis a new microRNA (miRNA) based layer of host-plant resistance (HPR) is hypothesized.

The first objective was to prove that selected miRNAs from *Nicotiana attenuata (N. attenuata)* cause a downregulation of mRNA levels from specific midgut genes in *Manduca sexta (M. sexta)*.

The second objective was to undermine the new emerging concept of cross-kingdom regulation (CKR), where miRNAs are considered as moving molecules which switch from one species to another.

Plants have evolved a broad range of adaptations to ensure their survival and reproduction capabilities against pathogens and herbivores, called host-plant resistance (HPR). Each type of defense is either constitutive or induced by stress, damage or elicitors. In response, plants may release secondary metabolites, proteins and enzymes.

In this context, miRNAs produced from *N. attenuata* upon attack by the specialist herbivore *M. sexta* are postulated as new direct defense molecules. Beyond their role in the stress response within the plant, these "direct" miRNAs are postulated to perform their relevant mechanism of action by knocking down specific targets in the midgut of *M. sexta.* The superior advantage of these tiny noncoding RNA's compared to other defense metabolites is their cost-efficient characteristic in terms of energy and putative flexibility to target every possible nucleotide sequence. Rather than intercepting a single target like in the case of selective protein inhibitors or siRNAs, miRNAs can modulate entire thousands of possible gene programs on a basic level, due to their promiscuity behavior. Based on their extraordinary modes of action, miRNAs will lead to a paradigm shift beyond plants. These aspects open up particular perspectives for new drugs, insecticides and knowledge.

The reverse genetics approach is applied here to elucidate gene function in insects. The conducted experiments were split into a dry lab phase where subsets of miRNAs from N. attenuata were selected in silico, based on sequence complementarity, to target specific mRNAs in *M. sexta*.

Successful candidates were transcribed in vitro by T7 in Vitro Transcription and then orally administered via feeding *M. sexta* on artificial diet. Simultaneously, a control group without miRNAs was fed. After 10 days, midgut RNA was isolated and qRT-PCR was performed with target specific primers. The results show a significant decrease of chitin synthase mRNA levels targeted by miRNA named: M6 and *MS*CYP6AN5 mRNA targeted by miRNA named: M25, respectively.

Consequently, our study adds to the increasing body of evidence, demonstrating that some miRNAs potentially serve as direct defense weapons and therefore support the superior concept of CKR.

Key Words: MicroRNAs, Host-plant resistance, Cross-kingdom regulation

1. Introduction

Since the commercial availability of Next generation sequencing (NGS) hand in hand with faster computers the accessibility, speed and amount of information have increased tremendously and gene expression studies went through enormous paradigm alteration. During my time in the Max Planck Society, a colleague formulated accurately: “Today we deal with information and possibilities what we couldn’t even imagine 6 months ago.” In the post NGS area, the human genome was sequenced, and it was considered that almost 95% of the DNA did not code for anything. Therefore, the term “junk DNA” was a commonly known and accepted expression which allegedly had no purpose. Mostly, the big molecules e.g. like proteins and their underlying genetic sequence stood in the center of attraction. The DNA sequence information itself only functions as a building plan from a library. Nowadays, as things get more sophisticated and scientists are able to look beyond the protein-horizon, the DNA and RNA are not playing only their role as a library “how to make proteins”. Instead these molecules create their sense by themselves.

Till the late 90’-s research was mainly focused on the important but only small tip of the protein iceberg. Proteins made only 2% of the genome and parts encoding for proteins are generally easy to find. Nevertheless, in human these are approximately 500.000 proteins. But one of the most highlighting results of the ENCODE project is the fact that 80% of the entire genome is transcribed, and therefore the question has to be answered, why the cellular machinery should invest these considerable expenditures. To say it metaphorically: The main challenge is now to breathe life into the 78% of the transcriptome in order to show structure and function instead of only to junk or random pattern. So the main question arouses: What to do with the 78% rest? (See Figure 1)

Figure 1

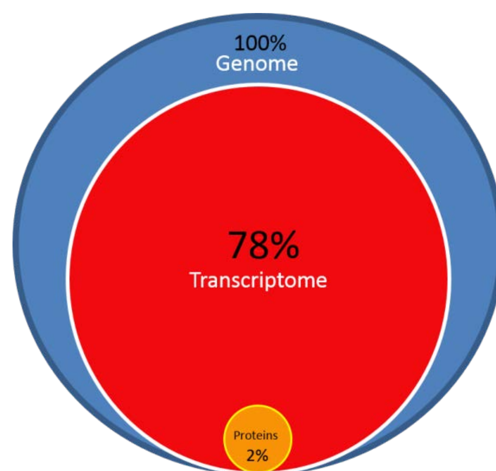


Figure by Marcus Horn

However the discovery of miRNAs as well as other non-coding RNAs (ncRNAs), which do not encode for proteins, indicates much more in the genome than protein coding genes. Indeed, miRNAs represent ~4% of the genes in the human genome. For plants, scientist also start to elucidate the role of miRNAs. This discovery suggests that the genome is far from being deciphered, and most importantly that miRNAs are likely to represent just the “tip of the (transcript) iceberg” with many other small non-coding RNAs to be discovered. It can be undermined by the relatively new identifications of e.g. piwi-interacting RNAs (piRNAs), endogenous siRNAs, and intron derived miRNAs (miRtrons) or cryomiRs. The complete list of new RNAs won’t be finished soon. In summary, the recent identification of novel regulatory RNAs has opened a new window to an important area of Biology which remained unexplored until now.

1.1 MiRNAs in HPR of *N. attenuata*

Figure 2

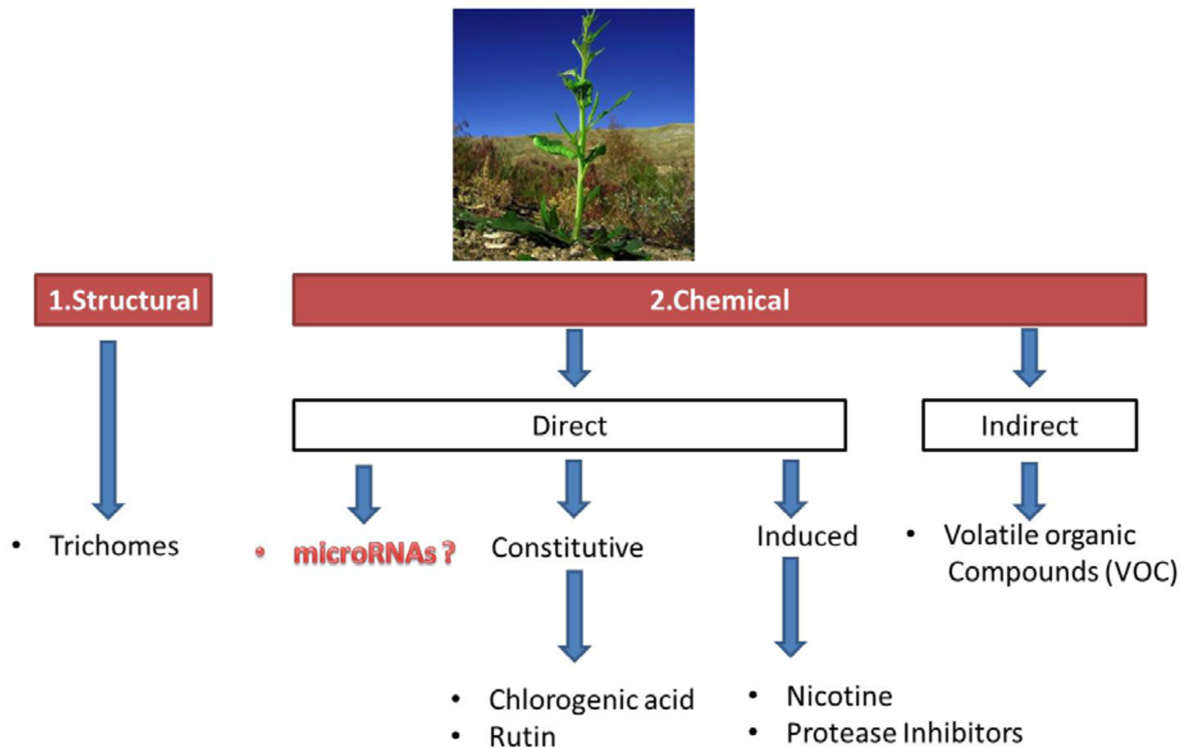


Figure by Marcus Horn

1.2 Hypothesis and Test

1. Hypothesis:

Selected *M. sexta*'s CDS levels are downregulated by MATURE or/and PRECURSOR miRNAs of *N. attenuata.*

2. Test:

1. Dry lab: In silico determination of miRNAs
2. Wet lab: Feeding MATURE and PRECURSOR miRNAs to *M. sexta* for 10 days
    - Measuring *Ms*CYP6AN5, *MS* Chitin synthase and *Ms*18-56 transcripts by qRT-PCR; comparison with the control

To achieve this aim it was necessary to assume a new concept of miRNAs in plants defense upon herbivore attack beyond their role as regulative molecules involved in the defense mechanism inside the plant. As a model system the interaction between *Manduca sexta (M. sexta)* and the wild tobacco *Nicotiana attenuata (N. attenuata)* is used to investigate the role of miRNAs in this context.

Moreover, it could be speculated that special tiny miRNAs are produced by *N. attenuata* as counterdefense molecules in sufficient amounts and then consequently transferred via oral uptake of leaf material to the caterpillar to perform their relevant mechanism of action by downregulating target genes in *M. sexta's* midgut. All in all the plant will benefit from this in vivo reverse genetic strategy.

However this hypothesis is attractive, it was not part of this thesis. The experimental objective was to provide a proof of concept and whether *N. attenuata* miRNAs are able to knock down transcripts the herbivore *M. sexta*.

1.3 What are microRNAs?

In my opinion, the felicitous expression: “Micromanagers of gene expression” is an excellent precise term to describe the nature of miRNAs to the point. MiRNAs are RNA molecules which originate from small non-proteinous coding sequences present in virtually all animals and plants and tend to be transcribed from various different loci in the nuclear genome.

These genes encode for RNAs with a hairpin structure that when processed by a series of RNaseIII enzymes (Drosha and Dicer) form a short miRNA duplex of approximately 22 bp long with 2nt overhangs on the 3'end.

They play their role in eukaryotic genomes and functions as transcriptional and post transcriptional regulators of gene expression. Nevertheless, the possibility cannot be ruled out that prokaryotes or Viruses also "use" them because miRNAs could be considered as a very cost effective multi-purpose tool.

In plants, miRNAs have a pivotal role during fundamental processes such as development, maintenance of genome integrity and abiotic stress responses. A good documented example is their involvement in leaf structure and morphogenesis. [1]

Figure 3

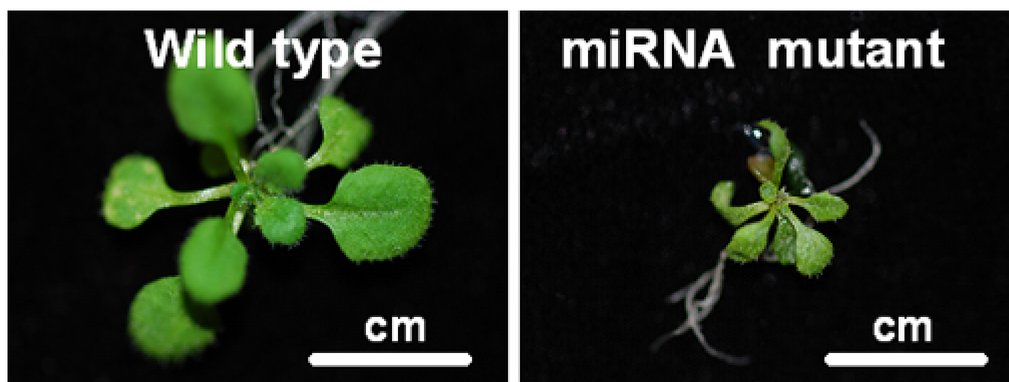


Figure by Pratik Shah, Seong Wook Yang

In insects research on miRNAs has been mainly limited to *Drosophila melanogaster*. In *D. melanogaster*, experimental methods, both *in vitro* and *in vivo*, have confirmed the essential and crucial roles of miRNAs for cellular functions. Indeed, miRNAs have been implicated in numerous biological processes ranging from cell proliferation and apoptosis during development, cell–cell interactions during development of the peripheral nervous system, to stress resistance and fat metabolism, from cellularization and segmentation on embryos to cardiogenesis and muscle growth. Unfortunately, the roles of miRNAs in non-drosophiloid insects have not been established clearly yet.

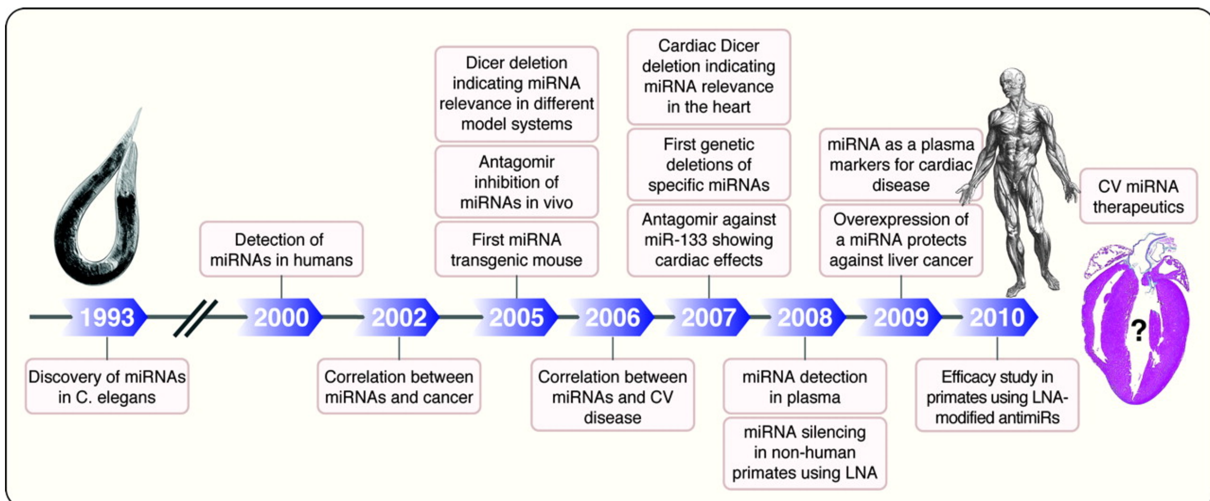
In general, miRNAs are not as precise, accurate or efficient as the big specialized enzymes but their outstanding advantage is that they are small, easy to produce and highly flexible. If an enzyme is considered as a specialist, miRNAs could be the generalist equivalent. Due to their metabolic low costs, the concept of small molecules like peptides, RNAs etc. is probably a strategy used to sound with trial and error characteristics. For example: a gene is knocked down primarily with miRNAs. If this gene turned out as a good candidate, a bigger expensive molecule like a protein comes into play. It is a kind of screening tactic like HTS-lead-discovery approaches in the pharmaceutical industry. Continuing this logic, an immediate phenotype effect in the larvae is therefore not strictly necessary. Sufficient enough for the plant is to find a good target for developing more sophisticated allelochemicals.

1.4 History

The recent discovery of microRNAs (miRNAs) took many by surprise because of their unorthodox features and widespread functions.

1993 Ambros et al. discovered that a gene called lin-*4*, did not encode a typical protein. This discovery itself is quite unexpected and revolutionary, because in the early 90's the concept of junk DNA and the idea that genes are only coding for proteins was widespread. Instead *lin-4*, gave rise to some small RNA molecules, 22 and 61 nucleotides in length, which Ambros named lin-4S (short) and lin-4L (long). Sequence analysis revealed that lin-4S was part of lin-4L and was predicted to form a stem-loop structure, with lin-4S contained in one of the arms. Moreover, lin-4S was partially complementary to several sequences in the 3'UTR of the mRNA coding for the LIN-14 protein. The *C. elegans* was used in this case for research whereas the repression of the protein LIN-14 is crucial for normal larva development. So nematodes with down regulated *lin-4* show mutant forms. Therefore, Ambros and colleagues hypothesized that *lin-4* could regulate LIN-14 through binding of lin-4S to these sequences in the *lin-14* transcript in a type of antisense RNA mechanism. [2; 3,]

Figure 4



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On this timescale it is easy to recognize a coincidence between the commercial availability of technically mature NGS technologies around 2005 and the increase by leaps of miRNA related breakthroughs.

1.5 Structure and Dicer cutting sites

Figure 5

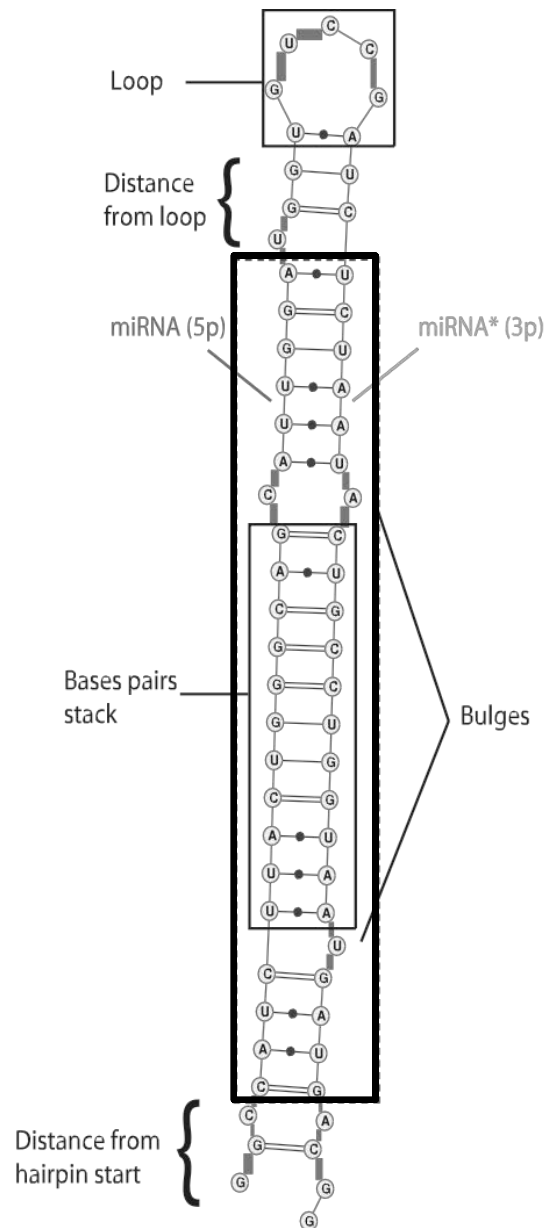


Figure by Meyer

This structure shows a typical miRNA precursor (pre-miRNA). As demonstrated here, the mature miRNA in the red rectangle is located between 2 secondary structure elements shown as bulges. The secondary structure of miRNA precursors frequently contains internal loop/bulge in those regions where one arm has extra inserted bases with no counterparts in the opposite arm. Those bulges serve as recognition site for a protein named DICER in addition to primary structure sequence motives. A typical pre-miRNA has a thermodynamically stable structure with a minimum free energy of ΔG = -25 kcal/mol for a thermodynamic ensemble.

A feature of mature miRNAs is the composition of typical Watson-Crick base pairings in a continuous stretch, with as far as possible, less mismatches; G:U base pairings; and bulges. Some papers also

suggest a typical sequence within the mature miRNA as primary structure motives e.g. an adenosine at the end, but there is no common rule for these findings.

One of the questions in this work was to define the exact DICER cutting site within the precursors in order to locate the exact length of the short mature miRNA.
Dicer is a multidomain enzyme that generates small RNAs for gene silencing in eukaryotes. Hereby, nucleotide composition and secondary structure information of Dicer cleavage sites determines the cutting properties of Dicer.

Figure 5.1

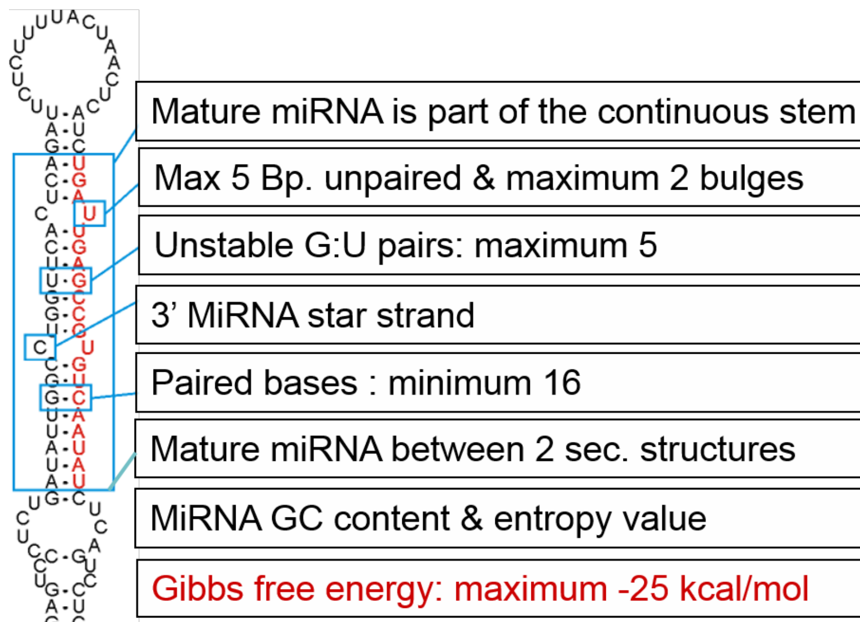


Figure by Marcus Horn

A common model posits that Dicer measures the distance from the 3' overhang of the double stranded RNA terminus and cuts somewhere ~22 bp apart. The 5' counting rule theory suggests a Dicer anchoring at the 5' prime end. A novel basic motif (5' pocket) in human Dicer recognizes the 5'-phosphorylated end and requires a 5'-terminal phosphate group. Studies indicate an altered cleavage if the 5'pocket is mutated. [42]

Those rules are not applicable everywhere, and are only valid for specific Dicers in human.

Unless Dicer plays an important role in processing pre-miRNA, several new studies revealed a non-canonical pathway including Argonaute2. By analyzing mutants in Dicer and Argonaute2 a catalytic

activity of Argonaute2 additional processing intermediates, termed Ago2-cleaved precursor miRNA or ac-pre-miRNA was observed. [43]

A method to predict Dicer processing sites at 5' arm of pre-miRNAs using support vector machine (SVM) is provided by Raghava et al.and was used in this thesis to obtain preliminary indications of the issues.

http://www.imtech.res.in/raghava/phdcleav/submission.html

Figure 6

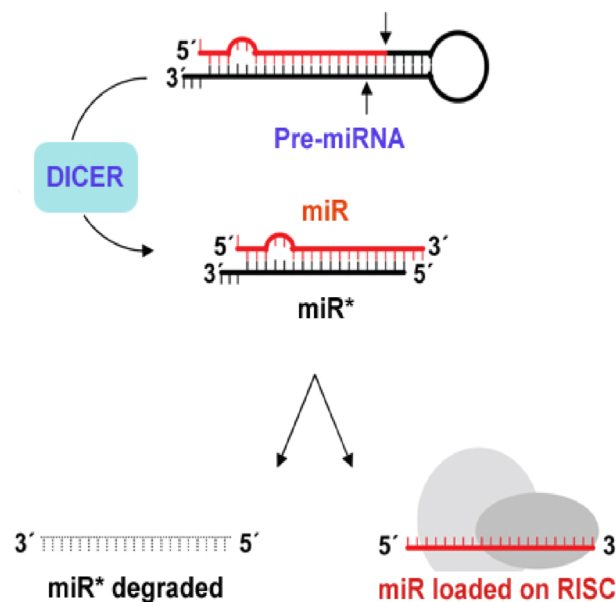
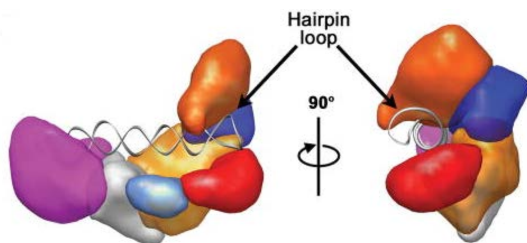


Figure by Dr. GPS Raghava et al.

To provide evidence of CKR-miRNA it is necessary to shed light on the Dicer molecule itself. One doubtful issue in this context is: Whether a Dicer from an insect is able to process a plant miRNA. In summary, it can be postulated that due to its hairpin recognizing structure and based on various publications, Dicer is able to deal with lots of different short (<200bp) ncRNA species. One common feature of a typical Dicer substrate is a hairpin motive (see figure 7). Impressively shown that mature aminoacylated tRNAs enter the small RNA pathway via Dicer processing. Dicer could produce two types of tRNAfragments (tRFs) from tRNAs. Meanwhile one type can be incorporated into argonaute proteins.

Another example is the processing of snoRNAs. [44] Additionally, lots of small RNA species can enter the canonical si/miRNA pathway or are involved in a cross-talk relationship.
The main statement at this point is the idea of a flexible Dicer which is able to handle numerous short RNA molecules, beyond the core repertoire. [45]

Figure 7



Picture by Lau PW et al.[46]

All those findings consistently add indications to a flexible processing of pre-miRNA with numerous degrees of freedom. Therefore, a diverse processing mechanism in insects with unexpected output of plant mature miRNAs appears assumable.

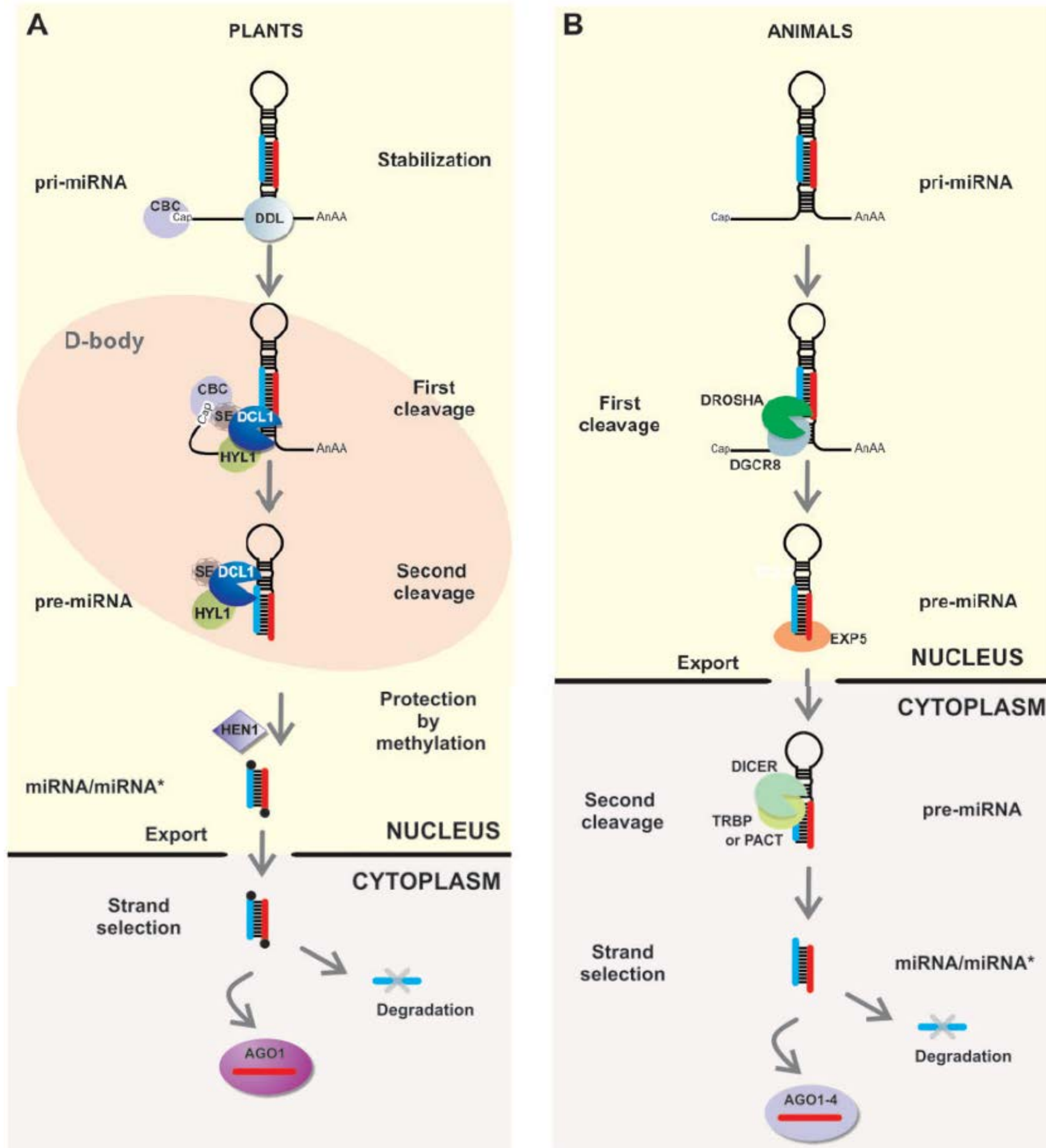
1.6 Plant and animal miRNAs biogenesis and differences

A focal point which has to be taken into consideration for this thesis is the arguable fact of a miRNA in two different phylogenetic kingdoms. Most of the literature allows only a functional concept in one species or at least in one kingdom. The question therefore arises, how far it is acceptable to hypothesize a functional miRNA in 2 different phylogenetic trees. At this point, little is known about the specific behavior, biogenesis and turnover of one given miRNA in different species. So therefore, it is necessary to put appropriate arguments which could be used to undermine this theory.

In the light of the information available, the major part of the literature differentiates between plants and animal miRNA. All the different stages like: biogenesis, structure and base composition, turnover, processing, transport, storage, target recognition, final mechanism of action and decay are subjects to discussion.

Profound knowledge is established about differences in the biogenesis pathway.

Figure 8



Picture by James C. Carrington, Victor Ambros

Additionally, in terms of target recognition, the finding of 100% base pair complementarity in plants and imperfect complementarity in animals has been considered as established. Nevertheless, there are obvious similarities between these 2 kingdoms from the early start. The basic characteristic principle of the biogenesis for instance is the same, as well as the key proteins which are in the majority of the cases coevolved isoforms with the same features and activities.

The final downstream effect of gene knockdown by an approximately 20 bp oligo strand incorporated in the RISC complex is the same.

Considering those findings it would not be at all that far-fetched to presume that plant miRNAs feature the potential to be processed by the animal miRNA machinery.

Target recognition

MicroRNAs regulate the expression of protein-coding genes in animals and plants. They function by binding to mRNA transcripts with complementary sequences and inhibit their expression by targeting one or multiple sites in the complete CDS. The 3'UTR was thought to be the only target site but many other miRNA target sites are possible. Once processed into their mature form, miRNAs generally can also bind to other regions of the gene including the 5 ' UTR and the coding region [4]. The only critical parameter is the target accessibility depending thermodynamic parameters, secondary structure and epigenetic factors (e.g. DNA binding proteins). [5]

Figure 9

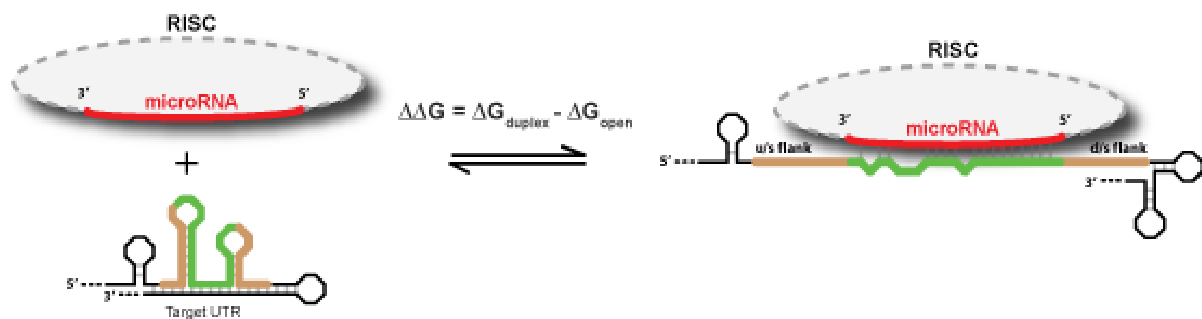


Figure by Kertesz et al.

The level of sequence complementarity between the microRNA and mRNA transcript varies between animal and plant systems. Owing to this subtle difference, it was initially believed that animal and plant microRNAs act in different ways. Recent developments revealed that, although differences still remain in the two kingdoms, the differences are smaller than first thought. It is now clear that both animal and plant microRNAs mediate both translational repression of intact mRNAs and also cause mRNA degradation.

Complementarity to the core region also known as "seed region" (positions 1–10) of miRNA is often sufficient for effective regulation. But latest findings suggest that even some bp meet the minimum requirements to cause an effect of downregulation. Therefore, one miRNA can affect transcript and protein levels of hundreds or even thousands of targets. Consequently, these miRNAs cover a wide scope of physiological and developmental processes in animals and in plants.

The sheer number of predicted targets for any given miRNA hints to the multitude of potential functions, of which only a limited number has been explored properly. [6] Consequently, an "off-target" effect is also a possible result of miRNA action.

1.7 MiRNA's silencing mechanism

The canonical function of miRNAs is to induce the degradation or translation repression by targeting on mRNA sequences.
Hereby, the prevailing doctrine is the concept that miRNAs fine tune protein synthesis from thousands of genes by direct or indirect effects. Mechanistic details of miRNA-mediated post-transcriptional regulation have been well documented in multicellular model organisms. A so called gene knockdown is achieved by 2 major principles:

1. First, the microRNA pair directly on their corresponding sides in the messenger RNA of protein coding genes which leads to direct cleavage, relocation or transcript destabilization of the targeted mRNA. This is performed by a set AGO proteins, which in turn are subcomponents of a multi-protein complex named RISC. And finally leads to a downregulation of these messages. This mechanism is named as **post-transcriptional silencing**.
2. Second, the miRNA is incorporated in RISC which in turn recruits several additional factors to inhibit the protein translation. This is named **translational silencing**.

Particularly the fact should be mentioned, that a single miRNA can repress hundreds of genes albeit in a mild manner. In this connection, the repression levels can be quantified by rule of thumb as 200-300 percent.

Based on different kinetic signatures, Morozova et al. [7] proposed nine major different mechanisms of action (see figure 10). So far, in vivo it is not possible to distinguish within the different mechanisms. No reliable experimental method has been established to track down the precise mode of silencing. However, in all cases an mRNA degradation of the target protein is observable and easy accessible by qRT-PCR.

Modes of silencing:

1. Cap-40S initiation inhibition;
2. 60S Ribosomal unit joining inhibition;
3. Elongation inhibition;
4. Ribosome drop-off (premature termination);
5. Co-translational nascent protein degradation;

6. Sequestration in P-bodies;
7. mRNA Decay (destabilisation);
8. mRNA Cleavage;
9. Transcriptional inhibition through microRNA-mediated chromatin reorganization following by gene silencing.

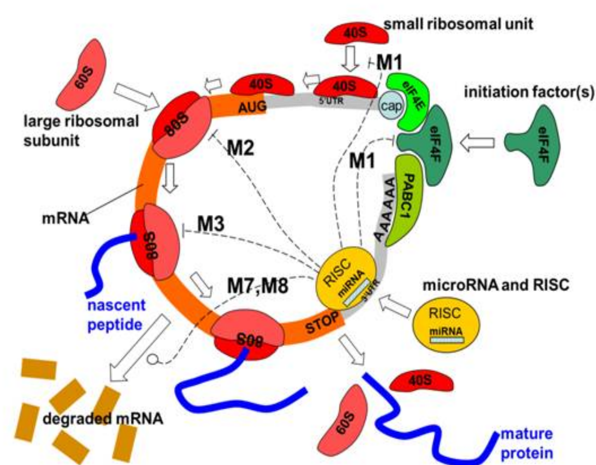
Additionally, it is shown that miRNA can directly target the coding region of a gene (CDS) or even up-regulates sets of genes. An emerging assortment of studies reveal that microRNAs and their associated protein complexes (microribonucleoproteins or microRNPs) can additionally function to post-transcriptionally stimulate gene expression by direct and indirect mechanisms. It is indicated that microRNA-mediated effects can be selective, regulated by the RNA sequence context, and associated with RNP factors and cellular conditions. Like repression, translation upregulation by microRNAs has been observed to range from fine-tuning effects to significant alterations in expression. [8]

So as a summary, miRNA action is more than downregulation of proteins by having 100% complementarity with 20 nt. in the 3'UTR.

For this work it has to be considered as an important factor because if we reveal a new layer of miRNA mechanisms it has to be stated that a miRNA is a highly diffuse molecule able to act in various environments and as a player in a concert depending on lots of factors and time points.

Figure 10

Several processes of posttranslational repression



M1) on the initiation process, preventing assembling of the initiation complex or recruiting the 40S ribosomal subunit;
M2) on the ribosome assembly;
M3) on the translation process;
M7, M8) on the degradation of mRNA.
Other mechanisms of microRNA action on protein translation (transcriptional, transport to P-bodies, ribosome drop-off, co-translational protein degradation and others) that are not visualized here.
The actual work of RNA silencing is performed by RISC (RNA-induced silencing complex) in which the main catalytic subunit is one of the Argonaute proteins (AGO), and miRNA serves as a template for recognizing specific mRNA sequences.

Picture by:
A. Zinovyev, N. Morozova, A. N. Gorban, A. Harel-Belan

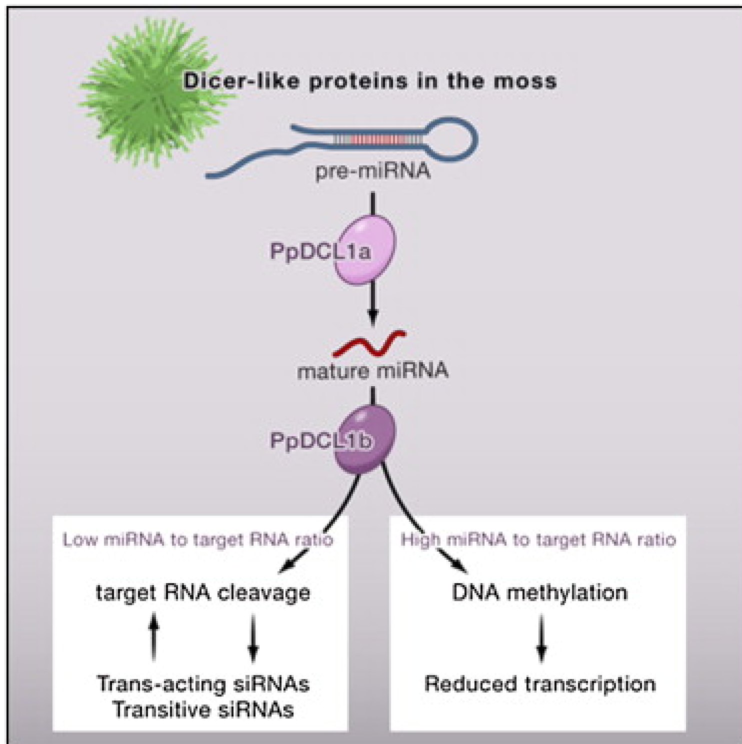
The consistently chameleonic nature of miRNAs, hand in hand with continuous improvement of knowledge, is one of the reasons to consider them as novel candidates for interspecies trespassing. To illustrate this, the exceptional steadily increasing understanding of the complex final cellular functions is a good example. Initially, it has been thought that a miRNA only target their associated mRNA and thus cause a RISC mediated fragmentation of it. Meanwhile, it is widely acknowledged that such easy mechanism of action is only the tip of the iceberg. The vast majority of miRNAs possible downstream action is hidden in a so far not clearly experimental accessible region. Mostly, the phenomenon of fragmented mRNA is only the observable effect with easy experimental access e.g. qRT-PCR. But it can be assumed that mRNA degradation is not the primarily effect of miRNAs.

As described above, miRNAs negatively regulate target gene expression basically via translational inhibition or transcript cleavage. In recent times, plenty new models of silencing are coming up e.g. histone modification, promoter methylation. Worthy of mention are another interesting “back channels” of regulation mediated by pseudogenes, pseudotargets and kinetic competition between miRNAs and ceRNAs (competing endogenous RNAs). Yet, those effects are still preferably deemed as “off-target effects” due to tricky and laborious experimental access.

Fortunately, scientists constantly add layer by layer of new modes of miRNA action. A good example to exemplify it in a more concrete manner is an interesting epigenetic one. In a paper published in CELL 2010 Khraiwesh, B. et al. propose a new pathway, demonstrating an influence of miRNA directly on the methylation pattern of a DNA and therefore revealing a novel **pre-transcriptional** level of interaction directly on the DNA itself. Normally miRNA are considered to act on the post-transcriptional and translational level.

In the canonical pathway DCL1b is responsible for the miRNA mediated target cleavage in concert with other proteins. Khraiwesh, B. et al. showed that in DCL1b- null mutants the methylation pattern of the genes including targets is higher due to a high miRNA - mRNA ratio. (See figure 11) These mutants accumulate miRNA-target-RNA duplexes and show hypermethylation of the genes encoding target RNAs leading to gene silencing. [9]

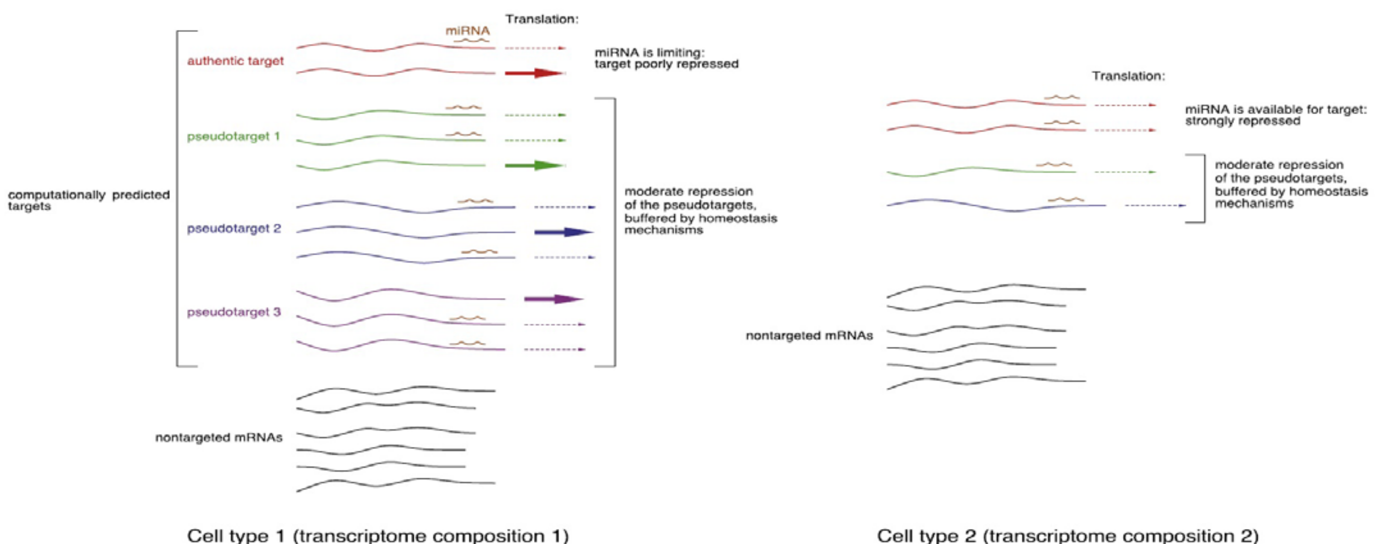
Figure 11



Khraiwesh, B. et al. Transcriptional control of gene expression by microRNAs. Cell 140, 111–122 (2010)

Another interesting example is a new hypothesis of computationally identified miRNA targets which are actually competitive inhibitors of miRNA function, preventing miRNAs from binding their authentic targets by sequestering them. (See figure 12) This theory may nicely explain the facts that off-target effects could contribute to miRNA action or why in some cases a computationally confirmed target shows no downregulation. [10]

Figure 12



Picture by H.Seitz et al.

MiRNA upregulation

An interesting paper clearly demonstrates the ability of miRNA to potentially upregulate gene's expression by targeting on gene regulatory elements. In this case, miR-373 targets the promoter of E-cadherin and enhances its expression in concert with Dicer. MiR-373 also induces the expression of CSDC2 in the same manner as E-cadherin. Moreover, RNA polymerase II was erected to be enriched on the promoter of E-cadherin and CSDC2 after miR-373 transfection. [11]

Deductively, miRNAs can also function in a similar way in recipient cells via CKR. Taking together those findings, they underline numerous degrees of freedom for the miRNA molecule. Ergo, it is conceivable that a miRNA acts in various ways in different organisms.

1.8 MiRNAs as immune defense weapons

Other results indicate that almost 30% of all genes in human are subject to regulation by only 60 miRNAs! Those and other findings impressively demonstrate the multi-tool nature and gives plenty of rope for speculation that the spectrum of miRNAs is not only limited to one cell or organism. Due to their simplicity, flexibility and under the light of energy aspects these molecules are perfect candidates to be considered as multi-purpose tools in nature and to cross the borders of species.

Meanwhile, beside their regulation of numerous biological processes described above, indications pointing to the potential connection of miRNAs and immune response against viruses or defense against pathogens. From this point it is not far to postulate miRNAs as a direct defense weapon against closely co-evolved species like *M. sexta and N. attenuata*.

Substantiated state of knowledge for this phenomenon is already described for their closely related relatives of miRNAs namely siRNAs. Building on those achievements it is therefore logical to consider miRNAs as an eligible aspirant in terms of immune/defense related issues.

According to their features it is entitled to presume that these flexible, tiny molecules paired with their low metabolic costs are perfect candidates to act as an early defense barrier against undesired external influences. Particularly with regard to their unlimited capacity to target every theoretical nucleotide sequence accompanied with the fact to act on an early stage of an infection.

A new review published in Nature provides some of the first cues that changes in cellular miRNA levels are a component of the mammalian innate immune response to viruses. Michael David and colleagues show that, in mammals, type I interferons (IFNα and IFNβ) upregulate several cellular miRNAs that can inhibit the replication of, or infection by, hepatitis C virus. [12]

In plants the literature describes that several miRNA families target genes encoding nucleotide binding site–leucine-rich repeat (NBS-LRR) plant innate immune receptors resulting in a transient downregulation of key defense-related molecules, such as NBS-LRR. Interestingly, the miR482/2118 superfamily generates secondary *trans*-acting siRNAs also providing a close relationship among those short RNAs. The authors demonstrate an in phase accumulation of cleaved mRNA with upregulation of miR482. In this scenario the pathogen triggers the suppression of the RNA silencing machinery resulting in an increase of NBS-LRR mRNA. Additionally to the already known mechanisms the authors propose another remarkable aspect that the miRNA regulations might create a role for NBS-LRR proteins in non-race-specific immunity. Shivaprasad et al. note that NBS-LRR proteins are normally associated with race-specific effector-triggered immunity. [13, 14]

Thus, miRNAs are the likely candidates for serving as regulators of defense responses to pathogens/ viruses and parasites, either by being part of the sensing network detecting the presence of the invader or by modulating expression levels of defense genes.

Most of the reciprocity shed light on the indirect defense reactions, but also direct mechanisms are possible but until now poorly understood. A nicely confirmed example of a CKR as a defense mechanism is described as a cellular miRNA effectively restricts the accumulation of the retrovirus primate foamy virus type 1 (PFV-1) in human cells. PFV-1 also encodes a protein, Tas, that suppresses microRNA-directed functions in mammalian cells and displays cross-kingdom antisilencing activities. Therefore, through fortuitous recognition of foreign nucleic acids, cellular microRNAs have direct antiviral effects in addition to their regulatory functions. [15]

These examples underline the options for a “direct use” of miRNAs against invaders. These principles are accompanied with a close adaptation and a molecular arms race of co-evolving species. It is very likely that the plant-herbivore system *N. attenuata- M. sexta* has developed a manifold relationship due to attack and defense, especially in the light of a specialist relationship of *M. sexta* to *N. attenuata*

Additionally, the existence of an RNA-based defense mechanism against invading elements (viruses, parasitic transposable elements, repetitive DNA) could be particularly important for organisms such as plants and invertebrate animals which lack protein-based adaptive immunity. In the dynamic move-countermove game played between host cells and invading genetic elements, cross-kingdom miRNAs could play their important role. It can therefore be hypothesized that amongst the thousands of conserved and non-conserved plant miRNAs, some of them will be revealed to be essential actors of viral and repeated sequence silencing. [16]

These and other data backup an involvement of miRNAs in the “molecular arms race” as new layers in the regulation of a defense reaction and provides first indication of a CKR in the system *N. attenuata-M. sexta,* as a role of an direct defense weapon.

1.9 Secondary (Non-target/off-target) Effects

The problematic of off-target effect is still present but in the context of miRNAs it could be wrong to talk about off-target effects as a negative by effect. “Off-target” does only mean that we just don’t know exactly what’s going on. The most famous off-target is for instance the blockbuster Viagra© from Pfizer, originally intended to treat vascular disorders.
The promiscuous characteristic of miRNA suggests that an effect of downregulation is particularly because of “off- target”. It is imaginable that isoforms or pseudogenes play their significant role to regulate the effective miRNA concentration on site or a generation of secondary siRNAs is responsible for the direct downregulation. So in this regard it appears better to name this as secondary effect.
Based on the simple principle of nucleotide sequence homology, it is anticipated that any transcript, or even a gene, which shares sequence homology with the effector ncRNAs, could become a candidate of silencing, leading to inadvertent secondary effects.

A good illustrated example for this is that ncRNAs originally intended to target western corn rootworm (WCR) effectively caused the silencing of other coleopterans like potato colorado beetle and southern corn rootworm (SCR) even though WCR and SCR shared 83 and 79 % sequence identity in vATPase A and vATPase E region, respectively [17].
Therefore it is deducible that a miRNA from a plant is able to affect a gene in an insect even though it doesn’t exhibit the same sequence.
Even if some miRNAs are relatively specific to their targets, they still suffer from off-target effects, which make it very difficult to predict the precise or major target. Generally all RNAi methods are hampered by this phenomenon making it difficult to winnow a “real” silencing from an effect with an unspecific randomized nature. Therefore, caution is warranted in interpreting gene function and phenotypes resulting from any RNAi experiments. In my point of view, considering the promiscuous nature of miRNAs, the actual mechanism of action is to target a complex multitude of transcripts/genes instead of only one target. And on the top, it is very likely that not only one miRNA is involved in an effective downregulation. It is rather a sophisticated orchestra of various miRNAs, targets and associated proteins which constitute the true nature. To back up this postulate Daqian Sun et al. have previously shown that a multi hairpin approach features superior benefits. [18]

Another point to mention is the average concentration of miRNA is high (100–30 nM) and low (3 nM) to cause knockdown effects. For human cells the number of miRNAs varies from 1-1000 per cell. The goal is now to find or create a miRNA with high specificity and functionality at the lowest concentration. This has not only an economic reason to conduct more experiments with a limited amount of (expensive) RNA, an even more significant benefit is to keep the RNA at lowest concentration as possible is to achieve better gene knockdown and fewer off-target effects—ultimately resulting in cleaner experimental output.

In contrast to small-molecule or big biological defense weapons that target properties of a gene product (e.g., enzymatic activity or cell surface localization), miRNAs are expected to interfere with the transcripts of genes on the basis of nucleotide sequence. Thus, they have a big potential to address all transcribed genes without regard to gene product properties.

1.10 MiRNA genes

Roughly only half of known miRNA genes are localized within previously annotated protein-coding regions ("intragenic miRNAs"). Approximately 20% of intragenic miRNAs were predicted to target their host mRNA transcript.

Interestingly miRNA containing genes manifest some common features like: three times length, more and longer 5' introns, host gene 3'-untranslated regions (3'-UTRs) were 40% longer and contained significantly more adenylate/uridylate-rich elements (AREs) compared to a randomly sampled gene cohort. [19] In fact, the current literature suggests that components of the *miRNA* biogenesis pathway are partly involved in ARE-mediated mRNA decay (AMD). [20]

Those miRNAs have been demonstrated to be coordinately regulated and/or co-expressed with their host genes, either with synergistic or antagonistic correlation patterns. This in turn could additionally indicate a CKR, because if a given miRNA is transcribed in a co-expressed manner a distinct relationship between certain genes in *M. sexta* and a miRNA in *N. attenuata* is assumable.

1.11 The concept of cross-kingdom regulation (CKR)

Latest results clearly demonstrate a direct and indirect involvement of smRNAs in the plant defence mechanisms. Information exchange is essential for the maintenance of the biological chain among different species.

To provide conceptual facts for CKR, I have to summarize some major underpinned bullet points as a solid base for further argumentation.

1. Herbivore attack causes tremendous changes in the smRNAnome of *N. attenuata*
2. Those small RNAs are mainly named as siRNAs and miRNAs
3. Orally administered smRNAs show the ability to silence specific genes in the midgut of *M. sexta* and in other insects
4. MiRNAs have a promiscuous behaviour what means that they can target various genes with different mechanisms and effects
5. MiRNAs stand out due to their low productive costs and flexible features to attack various genes
6. It has been demonstrated that miRNAs can be stably transferred over large distances, protected from degradation from abundant RNAses and other harmful conditions via extracellular vesicles: exosomes, microvesicles, and apoptotic bodies, by a broad range of cell types or even via microorganisms like bacteria.

7. Intriguingly, a large portion of extracellular miRNA is found outside of any lipid-containing vesicle, and instead is associated with RNA binding proteins like argonautes 1 and 2, which may aid in their protection from abundant nucleases in the extracellular space. [21; 22; 23]

Selected studies providing evidence for CKR by miRNAs

1.

A study by Kosaka et al. has revealed various stable miRNAs in human breast milk that are stable in the stomach's acidic conditions due to their enclosure in protective microvesicles. These can be taken up by the intestine, and are essential for the development of the child's immune system. [24]

2.

In 2010, miR-150 in the exosome was demonstrated to be selectively released from THP-1 cells and moves into human HMEC-1 cells to enhance recipient cell migration, which denotes that the moving miR-150 can be a long-range regulator of gene expression in recipient cells [25].

3.

A striking finding in this context is that pluripotent stem cells can be efficiently induced by miRNAs. So called induced-human pluripotent stem cells (iPS) were subject of the 2012 Nobel price. Other practices like lentiviral, episomal plasmid, transposon, protein or Site-Specific Recombinase Strategy have all their own advantages or disadvantages. The xenotropic RNA information supplied by the miRNA gene and the RNA gene information could expand to intracellular, intercellular, in- traorgan, interorgan, intraspecies and interspecies with unknown outcome, thitherto. So further studies and ethical thoughts have to be conducted to shed light on this kind of new "programmed evolution" and potential long term consequences. [26]

A hypothesized consequence to draw from these findings is that miRNA, acting on the fundamental stem cell level, should have a significant impact even at low concentrations. In order to continue this argumenta strain, therefore it could turn out that experimental access (e.g. specific target) is much more difficult to obtain. Consequently, a miRNA action might be considered as an off-target effect because a direct connectivity to a downregulation of a specific gene is not possible anymore.

4.

2012 Yao et al. identified GW182 protein which binds to argonaute (AGO) proteins and has a central role in the stability of transfected miRNA. They identified a 3′–5′ exoribonuclease complex responsible for the miRNA degradation only when GW182 is knocked down. Additionally, the half-life of utilized miRNA-mimics in these experiments is reduced. Same findings are demonstrated for TNRC6B, a paralogue of GW182. Immunoprecipitation reveals a presence of GW182 in RISC. Those results

underline the stability of miRNAs and give evidence for a stable transfer from one organism to another. [27]

5.

The most hyped study conducted in China provides indication of crop/food derived miRNAs in human. This hyped and highly discussed Nature-paper in this context is provided by Zhang et al. which shows that miR-168a from plants is verifiable and stable in the human blood stream, and in addition to it there is a knockdown activity of low-density lipoprotein receptor adapter protein 1 (LDLRAP1) by miR-168a [28]

6.

Another unique example of a host defense strategy is that erythrocytes with the HbS allele produce significant amounts of miR-451 and let-7i which in turn negatively affect parasite growth along with miR-223. Interestingly, those miRNA were found integrated in essential mRNAs of *P. falciparum* via impaired ribosomal loading, resulted in translational inhibition. The authors verified more than 100 intra-parasitic miRNA and proved these were fully intact mature human miRNAs. Differences in the maximum miRNA concentration within the parasite, based on the example of let7i and miR451indicate the uptake of miRNA might be an active, dynamic process. This example impressively demonstrates not only CKR but also the manifold nature of miRNAs as a direct defence weapon. [29]

7.

In the reversed mode of host-virus interaction, viruses could take advantage of the host cell miRNA machinery and establish a cellular environment conducive to viral replication. More interestingly, a handful of viral miRNAs exhibit homology to human oncogenic miRNAs. For example, Kaposi's sarcoma-associated herpesvirus (KSHV)-encoded miR-K12-11 is an ortholog of miR-155. [30]
Whereas the role of miR-122 in controlling hepatitis C virus (HCV) infection is the most interesting Example of the controlling of viruses through host miRNAs. MiR-122 stimulates HCV replication through unique interactions at two binding sites in the 5' UTR of the HCV genome, therefore it could be a target for new class of miRNA therapeutics. Indeed, a miR-122 inhibitor is known as miravirsen and is in phase II *clinical trials*. [31]

8.

Some miRNAs are conserved in their sequence but even more in the secondary structure across several species. Here, I hypothesize a model of mimicking miRNAs. In brief, it is supposable that plant miRNAs mimicking *M. sexta* miRNAs to achieve an evolutionary advantage.

In general all the RNAi technologies exhibit a potential as a new drug class. Therefore, directly after discovering the RNAi effect, it was soon followed by suggestions that this technology could be used for the development of a new class of therapeutics. The new paradigm of those drugs is to interfere very early and additionally in another dimension, namely the nucleotide dimension, in the chain of events which lead to disease. The same principle is postulated in this thesis: "It is beneficial for the plant to interfere at an early point in time and in an additional different plane, compared to other defense molecules, upon herbivore attack.

Figure 13

Interspecies miRNAs moving from different organisms to the human body.

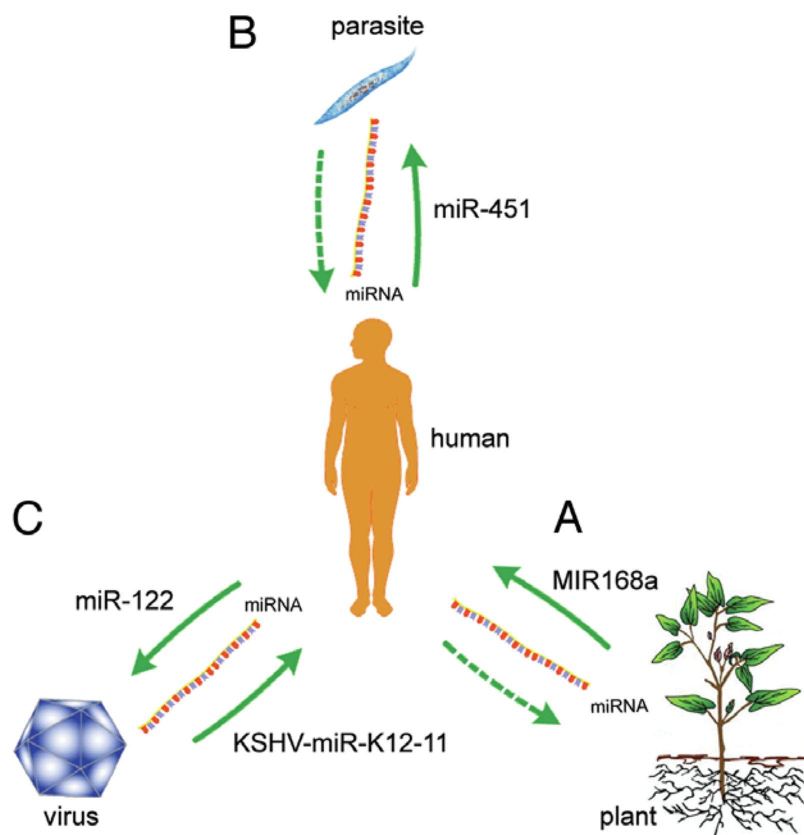


Figure by Hongwei Liang et al.

2. Material and Methods

The experiments were subdivided in a dry and a wet lab phase.

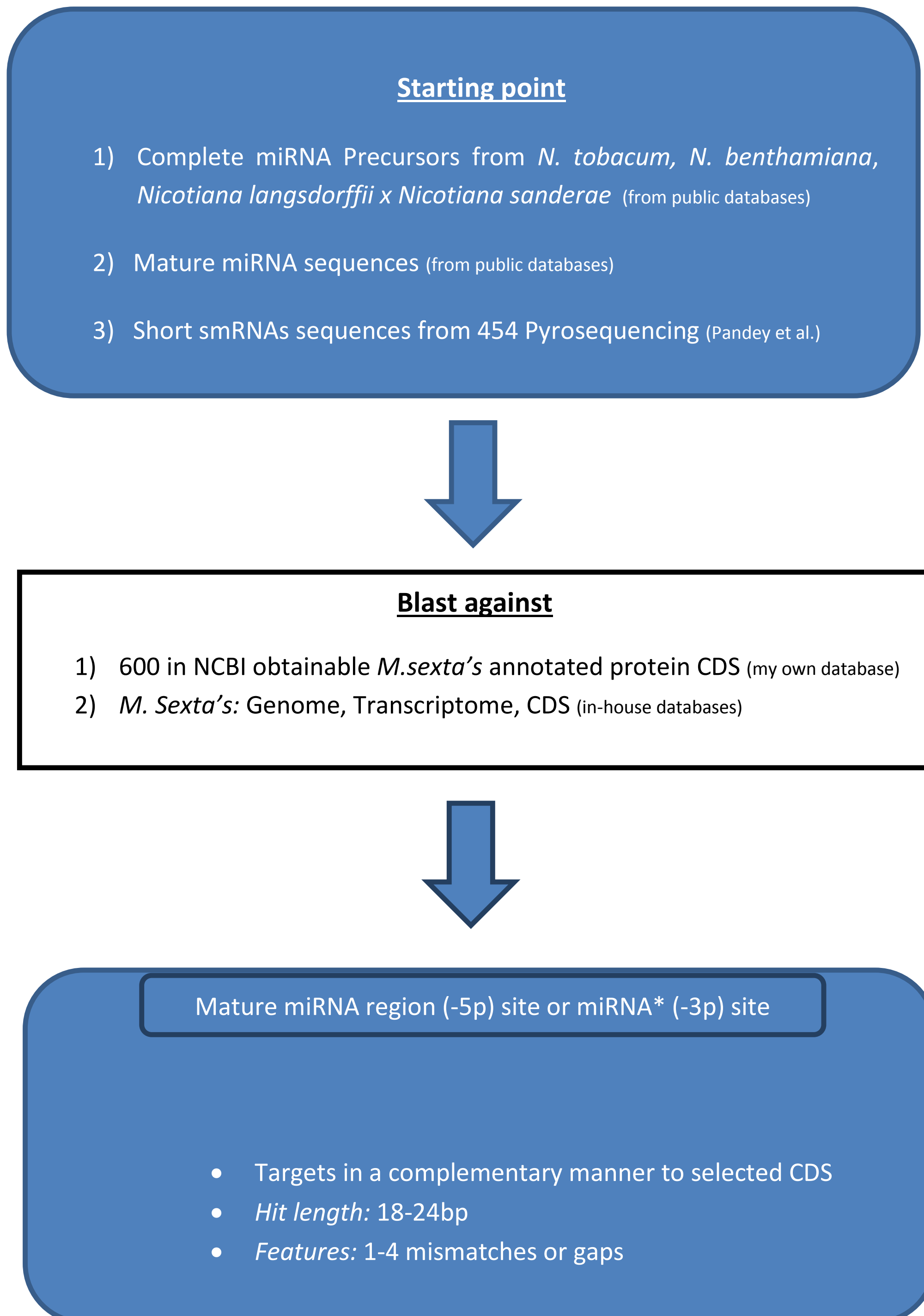
1. The objective of the dry lab was to find in silico appropriate miRNA candidates.
2. Whereas in the wet lab, promising miRNAs were administered with artificial diet (AD) to *M. sexta* and the transcript levels of the corresponding targets were measured by qRT-PCR after 10 days.

2.1 Materials

Table 2.1 List of chemicals

Chemicals	Manufacturer
cDNA synthesis KIT	Life Technologies
ssDNA oligos	Sigma-Aldrich
Primers	Sigma-Aldrich
Trizol reagent	Ambion
T7 IVT kit	Ambion, Thermo Scientific
SYBR Green I	Eurogentec
dNTP Mix	Thermo Scientific, FINNZYMES
Pfu polymerase, DreamTaq polymerase	Thermo Scientific

2.2 Overview dry lab



2.3 In silico prediction of miRNA candidates

Strategy 1

The first attempt was to perform an in-house BLAST search with all 50K sequences obtained from the smRNAnome of *N. attenuata* against the in house database of *M. sexta's* transcriptome and genome.

Parameters for a hit

1. ***A hit is confirmed only if 18-30 bp shows 100 % full complementarity***
2. ***No gaps or mismatches are allowed***
3. ***A hit is allowed within the complete transcriptome or genome of M. sexta***

After a hit was confirmed:

1. The hit-sequence in *N. attenuata* was cut by hand, with approximately 50-200 adjacent nucleotides in up and downstream direction to fold this sequence with RNA*fold* to proof a potential secondary structure in from of pre-miRNA. The source was preferably the *N. attenuata* transcriptome but genome hits were also used for folding.
   With the help of the Vienna RNA*fold* Web Server: http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi it was proved whether the short hit-sequence is part of a longer functional miRNA precursor. The minimum free energy (*MFE) ΔG <-25kj/mol* was chosen according to the literature, to consider the structure as a functional pre-miRNA. The shorter the size of the pre-miRNA the lower the free energy is. Additionally, the structure must exhibit the basic features like validated miRNAs, obtainable in public databases in regard to secondary structures and features of the mature miRNA region within the precursor (see introduction part 5).

2. The hit region in *M. sexta*'s transcriptome and genome was cut with circa 500 adjacent bp in up and downstream direction and annotated with NCBI Blast.

3. The anticipated mature hit region was checked whether it is part of an annotated precursor or an orthologous sequence present in one of the public miRNA databases:

MiRBase:
http://www.mirbase.org/cgi-bin/mirna_summary.pl?org=nta

miRNEST

http://lemur.amu.edu.pl/share/php/mirnest/browse.php?species=Nicotiana%20benthamiana

SolmiRNA:

https://genepool.kribb.re.kr/SolmiRNA/

Strategy 2

The second attempt was to conduct a NCBI BLAST search with all complete miRNA precursors from *N. tobacum, N. benthamiana*, *Nicotiana langsdorffii x Nicotiana sanderae* against my own database of annotated *M. sexta's* CDS. These annotated CDS sequences -600 in this case- are available in public databases like NCBI.

Parameters for a hit

1. ***Precursor must be present in a miRNA database***
2. ***Precursor transcript present in N. attenuata transcriptome***
3. ***Original mature region in the precursor must be experimentally and computationally confirmed***
4. ***Approximate mature region must be 18-25 bp complementary to M. sexta's CDS***
5. ***1-4 mismatches or gaps were allowed for the mature miRNA***
6. ***A hit is allowed within the complete CDS***

Previous experimental or computational validated mature miRNAs within the precursor are stretched a few base pairs up/downstream to be a potential miRNA because of the likelihood that mature precursor miRNAs are processed in a different manner in insects than in plants.

In general, an almost continuous stretch of base pairs with low thermodynamic free energy, among 2 secondary structure motives e.g. bulges and not located in the immediate proximity of the terminal stem-loop of the hairpin precursor are considered to fulfill the criteria of a potential mature miRNA in accordance with general rules of Dicer cutting sites. (See introduction part 5)

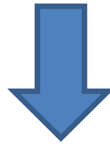
Strategy 3

Additionally, selected confirmed miRNA precursors from public databases were checked against the new in-house *M. sexta* transcriptome. Unfortunately, the in-house database was not available before august 2013.

2.4 Overview wet lab

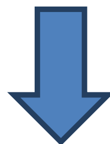
MiRNA production

1. Successful miRNA candidates (see list 1) ordered as ssDNA(+,- strands) from SIGMA or amplified from *N.att.* cDNA by Pfu-PCR
2. Sense and antisense ssDNA strands are heated up to 95° and cooled down to RT 8 hours for re association
3. T7-in vitro transcription (IVT)
4. dsRNA strands are heated up to 65° and cooled down to RT 8 hours for re-association
5. dsRNA stored in DEPC water



MiRNA feeding

- 20 *M.sexta* larvae are feed with artificial diet for 10 days at 30° degree with 10mg of miRNA/ 1g diet
- Pre-screening: All computationally miRNA candidates are feed in their normal hairpin conformation.
- MiRNAs showing successful downregulation are further feed with mature and mutated forms (AD without miRNA, normal + mut. pre miRNA ; mature miRNA)
- total amount of diet 5g is changed every 1-2 days



RNA isolation and qRT-PCR

1. After 10 days total RNA was isolated from midgut tissue and stored in TRI Reagent with 10 replicates
2. cDNA preparation
3. qRT-PCR with specific primers for the target genes of each miRNA candidate

2.5 MiRNA oligonucleotides (see appendix 1)

MiRNA nucleotides (long pre-miRNA and short mature RNA) up to 120 bp were ordered as ssDNA templates, containing the T7 promoter, from SIGMA. The complementary + and – strands were mixed in equimolar amounts, heated to 95° for 5 minutes in a heat block, covered with clean tissue papers and polystyrene for isolating and followed by slow cooling to room temperature (RT) in order to allow slow re-association of the strands.

For miRNA above 120 bp in this case: M25 and M6, it was necessary to conduct multiple PCRs for the purpose to yield enough dsDNA as template for IVT. In the case of M25 the template was obtained from *N. attenuata* cDNA. For M6 it was possible to order a 119 bp template, without promoter sequence, from SIGMA. The T7 promoter sequence (TAATACGACTCACTATAGGGG) was introduced afterwards via primers by Pfu-PCR.

2.6 Primers for PCR (see also appendix1)

Primer sequences were verified for secondary structures (hairpin loops and dimers) using the software AutoDimer v1.

M6F	GAAATAGAGAGTGGATTGCAGC
M6R	AGAGAGCGAAATGTAGCCAGG
M6iF	TAATACGACTCACTATAGGGGAAATAGAGAGTGGATTGCAGC
miR25F	TTTCTCTTCTCTTCCTCAACCTA
miR25R	TTTCTCCTTCTCACCCTCATC
miR25ivtF	TAATACGACTCACTATAGGGTTTCTCTTCTCTTCCTCAACCTA

2.7 Mutated miRNA oligonucleotides (see appendix 1)

1. Mutated oligos were used as a control in the feeding experiments. Mutations were inserted in the mature-seed region of the pre-miRNA. Whereas, 8 bases are mutated in this way, not to affect the Gibbs free energy of the thermodynamic ensemble or introduce, as little as possible, unwanted secondary structures in the hairpin. It has been shown that random mutations affect the structure in a negative way.
2. For the short mature oligos, 4 mutations were inserted on each end of the mature oligo in order to destroy the anticipated seed region.

Oligos up to 120 bp were directly ordered from SIGMA in this case miR6, miR25 and miR6150 mature oligos. Only pre-miR6150 was ordered from Sigma. (See list appendix 1)

For pre-M6 only the 119 bp template was ordered and mutations were introduced via Pfu-PCR primers. For M25 it was necessary to introduce the mutations in 2 steps from *N.att* cDNA with mutated primer by Pfu-PCR.

Primers for M25

Primers for introducing mutations	
M25mutiF	CAACATTCGGATCATCATCTTGGCTTTTCTCTGTCCTTCC
M25mutiR	CTTCTCACCCTTCGGATCCTCATCTGGCTCTTCATCTAC
Primers for generating the control template for IVT	
M25iCF	TAATACGACTCACTATAGGGCTAATAAATCAACATTCGGATCATC
M25iCR	CTTCTCACCCTTCGGATCCTC

2.8 In vitro transcription (IVT)

IVT was performed with T7 IVT kit from Ambion and Thermo Scientific according to manufacturer's protocol. Thereby, T7 produces in 5'-3' direction from dsDNA a single strand ssRNA. 1 ug of dsDNA template yields in approximately 200 ug of ssRNA. Due to the short size of oligos, the incubation time was adjusted up to 16 hours.

After purification the complementary ssRNA strands were diluted with 1ml of DEPC water and heated carefully to 65° for 4 minutes in a heat block, covered with clean tissue papers and polystyrene followed by cooling down to RT for 6-8 hours in order to allow slow re-association of the strands. After cooling, Nanodrop measurement of the RNA-concentration was conducted. Then, the RNA was aliquoted to 250 uL/tube for the purpose to avoid unnecessary thaw and freezing cycles, and immediately transferred to -20° to protect the RNA from degradation.

2.9 Primer design for qRT-PCR

All Primers for the PCR-amplification of *M. sexta* transcripts were designed using the software Primer 3. These primers were designed to anneal at 60°C and to amplify ≥ 150bp region of the target gene. The correct length and existence of the targets were verified by PCR.

Primer sequences were verified for secondary structures (hairpin loops and dimers) using the software AutoDimer.

2.10 *M. sexta* eggs

M. sexta eggs were collected from an in-house reared colony. These eggs were kept in a growth chamber (Snijders Scientific) at controlled light; 400- and 600-W sodium lamps (Philips) (16h light-8h darkness), temperature (26°C) and humidity conditions (65%). Once the eggs hatched, neonates were transferred on wheat germ based Artificial Diet (AD) at 30°C.

2.11 Insect rearing

AD was prepared as described by Waldbauer *et al.* and Grosse-Wilde *et al.* 20 larvae were reared on artificial diet (AD) containing 5mg miRNA precursor/1g diet. The total amount of diet was 5 g in a mountain shaped form. Insects were kept in plastic boxes. The diet was changed at the beginning every

second day and every alternate day during the later stages. Up to 4th day, 10 larvae were kept in one box with 2 mountains of 4g diet. Each day 700ul DEPC water was added to the diet mountain to guarantee enough moisture.

2.12 Dissection

Caterpillars were dissected to collect the midgut (Mg) of a 10 days old larva which is not in molting stage. The insects were anesthetized in ice for 3 min. and dissected under clean (RNAase free) conditions and a binocular microscope. Thereby, the insect was cut with a scissor between the 4th and 8th segment, gut contend was carefully pulled out with forcipes and midgut was immediately transferred in a drop TRIS/HCL buffer, for further dissection/cleaning steps, to protect against RNAases. Midgut should be gotten from larva en bloc and not ruptured. Finally, the tissue was cleaned with sterile water, to get rid of remaining malpighian tubules. Samples were transferred in pre labeled tubes containing 200 ul Trizol reagent for RNA isolation and stored at +4 degree or -20 degree (long term storage), until further use.

2.13 RNA extraction

Tissues stored overnight in Trizol reagent at 4°C were used for extracting RNA. These tissues were subjected to ceramic beads (0.9 g: Sili GmbH, Germany) mediated homogenizing in a Geno/Grinder 2000 (Elvatech, Ukraine). 200µL of chloroform was added for phase separation. The upper phase containing the RNA was carefully pipetted out with cut-tips in a separate tube. RNA was precipitated using 0.6 volumes of prechilled Isopropanol. The pellet was washed with 1.0mL of 70% ethanol and dissolved in 30µL DEPC treated water. For cleaner results it is strongly recommended that remaining ethanol (containing phenol) is pipetted out completely with a pipette of choice.
The final concentration of RNA was measured using nanodrop.

2.14 cDNA synthesis

All the reagents for cDNA synthesis were added in a cDNA plate. Each reaction (total 10µL) consisted of 4.0µL RNA (~700ng), 0.5µL dNTP's and 0.5µL Oligo(dT)18. The mixture was denatured at 65°C for 5 min. and snap frozen on a pre-cooled Aluminum rack. To this reaction mixture 5.0µL of 5X RT buffer, 1.0µL of 0.1M DTT, 2.0µL of 25mM MgCl2, 0.25µL of RNAse Out and 0.075µL of SuperScript II enzyme were added. It was then subjected to the following conditions: 42°C for 60 min, 70°C for 15 min. Finally, 90µL of sterile water was added to each reaction and the cDNA plate was kept and sealed at -20°C till further use.

2.15 qRT-PCR

3 miRNAs from all predicted candidates were selected for the final qRT-PCR. Those 3 have shown the best results from the initial pre-screening experiment.

For all qRT-PCR experiments, Mx3005P Multiplex qPCR system (Stratagene) and qPCR core kit for SYBR Green I were used. *Ms*Ubiquitin was used as an internal standard and mRNA transcript levels were relatively quantified by the comparative D cycle threshold (CT method). The following conditions were selected for all samples:

Step	Condition	Cycle
Initial denaturation	95°C for 30s	1
Denaturation	95°C for 30s	40
Annealing	60°C for 1min	40
	95°C for 30s	1
Final extension	60°C for 1min	1

2.16 Statistical analyses

One-way ANOVA was performed for all the quantitative data using StatView version 5 (SAS Institute Inc.) and statistical significance ($P <= 0.05$) was determined by Fisher's least significant difference *post hoc* test

3. Results

3.1 Dry Lab

The final goal was to identify miRNA candidates which meet all the selection criteria mentioned above.

3.1.1 Strategy 1

Blasting with the in-house Blast program unfortunately skips all the hits with more than 1 gap or mismatches. Due to the algorithm only 100% sequence complementary was considered as a positive match.

Result1

For the sequence from smRNAome of *N. attenuata*: cagataaaagaagaaggtt, a hit in the transcriptome of *M. sexta* named: Msex006316 was confirmed.

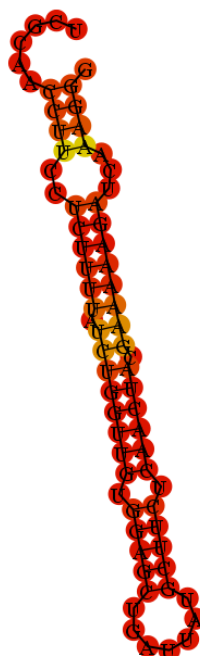
1. Annotation of Msex006316 :

Bombyx mori dynein heavy chain 7, axonemal-like (LOC101739511), mRNA
Length: 11763

Score	Expect	Identities	Gaps	Strand
6318 bits(3421)	0.0	7844/10035(78%)	81/10035(0%)	Plus/Plus

2. Folding with adjacent nucleotides by Vienna RNA*fold* Web Server results in a new pre-miRNA, which was not annotated in *N. attenuata* targeting Msex006316.

New Pre-miRNA sequence:
TCGCAACCTTCCTCTTTTATCTGGTTGTGGAGCTGATTATGCTTCTCAACTACGAAAAAGATCAAAGGG



MFE secondary structure

The free energy of the thermodynamic ensemble is **-21.89** kcal/mol.

<u>Result2</u>

For the sequence from smRNAome of *N. attenuata*: Cattcaaacgaacaaacaaac, a hit in the genome of *M. sexta* named: *M. sexta*_20110516_scaffold00145 was confirmed. The reverse complementary sequence is Gtttgtttgttcgtttgaatg.

Folding with adjacent nucleotides by Vienna RNA*fold* Web Server results in a new pre-miRNA, which was not annotated in *N. attenuata* targeting *M. sexta*_20110516_scaffold00145. (See next page)
Additionally, I blasted this sequence against my own database of *M. sexta* proteins because annotation in NCBI gives no outcome. The results are multiple hits in different proteins supporting the promiscuous multi-targeting nature of miRNAs.

1. >gi|219958087|gb|FJ530955.1| Manduca sexta pro-lebocin gene, complete cds

   Identities = 18/19 (94%) Strand = Plus / Minus

   ```
   Query: 3   ttcaaacgaacaaacaaac 21
              ||||||| |||||||||||
   Sbjct: 234 ttcaaacaaacaaacaaac 216
   ```

2. >gi|73913561|gb|DQ115323.1| Manduca sexta hemolymph proteinase 12 (HP12), hemolymph proteinase 24 (HP24), and prophenoloxidase activating proteinase-2 (PAP-2) genes. complete cds

   Identities = 18/19 (94%) Strand = Plus / Minus

   ```
   Query: 3    ttcaaacgaacaaacaaac 21
               ||||||| |||||||||||
   Sbjct: 5691 ttcaaacaaacaaacaaac 5673
   ```

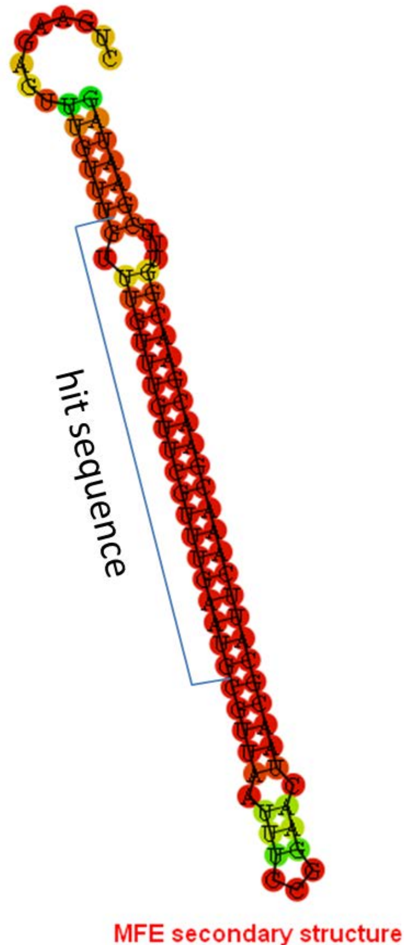
3. >gi|384482609|gb|JQ388324.1| Manduca sexta VMP30 (VMP30) gene, complete cds

   Identities = 18/19 (94%) Strand = Plus / Minus

   ```
   Query: 3    ttcaaacgaacaaacaaac 21
               ||||||| |||||||||||
   Sbjct: 2616 ttcaaacaaacaaacaaac 2598
   ```

New Pre-miRNA sequence:

GAAGAGTTTGTTTGTTTGTTTGTTCGTTTGAATGCGTTAATTTCCGGAACTAACGCATTCAAACGAACGAACGG
TTTCGAATAGCGCATTTATTGAGGAAGGCTG



The free energy of the thermodynamic ensemble is **-37.35** kcal/mol.

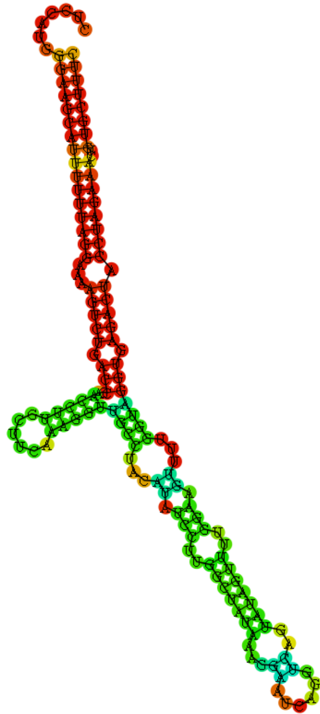
Result 3

The sequence: CTCCATGGGAAGCATTTTTTAG hits a 19 bp region within a transcribed sequence of Msex007141-RA_gene=Msex007141. Annotation of Msex007141 results in an orthologous sequence of *M. sexta* carboxylesterase.

Bombyx mandarina carboxylesterase mRNA, complete cds
Sequence ID: gb|EU328351.2|Length: 1823Number of Matches: 1
Related Information
Range 1: 174 to 1085GenBankGraphics Next Match Previous Match First Match
Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand	Frame
356 bits(394)	4e-94()	648/927(70%)	30/927(3%)	Plus/Plus	

New Pre-miRNA sequence:
CUCCAUGGGAAGCAUUUUUUAGGAAAAGUCUGACCGAACCUUGCUUCAAAGGUUGCCUACAUAUCCUU
GGCUAUAAAGGAAUCAGGUCAGUAUAGUUUUGGAAGUUUUGGUAGGUGAGACUACCUAGAAACUGUG
CUUUC



MFE secondary structure

The free energy of the thermodynamic ensemble is **-45.90** kcal/mol

In this case the structure features an acceptable Gibbs free energy but with 140 bp the value does not provide good information in terms of reliability of a potential precursor.

3.1.2 Strategy 2

In order to circumvent the problem in strategy 1 it was necessary to use the online NCBI-Blast server. Unfortunately, it was not possible to use the full in-house transcriptome and genome in NCBI due to its size and format. Moreover, the likelihood to find a good and relevant match was better with my own database of annotated 600 *M. sexta* CDSs. I found that previous annotated CDSs have proved to be a better source for targets of a given miRNA instead of the non-annotated transcribed or genomic sequences.

Result 1

Table 3.1: Results of blasted pre-miRNAs against 600 *M. sexta* annotated CDSs

Comments: Within the pre-miRNA sequences, only regions for potential mature miRNAs were considered as a positive match. (See also introduction part 1.5)

	miR sequence (mature)	**Pre-miRNA name**	**Normal strand/ ***	**Hit protein target (C/ RC)**	**Hit coverage (#/#)**	**Shree´s #**	**Precursor in N. att**
1	ACATCATCATCATCCTCTTCT ATGAGGATGAGGATGATGAGG STAR STRAND TCAACATCATCATCATCATCTTCGC Sequence in *N. attenuata*	M 25 MNEST001598 155bp	√ *	1. *MS*CYP6AN5-RC and C 2. VMP30-C 3. high-affinity serotonin transporter-RC 4. cadherin-related-protein receptor-RC	21/24 21/25 18/21 16/18 20/25	no	yes
2	ATCGGCAAGTTGTCCCTGGCTACAT	M 6 MNEST001605 112bp	*	1. chitin synthase-RC	21/25	no	yes
3	CAGATAAAAGAAGAAGGTT	Dynein miRNA (miRC554480)		1. Dynein	19/19	yes	yes
4	CAGATTTGTTTGATCGTCTTGG	nta-MIR6150	* √	1. *Ms*18-56 protein -RC 2. allatotropin receptor (ATR) -C 3. beta-fructofuranosidase 2 (Suc2)-RC 4. hemolymph juvenile hormone binding protein –RC 5. Manduca sexta moling mRNA-RC	18/22 20/23 17/18 18/22 19/23	no	yes
5	TTATTGTATTCGACTGTATTCAC	nta-MIR6152a	√	1. N-ethylmaleimide sensitive fusion protein and Hitcher protein (NSF)-RC 1. Cyp6B	20/23 17/18	no	yes
6	TGGCGTATGAGGAGCCAAGCA* GTGAATG-GCGTATGAGGA ATATGCCTGGCTCCCTGT	nta-MIR160a	* √	1. Manduca sexta receptor guanylyl cyclase GC-II-RC 2. Manduca sexta caspase-6-RC 3. Manduca sexta immulectin-3-RC	19/21 23/27 16/19 23/29 16/18	no	yes

3.2 Wet Lab

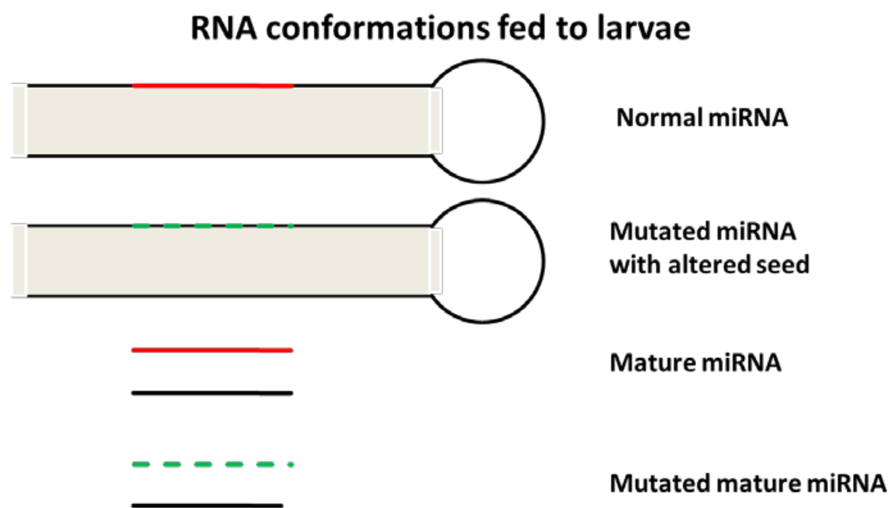
The objective of the wet lab was to show an mRNA knockdown with qRT-PCR of selected targets (see table3) and after feeding 3 selected miRNAs candidates in different conformations (see figure 3.2.1) (M25; M6; nta-miR6150). Initially, a pre-screening was made: All miRNAs were fed to larvae in their natural hairpin conformation and all predicted in silico targets were tested by qRT-pCR. Only those three show significant downregulation of one of their predicted targets.

Table 3.2.1

Overview tested targets by qRT-PCR

MicroRNA name	Targets predicted in silico	Target downregulation
1. M25	*MS*CYP6AN5	YES✓
2. M6	Chitin synthase	YES✓
3. Nta-miR6150	*Ms*18-56 protein	Only with pre-miRNA

Figure 3.2.1



Picture by Tucker and Horn

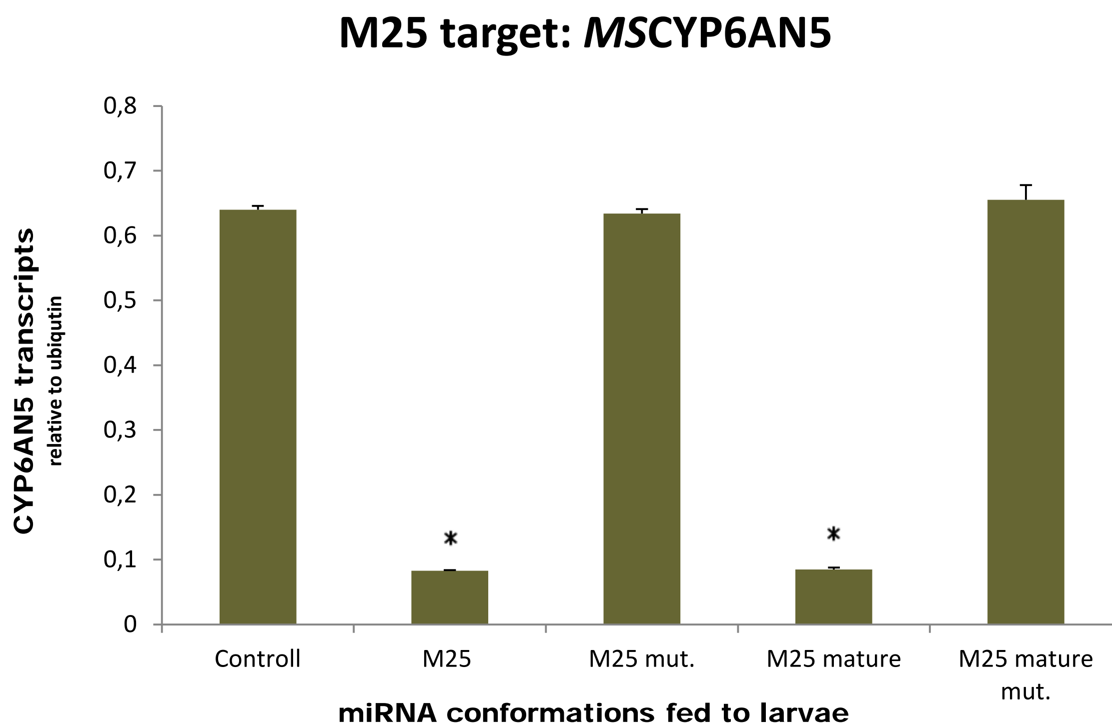
Results qRT-PCR

For all the 4 targets tested by qRT-PCR, a significant downregulation was observable for the normal (non-mutated) miRNA hairpin precursors. Consistent with the expectations, the mutated precursors with altered seed show no effect on the transcript levels. The short mature miRNAs derived from the hairpin show for *MS*CYP6AN5 and *MS* Chitin synthase a significant downregulation whereas for *Ms*18-56 no significant effect was observable. Here it has to be noted, that pre-miR6150 is problematic due to its short structure.

Summary:

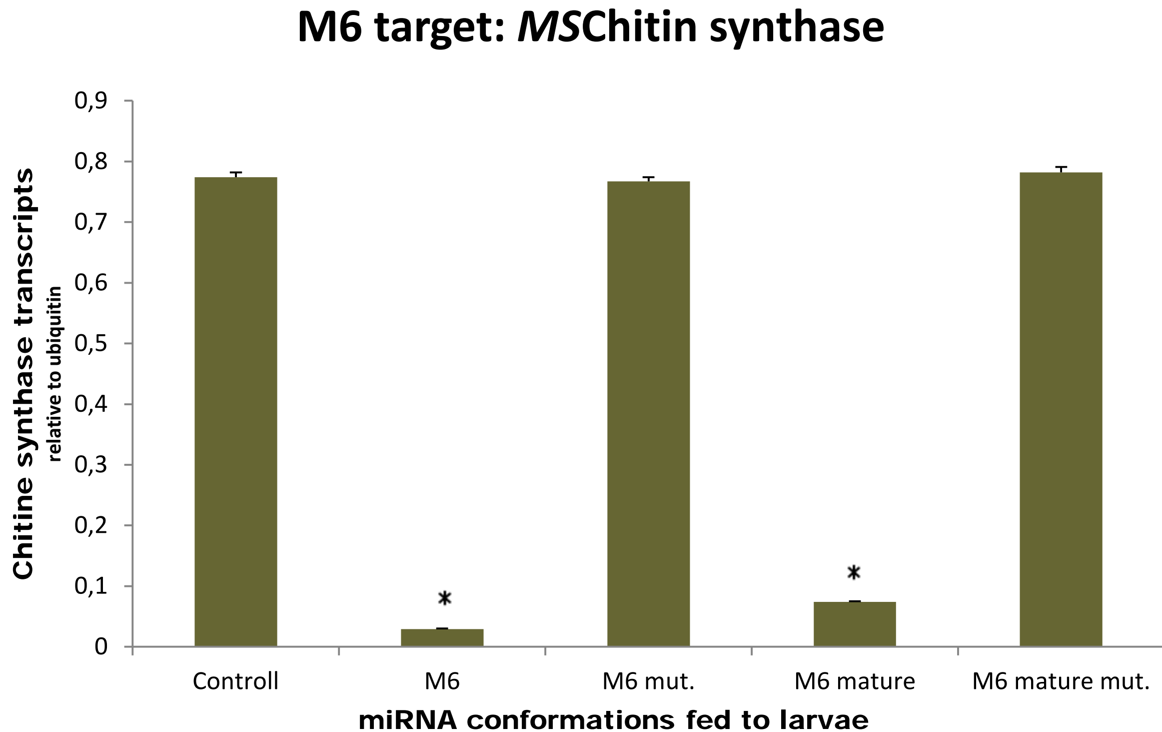
1. M25 and M6 non-mutated hairpin precursor are able to knock down foreign genes in *M. sexta*
2. Mature miRNAs represent a worse source of foreign RNA to silence genes in another organisms
3. Silencing effect is depends on the target
4. Short structures (miR6150) are susceptible to mutations and therefore they are not appropriate molecules for studying CKR.

Figure 3.2.2



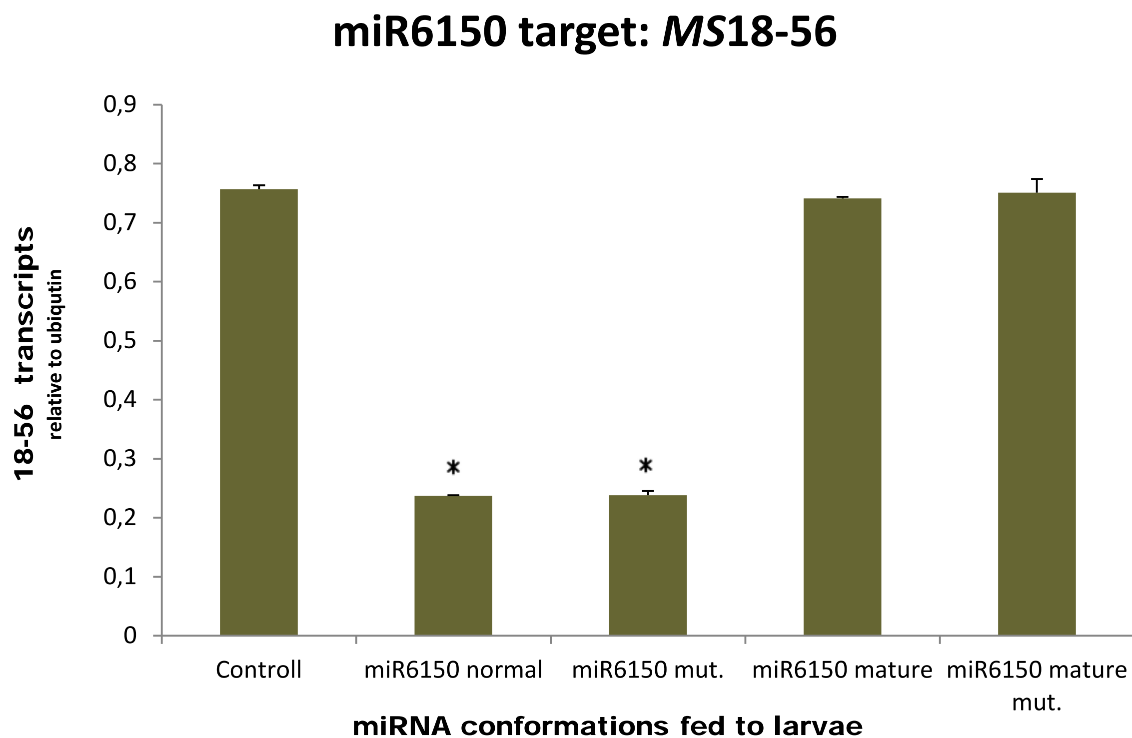
Transcript levels of *MSCYP6AN5* in the midgut of larvae fed with different miRNA conformations. Asterisk indicates the significant difference between control and M25 fed *M. sexta* larvae as determined by one way ANOVA (P≤ 0.05, n=10).

Figure 3.2.3



Transcript levels of *MS*CHS in the midgut of larvae fed with different miRNA conformations. Asterisk indicates the significant difference between control and M6 fed *M. sexta* larvae as determined by one way ANOVA (P≤ 0.05, n=10).

Figure 3.2.4



Transcript levels of *MS*18-56 in the midgut of larvae fed with different miRNA conformations. Asterisk indicates the significant difference between control and miR6150 fed *M. sexta* larvae as determined by one way ANOVA (P≤ 0.05, n=10).

4. Discussion

4.1. Dry Lab

The first objective was to verify whether a sequence from the smRNome is part of a miRNA or not. In this regard it was demonstrated that most of the considerable hits are not part of a computational predictable miRNA precursor.

Yet, no appropriate in silico application has been designed with the possibility of finding the 100% correct miRNA sequence automatically. By the help of the program *Softberry* it was possible to support the miRNA sequence folded by hand:

http://linux1.softberry.com/berry.phtml?topic=findmirna&group=programs&subgroup=rnastruct

Nonetheless, the only final possibility was here to fold the desired sequence manually one by one with the trial and error method. This particularly means, seeking for the best sequence by cropping or adding bases until the optimal sequence was found. In this respect, it has been shown that even one single base pair can critically change the structure. Due this biased approach, it is supposable that promising candidates were overseen.

The computational results of the strategy 1 where the goal was to show an occurrence of miRNAs in the smRNAnome of *N. attenuata* upon herbivore attack, indicate a direct involvement of miRNAs in the response to herbivores. Due to those findings, miRNAs could be eaten by the caterpillar in sufficient amounts, because the larva is capable of eating almost a large portion of the leaf material. Deductively, a function in the body of *M. sexta*. of those eaten miRNAs is also supposable.

In this work, only a few miRNAs were identified in silco from the smRNAnome which actually contains approximately 50k sequences. Therefore it appears very likely, to identify more miRNAs in the smRNAnome, with more accurate methods and custom tailed algorithms. At this point it is recommended to compare the miRNAnome/ smRNAnome of *M. sexta* while eating on *N. attenuata* with the miRNAnome of *N. attenuata*.

Due to the results of strategy 1, the next step should be, whether those upregulated miRNAs are acting only in the plant, in the caterpillar or both. Of course a proportion of miRNAs occur also in the stem or roots but nevertheless the likelihood of an interspecies transfer has to be considered as a given fact due to the consumption of almost the whole leaf material by the caterpillar. Considering the fast consumption of the whole plant, it appears logical for the plant to counter with a quick response and therefore to produce lots of miRNAs as ingredients in an allelochemical cocktail.

The pro of strategy 2 was the confirmed existence of the miRNA candidates in *N. attenuata* based on orthologous miRNAs in closely related tobacco species and computational folding studies. The previous annotated proteins in *M. sexta* were the guarantor for measurable targets in accordance with good investigated proteins in the literature. Nevertheless, it is very likely that miRNAs targeting various genomic regions and other RNAs, beyond CDSs, in still not estimable potential.
The obvious con in turn was the fact that it is not clear if these conserved miRNAs tested via qRT-PCR represent a direct response upon herbivore attack and hence could be considered as direct defense weapons. Nevertheless, a successful downregulation will provide strong support of the CKR concept, because at present no reliable and accepted example of a transferred functional miRNA from one organism to another has been documented, even though the experimental prerequisites in terms of artificial diet, miRNA type and quantities or growth setup are not directly comparable with natural conditions in the field.

4.2. Wet lab

The qRT-PCR results clearly demonstrate that miRNAs from *N. attenuata* exhibit the potential to knock-down specific genes in *M. sexta* and hence provide evidence to CKR. Whether those miRNAs are directly upregulated upon herbivore attack and subsequently occur in higher quantities in the caterpillar, is yet to be clarified.
The midgut is a good source tissue for testing the downregulation of genes by foreign RNAs. Here, it is not a coincidence that 2 abundant enzymes are significantly affected. For the other predicted target: *Ms*18-56 a developmental dependent or tissue specific expression is supposable so therefore no clear effect was observable.
In addition, feeding the natural hairpin precursors cause a stronger effect than the mature versions. Thus, these findings are in accordance to the literature and support the fact that those precursors are the natural transferred conformation. Moreover, it gives rise for speculations about the nature of the key proteins, most notably Dicer and RISC, involved in the processing pathway. As mentioned in the introduction Dicer and RISC exhibit the capability to process a wide scope of short RNAs independent of their species origin. So the potential and risks of stably transferred information in form of miRNAs is not estimable especially with a view of GMO crops for the environment.

Even though for some short mature miRNAs no effect was detectable, these findings doesn't automatically negate silencing under in vivo conditions but will leave room for interpretation that M25 and M6 are kind of strong "down-regulators" and target specific miRNAs for *MS*CYP6AN5 and *MS*CHS, respectively. As mentioned above, a downregulation of a target could also be realized by secondary or "off-target" effects. Nevertheless, I would interpret the results in this way that M25 and M6 target the *MS*CYP6AN5 and *MS*CHS -CDS directly as predicted in silico. On the other hand, M25, M6 and miR6150

could target in an indirect manner via secondary effects and alternate pathways. This question has to be clarified in future experiments and represents one major issue in the miRNA research.

All 3 miRNAs down-regulate their targets in the natural, in vivo pre-miRNA form. Effective downregulation in the natural conformation needs the processing and assistance from cellular proteins and stable transfer. Short mature RNAs in turn could be described as somehow instable, susceptible to degradation and correspond to a more artificial system as natural hairpin RNAs. Additionally, a higher abundance of *MS*CYP6AN5 and *MS*CHS as a target can be assumed. And in fact, *MS*CHS and *MS*CYP6AN5 are midgut specific transcripts compared to *Ms*18-56.

Regarding to their structure, M6 and M25 are thermodynamically more stable hairpins compared to the relatively short miR6150. Deductively, the mutations cause unwanted structures in miR6150 which in turn changes the ensemble in a negative way leading to a reduced or a nonsense silencing. In general, a short structure like miR6150 is not able to cope with mutations compared to bigger hairpins which are more thermodynamically resistant. Those results points to the conclusion, to strengthen the focus on thermodynamic criteria, while selecting the miRNA of choice. (See appendix 1, page 72)

Not surprisingly, *MS*CYP6AN5 and *MS*CHS show the most significant target susceptibility due to their abundance in the midgut of *M. sexta*. cytochromes represent a large number of genes with various isoforms and pseudogenes whereas MS chitin synthase 2 is highly expressed exclusively in the midgut. Whether *MS*CYP6AN5 and *MS*CHS represent the direct target or secondary effects are responsible, should be confirmed in further experiments such as Northern Blot, IHC or CLIP.

4.2.1 Coevolution provides the molecular basis for CKR

Why M25 and M6 target amongst dozens possible genes *MS*CYP6AN5 and *MS*CHS must be set in the context of coevolution. It should be noted, that only when two or more species evolve in response to each other it is named coevolution. If a specialist insect is able to utilize a toxic plant, it will gain an exclusive resource compared to a generalist. Henceforth, the specialist *M. sexta* with his corresponding host plant *N. attenuata* represent a perfect model (I.T. Baldwin and others).

A coevolutionary race at the molecular level between populations of coevolving flowering plant hosts and insect antagonists is supported by real-time experimental studies. Further, evo-devo studies using biochemically altered probes and microRNAs demonstrate the lability of flowers and insect bodies.

Established coevolutionary models are predominantly characterized a bit like an arms race, with each side evolving stronger and stronger weapons and defenses to match those of their opponent. For example, species 1 evolved toxicity in response to selection from the opponent species 2, and then the

species 2 would have evolved resistance in response to selection from 1, which allowed the 1 to evolve slightly greater toxicity — in response to which 2 evolved enhanced resistance...and so on.

The molecular basis of this close co-relationship is manifold but recent studies suggest mainly chemoreceptors and CYP_{P450} genes as multigene families as a theatre of war. Like chemoreceptors CYPs are considered as fast evolving genes, providing the evidence for a molecular key-player in terms of co-evolution. CYP_{P450} genes code for enzymes that oxidize hydrophobic compounds, therefore they are placed on the early start of a detoxification response. Secondary reactions are subsequent conjugation by sulfatases, attachment of glucuronic acid etc. In addition to the xenobiotic response CYPs are performing various metabolic reactions for synthesis of hormones, e.g. ecdysone. Baldwin et al. or Berenbaum et al. particularly address CYP_{P450} as the molecular pivotal point in a special HPR system.

In this thesis I hypothesize not a novel quantity of an existing attack and defense system, rather a new layer of a coevolutionary arsenal.
To support the concept of miRNAs in this context, the tradeoffs between the costs and benefits have to be taken into account.
Every species has to face an evolutionary tradeoff which finally results in a trait matching and/or a tie game. Therefore, equilibrium between those two parties has been established.
MiRNAs constitute a cheap metabolic weapon compared for example to a poison or proteins. As mentioned in the introduction, one effector mechanism of miRNAs is to buffer or titrate pseudotargets and isoforms in order to regulate the levels of a specific gene.
In a molecular arms race it is imaginable that one player has a timely edge before his competitor, and as the first to develop a stronger weapon. At this point, cheap miRNAs come into the play and could buffer the new superior defense molecule of one competitor at a genetic level. It could gain some time for the disadvantaged party. Similar to an antibiotic which gain approximately 4 days for the body to set on the cell mediated response of the adaptive immune system.
In fact, an adverse interaction between miRNAs and target genes has been studied across *Drosophila* species. [41]
In order to complete the picture of a possible miRNA involvement in the molecular arms race it should be mentioned that the rate of amino acid evolution in *Drosophila* is substantially elevated in genes related to antiviral RNAi function. A significantly accelerated evolvement compared to other genes is reported and reflects the characteristics of co-evolutionary pressure. Assumable genes which are already used in a host pathogen relationship are also utilized in host plant resistance because the key proteins in the RNAi/ miRNA cascade are generally the same.

Finally it must be stated that all the new proposed concepts mentioned are hypothetical and need further investigation.

4.2.2 *MS*CYP6AN5

The reason why *MS*CYP6AN5 represents an auspicious target for plant miRNAs is based on the relationships between herbivores and their host plants. The already mentioned reciprocal evolutionary change, called coevolution reflects a response which plants has evolved as allelopathic organisms producing allelochemicals with negatively effects the herbivore, is the supposable driving force in this context. Especially between specialists like *M. sexta* and their natural host plant, a particularly close connection with numerous tactics is highly assumable as compared to a generalist.

One of the most relevant mechanisms in this molecular arms race of “escape and radiation” finds expression in the quantity and rich facets of the CYP_{P450} genes. The evolutionary link between the capacity to metabolize, and resist, synthetic insecticides and the ability of insects to thrive on chemically well defended plants was recognized early on in the 1960s. Many cases of insecticide resistance were clearly attributable to alterations in the CYP_{P450} system, a quantitative and qualitative change.

The most extensively studied group of insect cytochromes is the CYP6 family, which is related to the major drug-metabolizing CYP3 family in vertebrates. Pauchet et al. revealed 36 new cytochromes, some of which have been implicated in the metabolism of host plant-derived nicotine [33].

Although this hypothesis is attractive, there have been very few studies that have examined the metabolism of natural substrates by individual cytochrome isoforms. For example, the CYP6B genes of papilionid caterpillars have been shown to metabolize host plant’s toxic natural substrate- furanocoumarin. CYP6A1 metabolizes the insecticides al-drin and heptachlor, and CYP6D1 has been linked to deltamethrin metabolism. Heterologous expression of *Drosophila melanogaster* CYP6A2 in Saccharomyces cerevisiae bioactivates some genotoxins (e.g., af latoxin B). This broad catalytic diversity is thought to have arisen as a result of coevolution between herbivorous animals and toxic allelochemical-producing plants [34, 35, and 36].

An impressive example to illustrate this molecular interplay and involvement of CYP isoforms in HPR was published by Kumar et al. where *M. sexta’s CYP6B46* represent the response to *N. attenuata’s* nicotine [37]. But that’s not all: The most remarkable fact on this is that the researches added another novel dimension to this interaction where the plant allelochemical nicotine is directly exploited from *M. sexta* as secondary insect chemical to deter their natural predators. The key player for this conversion in the insect’s body was identified as CYP6B46.

I mentioned this example because this could explain the hypothesis that downregulation of transcripts like CYP6B46 by plant RNA’s will negatively affect the larva’s nicotine protective shield. Continuative, this could also explain a missing negative larva performance in my experiments, because predators will significantly reduce the populations. This in turn requires higher altruistic patterns of behavior where some plant act unselfish and sacrifice themselves.

Taken together, those well-defined interrelationships provide an ideal model system with which to investigate cytochrome mediated resistance to toxic plant allelochemicals. A close coevolving relationship is assumed; therefore it could be hypothesized if *M. sexta's MS*CYP6AN5 is someway involved in detoxification of *N. attenuata* predominant allelochemical nicotine. It could present an alternate or complementary pathway to CYP6B46. Of course, those certain assumptions should be treated with care and need further experiments.
Over and above, CYP_{P450} family clans represent the 3rd largest group of genes in plants. As mentioned above, compared to mammals with ~57, the insect genome carries around hundred different CYP_{P450} genes, but still less than plants with approx. 300 in this case. MiRNAs are often evolutionally wired to their targets depending on the age of the miRNA gene. This phenomenon is known as co-expression. Deductively, it is also possible that plants developed a huge subset of miRNAs harmonized to their CYP_{P450} genes. Possessing this large miRNA arsenal, the plant is probably also able to interfere lots of other CYP_{P450} genes including isoforms in *M. sexta* due to the close specialist relationship.

4.2.3 Chitin synthase

Chitin synthase represents an attractive target due to its occurrence in the entire insect body. In addition, isoform *MS*CHS2 is special and occurs only in the midgut [40]. If a knockdown of *MS*CHS1 or midgut specific *MS*CHS2 was demonstrated here must be further investigated. It needs to be noted that the crucial importance of their product chitin is required in the complete insect. Chitin synthase itself is also mentioned in lots of publications as a model target for RNAi in flies and mosquitos in order to develop a new insecticide. [38]

Chitin is a structural polysaccharide, present in the insect exoskeleton and essential for survival. In general, this insoluble polysaccharide is found in two major extracellular structures in insects, the cuticle that overlays the epidermis and the peritrophic membrane (PM) that lines the midgut. Unique in *M. sexta* is the occurrence of only 2 isoforms *MS*CHS1 and *MS*CHS2. *MS*CHS2 is utilized exclusively for the synthesis of PM-associated chitin in the midgut. Interestingly, the anterior midgut shows the highest expression levels with tapering off in the medial and posterior midgut. The drop in the level of *MS*CHS2 expression is drastic between the anterior and the medial midgut sections. [39, 40]

In the mosquito fly, isoforms were both mainly expressed during the larval-pupal and pupal-adult transitions, so it can therefore be concluded that for *M. sexta* the highest susceptibility for foreign plant miRNA M6 is before developmental transitions. Developmental expression levels of *Ms*CHS should be therefore investigated.

4.2.4 *Ms*18-56

As mentioned above miR6150 is prone to mutations due to its short sequence. Therefore, the results must be investigated with other experimental methods. All in all, miR6150 is not a proper molecule for inserting mutations and for feeding in AD.

4.3. General Discussion

MiRNAs always orchestrate with other components and miRNA knockdown is additionally incorporated in a cellular network, therefore dependent on other pathways or molecules. Examples like cell cycle, cell type, delivering vesicles, presence of specific transporters e.g. SID protein, trafficking, larva state, gene expression, epigenetics, environmental stressors etc. are only some points to mention. Hereinafter, only some facts are mentioned to explain why or why not a miRNA leads to an effect in our plant-insect model system.

1. MiRNA quantities

The number of miRNAs, leading to a measurable effect, per cell cover a wide range of numbers (from 1-10000).

One crucial point is the question, which relevant concentrations of miRNAs on site are needed to cause an effect. There is no general rule to determine the quantity.

MiRNA levels depend on both biogenesis and turnover, so it is assumable that a miRNA produced in small quantities in *N. attenuata* is processed in the insect in a specific way to have a measurable impact. In insects compared to mammals the upside is the existing of confirmed amplification pathways to allow small amounts of an RNA taken up from the environment to trigger a larger response within the organism.

2. The role of Dicer

One of the most crucial lowdown is whether an alien miRNA is subject for processing by the insect Dicer machinery.

Dicer is an RNase III enzyme found in almost all eukaryotic organisms and responsible for the processing of pre-miRNAs into miRNAs, depending on primary and secondary structure motives. As described in the introduction, I hypothesize a Dicer enzyme with flexible features which is not restricted on the host species and therefore able to cope with foreign hairpin RNAs.

The way Dicer cuts the pre-miRNA is highly susceptible to interference, even one natural SNP or an A to I editing by ADAR (adenosine deaminase) can impair the correct processing of the pre-miRNA to a mature miRNA.

Mutations, as they are introduced in the control miRNAs, cause unwanted structures. Especially secondary structures can affect Dicer in a negative way which makes it impossible to cut in an appropriate manner. Depended on Dicer machinery some secondary structure motives are more/less subject for cleavage.
Considering the additional fact that animal stem–loop structures of miRNA precursors have highly uniform sizes of 65 nt., whereas there is a wide range of precursors from 50 to >500 nt in plant. This heterogeneity in the precursor structures might also reflect differences in the processing pathways especially if a plant miRNA is subjected for cutting by the insect DICER machinery. Therefore, it has to be discussed whether and in what extent this heterogeneity impacts the cutting.

3. Gene accessibility

Low gene expression depends on developmental stages. Hence, for a certain gene a miRNA is capable to target the mRNA but this gene is repressed at the moment. So synchronization between a miRNA and gene expression is essential. Appling this on the experimental setup means that a miRNA possibly shows no effect on a 3th instar larva stage but very well on other stages.

MiRNAs have been known for some time to shape cell type identity. This hypothesis also predicts each cell type, as a consequence of the composition of its transcriptome, modulates miRNA activity. Therefore a diverse susceptibility of each cell type to a miRNA candidate is conceivable. So the possibility of a knockdown in different tissues cannot be ruled out even though the probability of success in the midgut is highest.

In general, the chromatin accessibility and secondary structures to a certain time of a target gene must be embraced in the miRNA-target relationship.
Additionally, selected targets may exhibit dosage-dependent activity as described by H Seitz et al. So pseudogenes are involved and targeted by miRNAs which leads to an effect even though the actual target is not affected. Thus, a gene may be knocked down, but the qRT-PCR shows no result because the downregulation is caused by an alternative pathway. It could be argued if a qRT-PCR must include also primers for alternative targets related to the original ones.

4. Stability

Stability is one of the most arguable facts frequently mentioned in the discussions around CKR.
As described in several publications, a miRNA can travel over long distances in extracellular vesicles protected from degradation by abundant RNAases. Previous feeding experiments with hairpin precursor conducted by Pandit et al. didn’t show significant degradation of the RNA. Moreover they

found, feeding hairpin precursor is the method of choice and superior compared to other delivery techniques.
A critical point is the correct downstream processing of a miRNA. Accurate methylation, for instance, affects not only the durability but also the target mechanism itself via differential truncation and uridylation patterns.

The methyltransferase HEN1 for instance stabilizes plant miRNAs, animal piRNAs, and siRNAs in both kingdoms via 3' terminal methylation. A HEN1 knockout in plants results in non-templated oligo-uridylation and accelerated degradation of miRNAs. In those mutant plants a truncated miRNA is uridylated to their original size depending and differing among the miRNA families. Such patterns for the same miRNA are conserved between species interestingly indicating common protein machinery and so supporting the concept of CKR. [Blake C. Meyers et al. 57]

Beyond that, it gives rise to the assumption that miRNAs per se are regulated by a superordinate system of RISC and argonaute proteins. This is comparable with the posttranscriptional events (e.g. splicing) in the mRNA processing, in order to multiply the regulatory information within one miRNA.

5. Others

Some findings suggest a regulated gene knockdown only by a polycistronic expression pattern of identical or different miRNAs. Reporting indicates an artificial multi miRNA approach is superior in knockdown efficacy compared to a single hairpin construct. This in turn gives some evidence for a complex miRNA network. Therefore it is possible that for some specific genes, only a complex polycistronic multi miRNA approach will show a significant downregulation.

However, all studies dealing with CKR suffer greatly from potential contamination artifacts and undesired off-target effects and have thus proved controversial.

Future prospects of this project could be:

1. A complete miRNAnome of *N. attenuata* and *M. sexta* should be established by several new techniques like RNA-seq. (Rui Yi et al.) or microarray to investigate which miRNA is upregulated upon herbivore attack.

2. Presence or absence of candidate miRNAs in *M. sexta* with corresponding proteins must be confirmed by CLIP or dual Luciferase assay.

3. Comparison of *N. attenuata* and *M. sexta* miRNAs based on sequence and secondary structures

4. Candidate miRNAs which successfully regulate *M. sexta* genes will be used to create transgenic plants that will be uptake to Utah to determine the effects of such regulation and its ecological impacts.
5. Investigation of the trafficking system utilized from exogenous miRNAs for horizontally transfer in different species.

The general lack of information about the CKR phenomenon is to explain with the complexity, the relative novelty and the research focus, respectively. The focus of miRNA research is predominant in animals because it shows great promise for a new class of drugs against various diseases. But in recent times, scientists start to discover the potential of novel specific RNA based pesticides.

Since very recently, miRNA research will open lots of gates in the future. The concept of RNAi therapeutics is not new but unlike short interfering RNAs/short hairpin RNAs (siRNAs/shRNAs), which are designed to target a single transcript, miRNAs will affect hundreds of transcripts and so would potentially be capable of shutting down entire deregulated pathways. Of course, specific targeting of disease associated transcripts is probably more required than a completely random modulation of hundreds of transcripts without reliable predictably beyond a black box concept.

In general, miRNAs and their targets not only provide an invaluable source of novel transgenes, but also inspire the development of several new GM strategies, allowing advances in breeding novel crop cultivars with agronomically useful characteristics. Food security is one of the most important issues challenging the world today. Any strategies to solve this problem must include increasing crop yields and quality. MiRNA-based genetic modification technology (miRNA-based GM tech) can be one of the most promising solutions that contribute to agricultural productivity directly by developing superior crop cultivars with enhanced biotic and abiotic stress tolerance and increased biomass yields. Indirectly, the technology may increase usage of marginal soils and decrease pesticide use, among other benefits. Those new strategies do not only allowing advances in breeding novel crop cultivars they will also hopefully help to uncover valuable novel plant-animal interactions. [32] In contrast to the advantages the phenomenon of moving RNA hosts various and to date not controllable consequences. So in my point of view it is a fly-by-night to sow GMO crops with unpredictable RNA messages to the environment.

The reason why short RNAs haven’t been studied up to the year 2000 is simply that nobody was able to look at them due to their small size. Faster computers accompanied with the commercial availability of next generation sequencing technology open a window in science not only in terms of miRNAs but also for lots of other alternate ways of thinking and applications. It’s a matter of fact that you can only find what you are looking for. Over and above, psychological reasons play definitely their role. It must

be kept in mind, that 15 years ago, the genetic informations outside protein coding regions, where not only miRNAs are originated, were considered as junk material. The hegemonic concept of: “one gene one protein” was prevalent for decades. Constituently to this analogy it is stirring to consider RNA as a cross-kingdom messenger. Interspecies communication is essential to understand the whole nature of organisms. Henceforth, it is fascinating to explore why and how RNA is able to move out of the cell and switch to another species. In my opinion, special focus should be set on mechanisms responsible for the packaging and moving.

Therefore, I am definitely convinced that RNAs trespass from one species to another widely exist in the biological world and satisfy the genetic information exchange between different organisms, which can regulate physiological and pathological processes.

In essence, CKR miRNAs and its biological function may be more significant in the biological world than our callow expectations. They present an exciting avenue for future research with far-reaching implications.

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6. Abbreviations in alphabetical order

AD-artificial diet	*M. sexta*- Manduca sexta
ADAR- adenosine deaminase	*N. attenuata*- Nicotiana attenuata
AGO- argonaute protein	NcRNAs - non-coding RNAs
bp- base pair	NGS- next generation sequencing
CDS- coding DNA sequence	Nt.- nucleotide
cDNA- complementary DNA	P-Body- processing bodies
C. elegans- Caenorhabditis elegans	PCR- polymerase chain reaction
DEPC- Diethylpyrocarbonate	Pre-miRNA- Precursor micro RNA
*MS*CHS- Manduca sexta chitin synthase	Pri-miRNA- primary micro RNA
Dicer- endoribonuclease in the RNase III family	qRT-PCR- quantitative real-time PCR
CKR- cross-kingdom regulation	RBP- RNA-binding protein
Drosha- Class 2 RNase III enzyme	RNP- ribonucleoprotein particle
ds DNA- double strand DNA	RISC- RNA induced silencing complex
G:U- guanine-uracil base pair	RT- room temperature
HPR- host-plant resistance	SNP- single nucleotide polymorphism
IVT- in vitro transcription	ssDNA- single strand DNA
Let7- lethal 7	ssRNA- single strand RNA
Lin4- name of a micro RNA	tRF’s- tRNAfragments
MiRNA –Micro Ribose Nucleic Acid	tRNA- transfer RNA
mRNA- messenger RNA	3’UTR- 3' untranslated region

7. Sworn Statement

I declare in lieu of oath that I have researched and written the diploma thesis myself (statement of authorship), no passages of text have been taken from third parties or own exam papers without having been identified as such and that all tools, personal notifications, and sources used by the applicant have been indicated in the Master thesis. The persons, who have supported the applicant in selecting and analyzing the material and preparing the manuscript, are declared completely. The assistance of a professional consultant has not been utilized and no third parties have either directly or indirectly received monetary benefits from the candidate for work related to the contents of the submitted Master thesis. The Master thesis has not yet been submitted as an examination paper for state or other academic examinations.

Jena, 24th February, 2014 Marcus Horn

8. Appendix

Appendix 1

MicroRNA M6

M6 target: Manduca sexta chitin synthase mRNA

gi|24762311|gb|AY062175.2| Manduca sexta chitin synthase mRNA, complete cds
24.7 bits(26) 0.018 21/25(84%) 2/25(8%) Plus/Minus

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Query  79   ATCGGCAAGTTGTCCCTGGCTACAT  103
            |||||  ||||||| |||||| |||
Sbjct  195  ATCGG--AGTTGTCGCTGGCTTCAT  173
```

Mutated form mature miRNA:

M6siF (mature siRNA)
TAATACGACTCACTATAGGGATCGGCAAGTTGTCCCTGGCTACAT

M6siFRC
ATGTAGCCAGGGACAACTTGCCGATCCCTATAGTGAGTCGTATTA

M6siR (mature siRNA*)
TAATACGACTCACTATAGGGGGAGCCGUUCAGUAGGGACCGACG

M6siRRC
CGTCGGTCCCTACTGAACGGCTCCCCCTATAGTGAGTCGTATTA

==

M6siCF (mature control siRNA)-mutated
TAATACGACTCACTATAGGGAAGCCCAAGTTGTCCCTCCGAACAT

M6siCFRC
ATGTTCGGAGGGACAACTTGGGCTTCCCTATAGTGAGTCGTATTA

M6siCR (mature control siRNA *)
TAATACGACTCACTATAGGGGGAGCCGUUCAGUAGGGACCGACG

M6siCRRC
CCAGCCCAAGTCATCCCTCCGATGCCCCTATAGTGAGTCGTATTA

==

RCM6siR (exactly complementary * strand)
TAATACGACTCACTATAGGGATGTAGCCAGGGACAACTTGCCGAT

RCM6siRRC
TAATACGACTCACTATAGGGCCAAGACGATCAAACAAATCTGCTA

Mutated form pre-miRNA:

Mir6CR [please use this primer with the M6iF primer (containing T7 promoter) on the mutated template]

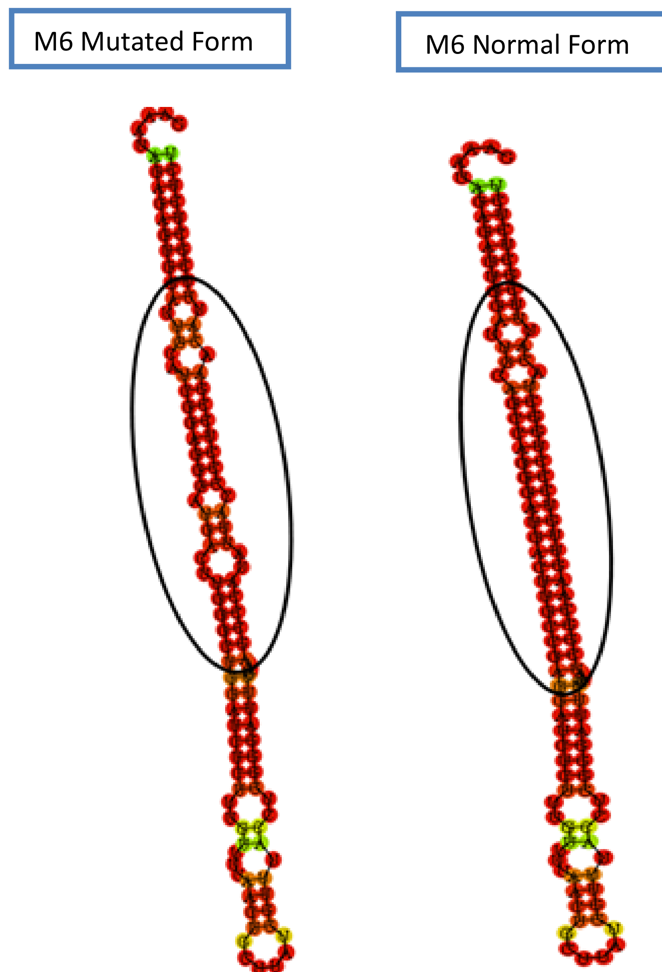
>M6iCF
TAATACGACTCACTATAGGGGAAATAGAGAGTGGATTGCATC

>M6iCR
AGAGAGCGAAATGTTCGGA

Mir6C (mutated template from SIGMA)

GAAATAGAGAGTGGATTGCATCGGAGGGAUGACUUGGGCTGGATCTCTTTGTTTTAACTGCTTATGGTTTACCTGGGGAT TTGAAAGCCCATATTACCCCTCCGAACATTTCGCTCTCT

This picture illustrates that introducing mutations in the pre-miRNA cause new secondary structures.



The free energy of both thermodynamic ensembles is **-52.98** kcal/mol.

MicroRNA M25

M25 target 1: Manduca sexta cytochrome $_{P450}$ 6AN5 (*MS*CYP6AN5)

gi|291464094|gb|GU731535.1| Manduca sexta cytochrome $_{P450}$ 6AN5 (*MS*CYP6AN5) mRNA, complete cds

Already fitted sequence for *N.att* M25:

Score	Expect	Identities	Gaps	Strand
26.5 bits(28)	0.005	20/21(88%)	1/24(4%)	Plus/Plus

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Sbjct  2365  CATCATCATCATCAT-TTCGC  2387
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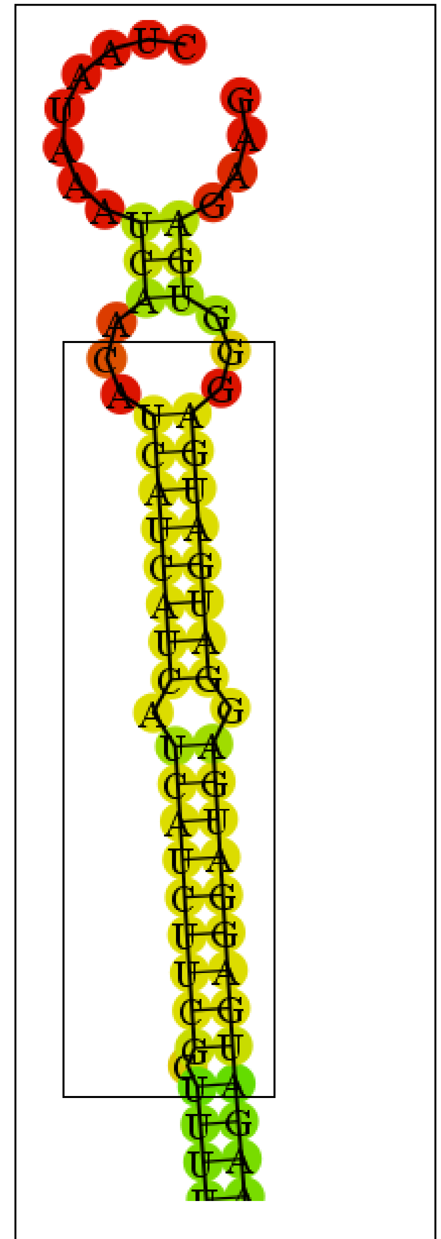


Normal Mir25 pre-mirRNA sequence Hits for *MS*CYP6AN5

CTAATAAATCAACATCATCATCATCATCTTCGCTTTTCTCTGTCCTTCCTCCGCCCAAA
ACCACTCCTTTGTCTTCGTCAGATCCTAAACCTAAAAGAAAGGTTGTTCAGTTCAAGC
CTCCTGCCAACCCTTTTTCTGTAAAGTCCAATAGAAGTGTAGA
TGAAGATGAGGATGAGGATGATGAGGGTGAGAAG

Due to the big structure, only a part with the predicted mature region of M25 is shown on the right side.

The free energy of the thermodynamic ensemble is **-47.49** kcal/mol.

Mutated form mature miRNA:

CATCATCATCATCATCTTCGC-hit sequence

M25 *MS*CYP6AN5siF

TAATACGACTCACTATAGGG AACATCATCATCATCATCTTCGCUU

M25 *MS*CYP6AN5siFRC

AAGCGAAGATGATGATGATGATG CCCTATAGTGAGTCGTATTA

M25 *MS*CYP6AN5 siR (mature siRNA*)

TAATACGACTCACTATAGGG AUGAGGAUGAGGAUGAUGAGGGUGA

M25 *MS*CYP6AN5siRRC

TCACCCTCATCATCCTCATCCTCAT CCCTATAGTGAGTCGTATTA

M25 *MS*CYP6AN5siCF (mature control siRNA)

AACATCATCATCATCATCTTCGCUU

TAATACGACTCACTATAGGG AACATCATGTCGATCATCTTCGCUU

M25 *MS*CYP6AN5siCFRC

AAGCGAAGATGATCGACATGATGTT CCCTATAGTGAGTCGTATTA

M6siCR (mature control siRNA *)

TAATACGACTCACTATAGGG AUGAGGAUGAGCGACAUGAGGGUGA

M6siCRRC

TCACCCTCATGTCGCTCATCCTCAT CCCTATAGTGAGTCGTATTA

Mutated form pre-miRNA:

CTAATAAATCAA CAT GGC CAT CAT CAT CTT CGC TTTTCTCTGTCCTTCCTCCGCCCAAAACCACTCCTTTGTCTTCGTCA

GATCCTAAACCTAAAAGAAAGGTTGTTCAGTTCAAGCCTCCTGCCAACCCTTTTTCTGTAAAGTCCAATAGAAGTGTAGA

TGAAG AUGAGGAUGAGGAUG GCC AGG GTGAGAAG

The free energy of the thermodynamic ensemble is **-51.41** kcal/mol.

M25 target 2: Manduca sexta cytochrome $_{P450}$ 6AN5 (*MS*CYP6AN5) with "star" (-5p; opposite) strand

Normal Mir25 mirRNA sequence Hits for *MS*CYP6AN5

CTAATAAATCAACATCATCATCATCATCTTCGCTTTTCTCTGTCCTTCCTCCGCCCAAAACCACTCCTTTGTCTTCGTCA

GATCCTAAACCTAAAAGAAAGGTTGTTCAGTTCAAGCCTCCTGCCAACCCTTTTTCTGTAAAGTCCAATAGAAGTGTAGA

TGAAGATGAGGATGAGGATGATGAGGGTGAGAAG

26.5 bits(28) 0.005 17/19(89%) 0/19(0%) Plus/Minus

```
Query   169    ATGAGGATGAGGATGATGA   187
               |||| ||||| ||||||||
Sbjct   2382   ATGATGATGATGATGATGA   2364
```

M25 ***MS*****CYP6AN5siF**

TAATACGACTCACTATAGGG ATGAGGATGAGGATGATGA

M25 ***MS*****CYP6AN5siFRC**

TCATCATCCTCATCCTCAT CCCTATAGTGAGTCGTATTA

M25 ***MS*****CYP6AN5 siR (mature siRNA*)**

TAATACGACTCACTATAGGG UCAUCAUCAUCAUCUUCGCU

M25 ***MS*****CYP6AN5siRRC**

AGCGAAGATGATGATGATGA CCCTATAGTGAGTCGTATTA

M25 ***MS*****CYP6AN5siCF (mature control siRNA)**

TAATACGACTCACTATAGGG A GCCA GATGAGGAT CCGA A

M25 ***MS*****CYP6AN5siCFRC**

TTCGGATCCTCATCTGGCT CCCTATAGTGAGTCGTATTA

M25siCR (mature control siRNA *)

TAATACGACTCACTATAGGG U UCGG AUCAUCAUCU UGGC U

M25siCRRC

AGCCAAGATGATGATCCGAA CCCTATAGTGAGTCGTATT

Mutated form pre-miRNA:

CTAATAAATCAACA U UCGG AUCAUCAUCU UGGC UTTTCTCTGTCCTTCCTCCGCCCAAAACCACTCCTTTGTCTTCGTCA

GATCCTAAACCTAAAAGAAAGGTTGTTCAGTTCAAGCCTCCTGCCAACCCTTTTTCTGTAAAGTCCAATAGAAGTGTAGA

TGAAG A GCCA GATGAGGAT CCGA A GGGTGAGAAG

The free energy of the thermodynamic ensemble is **-54.00** kcal/mol.

Summary

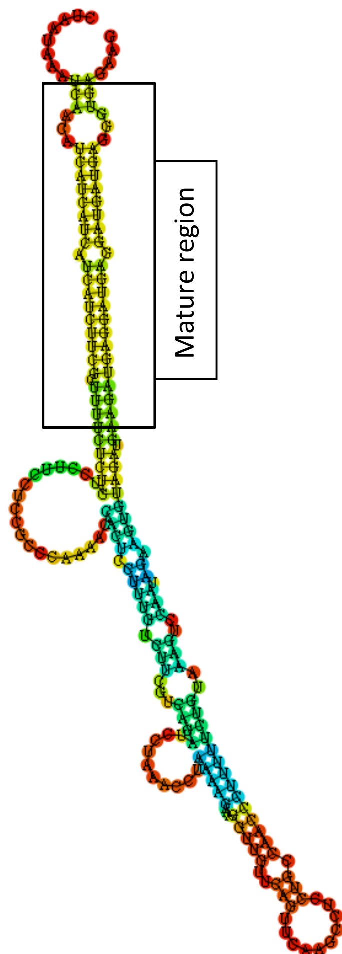
Normal Mir25 mirRNA sequence Hits for *MS*CYP6AN5

CTAATAAATCAACATCATCATCATCATCTTCGCTTTTCTCTGTCCTTCCTCCGCCCAAAACCACTCCTTTGTCTTCGTCA

GATCCTAAACCTAAAAGAAAGGTTGTTCAGTTCAAGCCTCCTGCCAACCCTTTTTCTGTAAAGTCCAATAGAAGTGTAGA

TGAAGATGAGGATGAGGATGATGAGGGTGAGAAG

Due to the big structure M25 is not affect by inserted mutations.



MFE secondary structure

The free energy of the thermodynamic ensemble is **-47.49** kcal/mol.

MicroRNA nta-miR6150

M6150 target: Manduca sexta *Ms*18-56 protein

Hit in original seq: 11 - agauuuguuugaucgucuuggc- 32

Mature region predicted in mirBASE experimental and in silico.

gi|1167962|gb|U43728.1|MSU43728

Manduca sexta *Ms*18-56 protein mRNA, complete cds

Score	Expect	Identities	Gaps	Strand
22.9 bits(24)	0.011	18/22(82%)	0/22(0%)	Plus/Plus

```
Query  10    CAGATTTGTTTGATCGTCTTGG  31
             |||| ||||||||    ||||||
Sbjct  530   CAGAGTTGTTTGACGCTCTTGG  55
```

MiR6150 original

CCTTTGATAGCAGATTTGTTTGATCGTCTTGGCCAGGCTAAGGTGTTTAGCAAGATGGATTTGAGGAAAAGA

MiR6150 mutated

CCTTTGATAGCAGATTTGCTTGATCGTGTTGGCCAGGCTAACGTGTTTAGCAAGATGGATTTGAGGAAAAGA

MiR6150 with IVT promoter

TAATACGACTCACTATAGGGTAGCAGATTTGTTTGATCGTCTTGGCCAGGCTAAGGTGTTTAGCAAGATGGATT
TGAGGAAAAGATACTATCAAG

*****************Purple bases are dangling 3'overhangs

M6150siF

TAATACGACTCACTATAGGGTAGCAGATTTGTTTGATCGTCTTGG

M6150siFRC

CCAAGACGATCAAACAAATCTGCTACCCTATAGTGAGTCGTATTA

M6150siR (mature siRNA*; original * strand)

TAATACGACTCACTATAGGGAAGGUGUUUAGCAAGAUGGAUUUGA

M6150siRRC

TCAAATCCATCTTGCTAAACACCTTCCCTATAGTGAGTCGTATTA

RCM6150siR (exactly complementary * strand)

TAATACGACTCACTATAGGGCCAAGACGATCAAACAAATCTGCTA

RCM6150siRRC

TAATACGACTCACTATAGGGCCAAGACGATCAAACAAATCTGCTA

M6150 *Ms*18-56 siCF (mature control siRNA)

TAATACGACTCACTATAGGG CAGATCCACTTGATCGTCTTGG

M6150 *Ms*18-56 siCFRC

CCAAGACGATCAAGTGGATCTG CCCTATAGTGAGTCGTATTA

M6150siCR (mature control siRNA *)

TAATACGACTCACTATAGGG AAGGUGUUUGGUGAGAUGGAUU

M6150siCRRC

AATCCATCTCACCAAACACCTT CCCTATAGTGAGTCGTATT

Comparison between mutated and normal pre-miRNA