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Function and biogenesis of small RNAs in Dictyostelium discoideum

Ilona Urbárová

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Biology Education Centre, Uppsala University, and Department of Molecular Biology, Swedish University of Agricultural Sciences
Supervisors: Assoc. prof. Fredrik Söderbom and Lotta Avesson

Table of Contents

Summary ..... - 4 -

Introduction ..... - 5 -

Small non-coding RNAs ..... - 5 -

First to be discovered - microRNAs ..... - 5 -

Another type of small non-coding RNAs : small interfering RNAs ..... - 7 -

Different ways of silencing the gene output ..... - 8 -

The unique model organism –Dictyostelium discoideum ..... - 9 -

My interests and aims in this project ..... - 10 -

Results ..... - 12 -

Validation of predicted microRNA targets ..... - 12 -

Subcellular localization of small RNAs ..... - 13 -

Enrichment for small RNAs ..... - 14 -

Biogenesis of microRNAs and small interfering RNAs - 5´end analysis ..... - 15 -

Biogenesis of microRNAs and small interfering RNAs - 3´end analysis ..... - 17 -

Discussion ..... - 18 -

Why study RNAi in Dictyostelium discoideum? ..... - 18 -

Post-transcriptional silencing in Dictyostelium discoideum ..... - 18 -

Localization studies ..... - 18 -

Biogenesis of small RNAs ..... - 19 -

The importance of this study ..... - 20 -

Materials and Methods ..... - 21 -

Description of strains & plasmids ..... - 21 -

Oligonucleotides used ..... - 21 -

How to handle RNA ..... - 21 -

Electrophoresis ..... - 21 -

Phenol extraction ..... - 21 -

Ethanol precipitation ..... - 21 -

Solutions used ..... - 21 -

Total RNA preparations ..... - 22 -

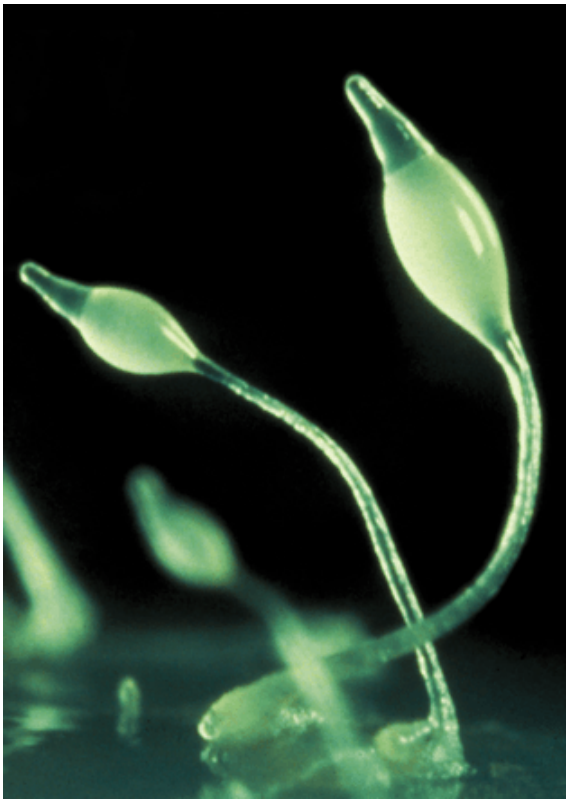
Northern blots ..... - 22 -

5´RACE ..... - 23 -

Cloning ..... - 24 -

Nuclear RNA preparations ……- 25 -
Enrichment for the small RNAs ……- 25 -
Dephosphorylation assay ……- 25 -
β-elimination assay ……- 26 -
Acknowledgements ……- 27 -
References ……- 28 -
Appendix ……- 31 -

(Figure on the cover page : Scanning electron microscope picture of spore towers of the slime mold *Dictyostelium discoideum* by David Scharf/ Photo Researchers, Inc, reproduced/adapted with permission from online journal The Scientist, Volume 22, Issue 7, Page 30; the article The cheating amoeba)



The development of the fruiting body of *Dictyostelium discoideum* (reproduced/ adapted with permission from the cover page of Journal of Cell Science (2001), Volume 114, Issue 24 – by Richard L. Blanton, North Carolina State University)

Summary

Small RNAs have a higher impact on the life of the organisms than most of us would have guessed twenty years ago. They are studied nowadays all over the world in different organisms and new discoveries surprise us almost everyday. The process central to two classes of small RNAs (microRNAs and short interfering RNAs, miRNAs and siRNAs respectively) was named RNA interference (RNAi), reflecting the interaction of small RNAs with messenger (m)RNA. miRNAs can interact with mRNAs in two ways, either by perfect base pairing resulting in cleavage of targeted mRNA or by so called ´seed´ pairing leading to translational silencing of targeted mRNA. The way of silencing is known for many organisms, but had not been studied in *Dictyostelium discoideum (D. discoideum).*

D. discoideum is a very interesting model organism, which stands in the evolutionary tree between plants and animals. Since its life cycle is quite short and simple and it is easy to construct gene knockouts, this model organism has become valuable in many genetic and developmental studies. It seems to be a very good model for studying small RNAs, since small interfering RNAs and recently also putative microRNAs have been found in this organism.

The aim of this study was to elucidate the silencing mechanism of targeted mRNA by four putative microRNAs in *D. discoideum.* Two different approaches were used, 5´Rapid Amplification of cDNA Ends (5´RACE) and construction of strains overexpressing the microRNAs and their targets. From this study, the silencing was predicted to be more related to animals, where the targeted mRNA is not cleaved, but is translationally silenced. However, that is a preliminary deduction based on the observation that we could not gain any cleavage products.

Biogenesis of these small RNAs was also studied, since the localization and the composition of the ends of these molecules can suggest the way of their formation and give a clue how the RNAi machinery in *D. discoideum* works. For these analyses Northern blots were used. The biogenesis of one siRNA was found to be more similar to those in animals, supporting further the possibility of an animal-like RNAi pathway in *D. discoideum.* But exceptions exist within both plant and animal kingdoms and the RNAi silencing mechanism may also function in both ways in *D. discoideum*. The process is now under investigation.

Introduction

Small non-coding RNAs

Gene expression is a complicated process that results in a functional gene product. Generally, the final gene product is a protein, but in some cases (as tRNA-, rRNA- and small silencing RNA genes) the final product is a functional RNA. The process is universal for all organisms, including viruses. Since gene expression is crucial for life, it needs to be tightly regulated. Two main steps leading to a protein product are transcription (produces RNA copies of DNA in the form of messenger (m)RNAs) followed by translation (produces one or more proteins from mRNA). That is not the case for non-coding (nc)RNA genes. Large ncRNAs (tRNAs and rRNAs) are known already for many years, but quite surprising was the discovery of small silencing RNAs almost two decades ago. The first small silencing RNA (lin 4 micro (mi)RNA) was found in 1993 by Victor Ambros, when screening for genes required for post-embryonic development in *Caernohabditis elegans* (Lee et al., 1993).

These approximately 20 nucleotides (nt) long stretches of RNA revolutionized the view on the regulation of gene expression. Since then many research groups targeted their scientific interests on this field and even though it is almost twenty years since the first miRNA was reported, there is still a lot to discover. The small silencing RNAs are of a huge importance, they regulate many different cellular pathways in development by regulation of gene expression and defects in their production can lead to death.

The small silencing RNAs are subdivided into different classes depending on : 1) which enzymes are involved in their biogenesis and processing, 2) their final size and 3) what they regulate. So far the following classes were found : small interfering RNAs (siRNAs), microRNAs (miRNAs), Piwi-interacting RNAs (piRNAs), endogenous siRNAs (endo-siRNAs), cis-acting siRNAs (casiRNAs), trans-acting siRNAs (tasiRNAs), natural antisense transcript-derived siRNAs (natsiRNAs) and probably more to come! This study was concentrating on siRNAs and miRNAs.

First to be discovered – micro RNAs

Micro (mi)RNAs are endogenous small silencing RNAs; almost 15000 different miRNAs are known today (Griffiths-Jones, 2010). miRNAs had always been thought to exist only in multicellular organisms, but their discovery in unicellular green alga *Chlamydomonas reinhardtii* (Zhao et al., 2007; Molnár et al., 2007) changed this dogma.

miRNAs are generated from single-molecule precursors with an imperfect secondary hairpin structure called primary miRNA transcripts (pri-miRNAs). Each processed precursor results in production of one miRNA molecule from one arm of the hairpin precursor. Pri-miRNAs are transcribed by RNA polymerase II in the nucleus. In animals, the maturation of miRNAs occurs in two steps. Pri-miRNA is processed by two RNase III endonucleases (with a help of their double-stranded RNA-binding domain (dsRBD) partner proteins) resulting in ~21nt long miRNA. First, the pri-miRNA is processed by ~650kDa protein Drosha (with the help of a dsRBD partner, called Pasha in flies (reviewed in Ghildiyal and Zamore, 2009) and DGCR8 in mammals (Han, J. et al., 2004)) into a 60-70nt long hairpin precursor miRNAs (pre-miRNAs). This pre-miRNA is then exported out of the nucleus and processed by an enzyme called Dicer. Dicer (with the help of a dsRBD partner, TRBP in mammals (Chendrimada et al., 2005) and Loqs in flies (Saito et al., 2005) generates miRNAs duplexes

(miRNA : miRNA*). The double-stranded structure carries two nucleotide overhangs at 3´ends and each strand has one phosphate at the 5´end and a hydroxyl group at the 3´end, a sign of processing by Dicer (Lee et al., 2003). The miRNA strand from the duplex is the one with the lower internal stability of the 5´terminus (Khvorova et al., 2003 and Schwarz et al., 2003). The 5´end of the miRNA strand seems to be unpaired compared to miRNA* strand; the difference is in the base pairing of the first five nucleotides. Because of these mismatches, the miRNA strand seems to be the one incorporated into RNA-induced silencing complex (RISC) through the help of ATPase/RNA helicase activity of a Dicer. The miRNA* strand is then destroyed. This leads to the activation of the RISC complex, which then can direct the small RNA to its target enabling silencing of the targeted mRNA (Fig. 1). This pathway is known as RNA interference (RNAi) and was first discovered in *C. elegans* (Fire et al., 1998). Quite soon after this discovery researchers realized that it is a universal process present in most eukaryotes.

The RNA-induced silencing complex (RISC) contains Argonaute proteins and possibly also some auxiliary proteins that promote or change the function of the whole complex. The ´minimal´ active RISCs contain just Argonautes with siRNAs, as shown for Ago2, purified from *Drosophila* extracts (Rand et al., 2004), indicating that Argonautes are probably responsible for the cleavage activity. The composition of RISC complex, especially concerning Argonaute proteins and the level of complementarity of the miRNA to the target messenger (m)RNA sequence, determines the type of mRNA silencing.

There is one class of miRNAs, which is not processed in the same way, mirtrons. In that case, miRNA resides in introns of mRNAs and the pri-miRNA is excised by the spliceosome and forms into Dicer

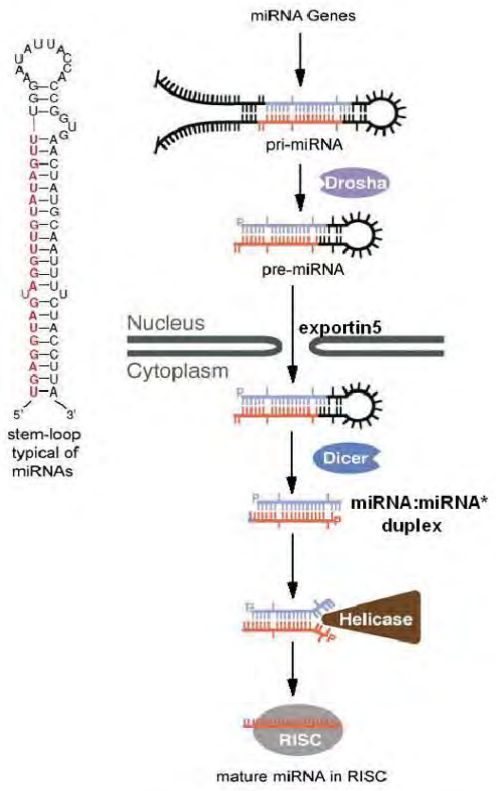


Fig. 1. Processing of miRNA within RNAi pathway. A typical stem-loop secondary structure of the miRNA precursor on the left (Bartel, 2004).

substrates (Ruby et al., 2007). Mirtrons have so far been found just in animals.

In plants, the miRNA-dependent gene silencing pathway (called post-transcriptional gene silencing, PTGS in this case) is somewhat different. Plants have a specific RNase III endonuclease called DCL-1 (with a dsRBD partner HYL1 - Vazquez et al., 2004), which localizes in the nucleus. It functions as both Drosha and Dicer, so it is responsible for processing the pri-miRNAs to pre-miRNAs and after that also to miRNAs (Kurihara and Watanabe, 2004). Plant miRNAs (Yang et al., 2006) have been found to be 2´-O-methylated at their 3´ends by HEN1 S-adenosyl methionine-dependent methyltransferase as a protection against degradation.

In general, miRNAs regulate different genes than they are derived from. miRNAs

are believed to regulate many different biological processes, either under normal or stress conditions. Many miRNAs also act just in specific tissues and at specific times. miRNAs are usually conserved in related organisms.

Another type of small non-coding RNAs : small interfering RNAs

Although it seems that small interfering (si)RNAs are very similar to miRNAs (especially after the discovery of endogenous siRNAs : Hamilton et al., 2002; Ambros et al., 2003), there are quite a few differences one has to consider when defining them.

In contrast to miRNAs, siRNAs always bind to their target with complete complementarity, resulting in cleavage of targeted mRNA. siRNAs come from long exo- or endogenous perfectly base-paired dsRNA molecules, which are either extended hairpins or long bimolecular RNA duplexes (Ambros et al., 2003). Processing of those precursors by Dicer results in numerous siRNAs from both strands. They also silence the same genes they are derived from (mRNAs, transposons, virus DNA/RNA, nuclear DNA) (Bartel, 2004) and are rarely conserved between organisms compared to miRNAs. siRNAs in *Drosophila* and plants (Horwich et al., 2007; Pelisson et al., 2007) are 2´-O-methylated at their 3´ends by HEN1 methyltransferase.

The siRNAs share many features with the miRNAs, i.e. they are both Dicer products (20-25nt in length, having 2nt 3´overhangs, a 5´phosphate and a 3´hydroxyl group) and being part of the same (RNAi) pathway. However, these two different classes of small RNAs can be separated based on their origin and biogenesis. Some criteria for the annotation of novel miRNAs also exist (Ambros et al., 2003). In contrast to miRNAs, siRNAs can be introduced into the cell by transfection of artifically synthesized 20-25nt stretches of dsRNA complementary to the targeted mRNA. In this way, siRNAs can be used for specific knockdown of a gene of interest.

The siRNAs mentioned above are referred to as primary siRNAs, because another type of siRNAs have been found. Those siRNAs are called secondary siRNAs. They are predicted to be produced by RNA-dependent RNA polymerases (RdRPs), since they have been found in plants and worms, which contains RdRPs. However, recent discovery of these secondary siRNAs in flies and mammals, where RdRPs are not present, making this process slightly mysterious. RdRPs can synthesize strands complementary to a mRNA template. In case of plants, secondary siRNA synthesis appears to be rather unprimed, derived from both directions and result of Dicer activity (Petersen and Albrechtsen, 2005; Axtell et al., 2006). In *C. elegans*, primary siRNAs probably act as primers for secondary siRNA synthesis and recruit RdRPs to the targeted mRNA (Pak and Fire, 2007). Secondary siRNAs seem to be found only in antisense polarity, mostly upstream of the dsRNA inducer sequence and not products of Dicer activity, since they contain three phosphates at their 5´ends (Sijen et al., 2001) (Fig. 3). It is not known, if the secondary siRNAs are transcribed already as short stretches of ~21nt or if they are cleaved by a different endonuclease from a longer transcript. Since endogenous siRNAs are expressed at very low levels in the cell, production of so called secondary siRNAs might be the way how to amplify the signal (Sijen, T. et al., 2001). siRNAs have been found to be recycled and acting in multiple rounds compared to miRNAs (Seitz, 2010), so it seems as if they do not actually need the amplification step.

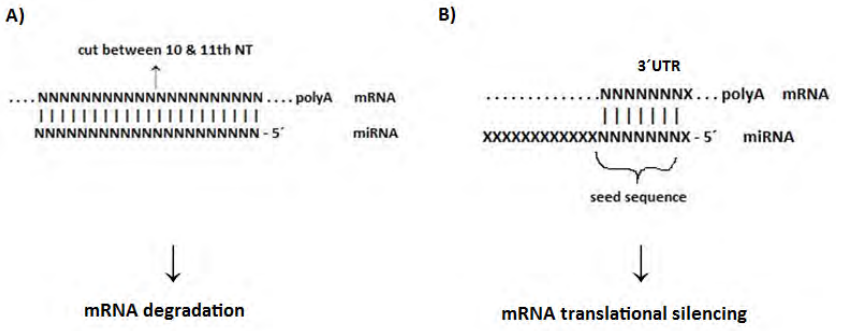


Fig. 2. Post-transcriptional silencing.

A. Target mRNA cleavage in case of complete complemetarity miRNA-mRNA.

B. Translational silencing of targeted mRNA in case of imperfect base-pairing of miRNA-mRNA

Different ways of silencing the gene output

Small RNAs can silence protein synthesis in many different ways : by cleavage of targeted mRNA; by translational silencing; by RNAi-mediated chromatin silencing via DNA/histone methylation (Lippman and Martienssen, 2004); by DNA elimination or rearrangements (Matzke and Birchler, 2005); or by transitive RNAi phenomena (Sijen et al., 2001), which is connected to the production of secondary siRNAs and spreading to the regions upstream and downstream of targeted mRNA (Petersen and Albrechtsen, 2005).

There are basically two different types of mRNA silencing by miRNAs. When miRNA in the RISC complex binds to its target with a full complementarity, the targeted mRNA is specifically cleaved at the phosphodiester bond between nt 10 and 11 of the miRNA, counting from its 5´end (Elbashir et al., 2001). This is the common mode of miRNA action in plants. Cleavage itself does not require ATP (Nykänen et al., 2002) (Fig. 2a).

When miRNA base pairs with the targeted mRNA through a small 5´proximal ´seed´ region (2.-8. nt of the miRNA) and lacks complementarity in the central part of the miRNA (Doench and Sharp, 2004), translational inhibition occurs. This is the common action of miRNAs in animals (Fig. 2b).

Since the seed sequence is short, the specificity of the binding is quite low. The same miRNA can bind to many different targets and therefore downregulate the levels of many different mRNAs (Lim et al., 2005). Deep sequencing experiments show evolutionary conservation between animal and plant miRNAs and suggest that the miRNA genes arose at least twice in evolution. It seems that in case of plants, targeted mRNA is mostly cleaved and degraded and animals show mostly mRNA target inhibition, but exceptions exist and the division according to the species is not appropriate.

Generally, organisms have different number of Argonautes (the effector proteins of small RNA induced silencing) and Dicer-like proteins. While *Drosophila* and *Arabidopsis* have more Dicer enzymes with specialized functions (Lee et al., 2004; Tang et al., 2003), mammals and *C. elegans* have just one Dicer protein responsible for both miRNA and siRNA production, meaning that Dicer has to interact with additional proteins to gain the specificity of its function. The composition of RISC complex determines the targeted RNA/DNA.

The composition of the mi- and siRNA ends differs between species, depending on the way of small RNA biogenesis. In case of Dicer processing, the small RNAs contain one phosphate at the 5´end and a hydroxyl group at the 3´end. Another case are RNA-dependent RNA polymerases (RdRPs). These enzymes also leave a hydroxyl group at the 3´end, but can leave three phosphates at the 5´end (the synthesis by RdRPs differs between different species).

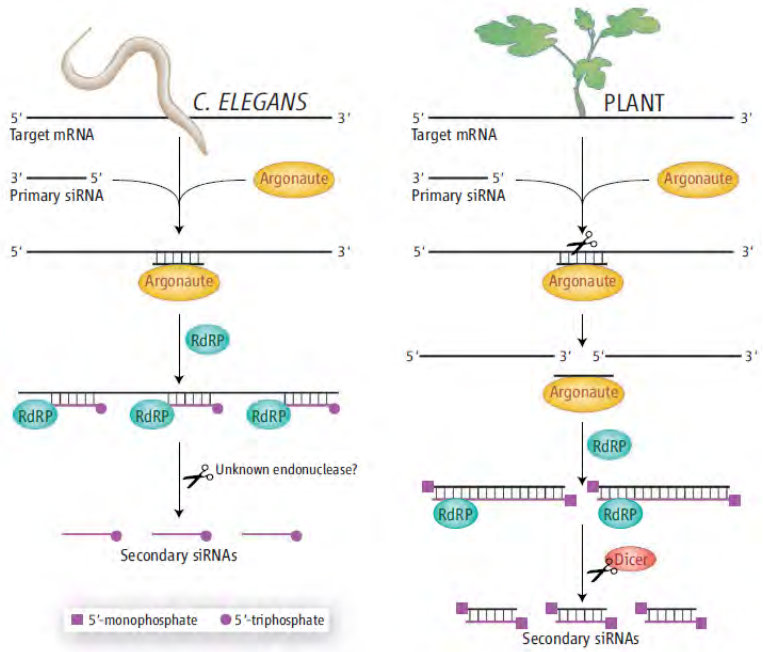


Fig. 3. Mechanism of RdRP function. In case of *C. elegans*, secondary siRNAs are produced by primed RNA-dependent RNA polymerase (RdRP) synthesis and contain 5´triphosphates. Concerning plants, the reaction is unprimed and resulting secondary siRNAs contain 5´monophosphate, as a product of Dicer cleavage **(**Baulcombe, 2007).

The unique model organism – Dictyostelium discoideum

Dictyostelium discoideum (D. discoideum hereafter*)* is a unicellular eukaryotic model organism used to study fundamental cellular processes. The first description of *D. discoideum* dates back to the 19th century (Brefeld, 1869).

This social amoebae (phylum *Mycetozoa*) lives in soil and feeds on bacteria. In laboratory conditions the cells are grown either on a plate or in a shaking liquid culture at 22-27°C. *D. discoideum* undergoes a vegetative cycle and divides mitotically, but under starvation conditions, it enters a developmental cycle, either social or sexual. There is only little known about the latter one and it is not usually studied in laboratory conditions (Fig. 4).

In the social cycle, individual cells aggregate together by chemotaxis upon attraction by cAMP (although the amoeba does not have any sensor cells or organs) and with help of glycoprotein adhesion molecules form a structure called slug. The slug is about 2-4mm long and can move around in a forward-only direction towards light, heat and humidity. Once it finds a suitable environment, it differentiates into a multicellular fruiting body with its anterior part forming stalk cells and the posterior part developing into spore cells. The anterior part is raised in the air, firstly

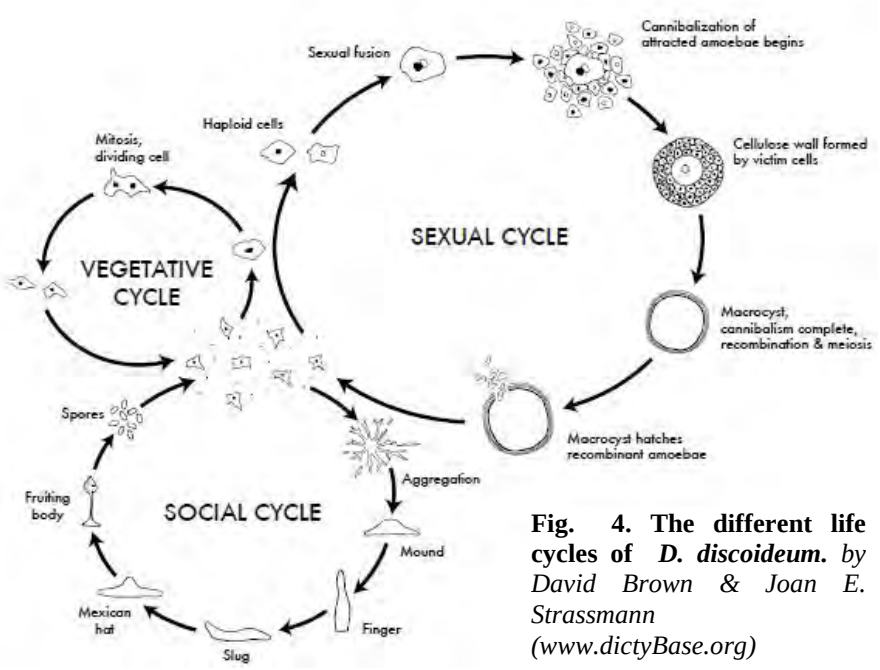


Fig. 4. The different life cycles of *D. discoideum.* *by David Brown & Joan E. Strassmann (www.dictyBase.org)*

forming a structure called the "Mexican hat" and then a tube through which the pre-spore cells can move up to the top and form mature spores. There are usually 4 times more spore than stalk cells. This developmental cycle happens within 24 hours under laboratory conditions (Kessin, 2001) (Fig. 5).

The growth and developmental stages are strictly separated and genes induced during the development are mostly not needed during the mitotic growth (Kessin, 2001). It also seems that levels of small RNAs are upregulated in developing cells (Hinas et al., 2007). The life cycle of *D. discoideum* is relatively short and simple, which makes the amoebae a valuable model organism to study different life

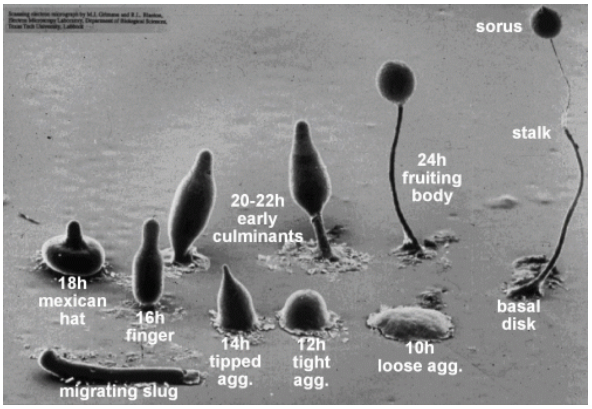


Fig. 5. The developmental cycle of *D. discoideum*. Under starvation conditions the amoebae undergoes unicellular to multicellular transition resulting in production of spores. (M. Grimson, R. Blanton, Texas Tech University, www.dictyBase.org)

processes. Several *D. discoideum* genes are homologous to human genes; these can be easily studied since the genome of *D. discoideum* is haploid and it therefore is easy to make gene-knockouts and observe the effect on the organism.

D. discoideum has a 34Mb genome, which contains approximately 12500 genes (Elchinger et al., 2005). The genome is rich in AT-bases and transposable elements. Small silencing RNAs are present in *D. discoideum* during growth and development and recently also miRNAs were found in this species, which seem to be preferably 21nt long (Hinas et al., 2007). Two genes encoding Dicer-like proteins were identified – drnA, drnB (Martens et al., 2002) and five genes were predicted to encode putative Argonaute proteins (Cerrutti and Casas-Mollano, 2006).

D. discoideum is a close relative of higher metazoans. It branched out after plants, but before the fungal and animal lineages (Fig. 6), therefore some cellular processes are animal-like, but some other pathways are more similar to fungi and plants. Since the way of translational silencing by miRNAs in *D. discoideum* is still not known, it would be interesting to see, if it functions more like in plants or in animals.

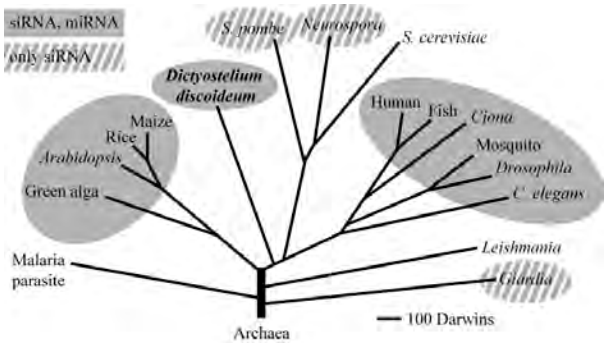


Fig. 6. The evolutionary position of *D. discoideum* between plants and animals. In species with shaded circles only siRNAs have been found so far.

My interests and aims in this project

Not so much is known about small silencing RNAs in *D. discoideum*, therefore, the aim of my thesis work was to study the biogenesis and function of small RNAs in this species. I was trying to elucidate in which way the translational silencing works, since in other organisms the targeted mRNAs are either cleaved (siRNAs and miRNAs in plants) or post-

transcriptionally silenced (miRNAs in animals). I was also trying to investigate the biogenesis of the small RNAs (the identity of the 5´ and 3´ends of siRNAs and miRNAs), since it varies between different organisms depending on their biogenesis. To be able to better assess the function of individual small silencing RNAs in *D. discoideum*, I was also looking at their subcellular localization within the cell.

I have shown that siRNAs in *D. discoideum* are probably not modified at the 3´end and contain three phosphate groups at their 5´end, which indicates their synthesis by RNA-dependent RNA polymerases (RdRPs), independent of Dicer processing. These data suggested that siRNA biogenesis is more similar to that in animals, i.e. *C. elegans* than to the process in plants.

Results

Validation of predicted microRNA targets

The main question of this study was whether silencing by small RNAs in *D. discoideum* is more plant- or animal-like. Because the way of posttranscriptional silencing by RNA interference (RNAi) pathway differs, also the way of searching for small RNA targets must be different. Two different approaches were used to elucidate the type of posttranscriptional silencing in *D. discoideum*.

In plants, binding of micro (mi)RNA to the target with perfect complementarity results in cleavage of the targeted mRNA. This can be experimentally validated by 5´RACE (Rapid Amplification of cDNA Ends), which detects site-specific mRNA cleavage. The method is based on ligation of an RNA adapter to the cleavage site followed by RT-PCR and sequencing of the gained products. I have studied two putatively cleaved targets. Neither of them indicated miRNA-induced cleavage. Since other putative targets had been already studied earlier by this method without any identified cleavage sites, it seems that the targeted mRNAs are probably not cleaved.

In case of animals, when just the ´seed´ sequence of miRNA binds to the 3´UTR of the targeted mRNA, translational inhibition occurs. To analyze this type of RNAi silencing, vector constructs can be made, where the pre-miRNA hairpin structure is placed on one vector and reporter genes with one or more miRNA target sequences in their 3´UTR (that is where the ´seed´ region of the miRNA binds) are placed on the other vector (our setup in the Fig. 7). First, construction of vectors with the four putative pre-miRNA hairpins (mi*1*, mi*2*, mi*1129* and mi*polB*, see supplementary material for sequences and structure) and their transformation into *D. discoideum* was performed. Three of the hairpins (mi*1*, mi*2*, mi*1129*) were successfully cloned into a vector with constitutive expression – pDM304 (Veltman et al., 2009a) and two of them (mi*1* and mi*2*) under an inducible promoter in vector pDM310 (Veltman et al., 2009b). (The timespan of the project did not allow me to transform the other pre-miRNAs to the respective vectors). Total RNA was prepared only from *D. discoideum* cells carrying mi*1*-pDM304, mi*2*-pDM304 and mi*1129*-pDM304. The expression of the miRNAs was analyzed

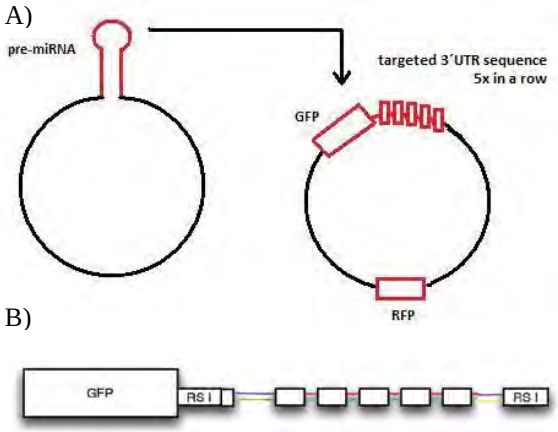


Fig. 7. The cloning setup.

A. The setup. Predicted hairpin precursor microRNAs (pre-miRNAs) on one vector with predicted 3´UTRs of targeted mRNA under GFP reporter on the other one (RFP serves as an internal control).

B. Close look at the setup from A. Five 3´UTR sequences of targeted mRNA in a row with two different restriction cloning sites (RSI and RSII) under GFP reporter.

(Avesson, Reimegård and Söderbom, unpublished data)

by Northern blot and compared to the expression in a wild-type (wt) *D. discoideum* strain called AX2. Much higher signal was observed for AX2 mi*2* strain compared to wt (Fig. 8) and could have been detected already after one day of exposure. No band was seen for the DrnB$^-$ strain, which confirms that the miRNA is processed by Dicer. Similar result was observed for the two other strains (AX2 mi1 and AX2 mi1129 strain), data not shown. The strains need to be analyzed

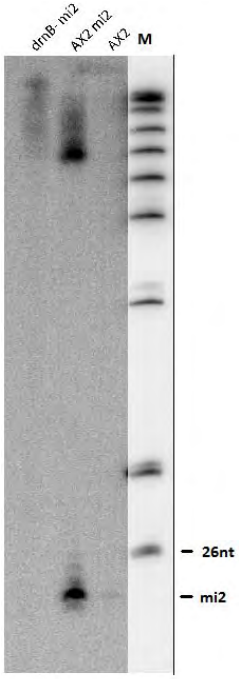


Fig. 8. Northern blot analysis of mi2 overexpression from vector transformed into *D. discoideum* cells. *drnB*$^{-}$ *mi2*, Dicer knockout with mi2 on pDM304 vector; *AX2 mi2*, AX2 strain with mi2 on pDM304 vector; *AX2*, wt strain; *M*, DNA marker; (10% PAA gel)

further to be able to validate their correctness before proceeding with other clonings.

Cloning of the 3´UTRs of predicted mRNA targets was started, but it will take a few more weeks to see results.

Subcellular localization of small RNAs

To investigate the subcellular localization of mi- and siRNAs in *D. discoideum*, total and nuclear RNA preparations were made for comparisons. Total RNA was chosen rather than the cytoplasmic fraction, since it is very difficult to separate the cytoplasm from the nucleus. The purity of the nuclear separation was checked on both an agarose gel (Fig. 9) and by Northern blot (Fig. 10). Low levels of contamination of the cytoplasm in the nuclear fractions was observed, which was satisfactory for further analyses.

Nuclear RNA preparation should not contain any tRNAs (they are present only in the cytoplasm) and might contain some larger ribosomal (r)RNA precursors (since they are processed in the nucleus). We can see both these characteristics in Fig. 9.

Total RNA and two different nuclear RNA fractions were analyzed by Northern blot to check for the presence of small RNAs (Fig. 10). The nuclear RNA fractions were prepared at two different occasions; the time of the cell lysis differed. According to the quantification from the Northern blot analysis (Fig. 10) it seems that prolonging the lysis to 6

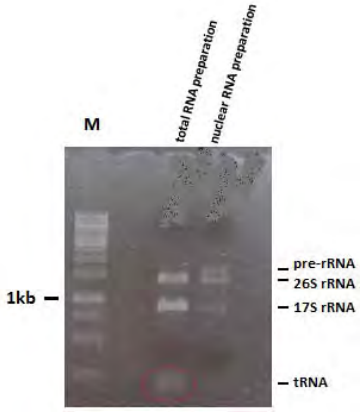


Fig. 9. The purity of nuclear RNA preparations. The composition of total and nuclear RNA preparation was compared on an agarose gel. *M*, DNA marker.

minutes (compared to initial 3 minutes) resulted in at least twice as high purity of the nuclear fraction. Any negative impact of prolonging the lysis was not detected. 3.7 µg of nuclear RNA fraction was loaded on Northern blot compared to 20 µg of total RNA to ensure the equality of nuclear RNAs in both samples (quantification from Hinas, unpublished data).

The membrane in fig. 10 was probed for siRNA from the most abundant retrotransposon in the *D. discoideum* cells, DIRS-1 (Elchinger et al., 2005; Fig. 11) and then for $tRNA^{Arg}$ as a purity and U6 RNA as a loading control (Fig. 10). If the nuclear fraction is pure, there should be no tRNA is this fraction. U6 RNA serves to check for an equal loading, since it should be present both in the nuclear and total RNA fraction. If the miRNAs and/or siRNAs are located only within the cytoplasm, it would be possible to see them only in the total RNA preparation, but if they are present only in the nucleus, bands of approximately same amounts

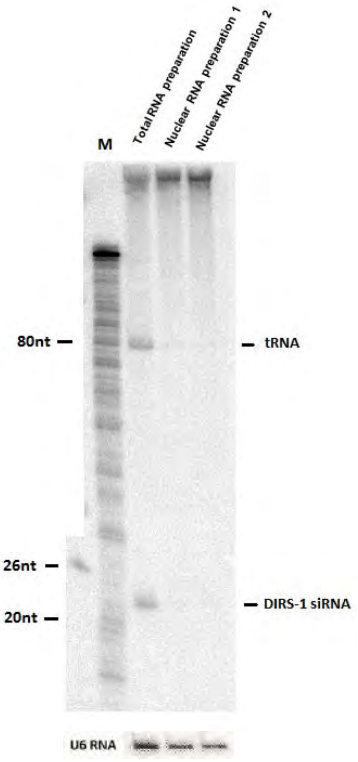


Fig. 10. Northern blot analysis of subcellular localization of small RNAs in *D. discoideum*. 20 µg of total and 3.7 µg of nuclear RNA preparations were loaded on a 10% polyacrylamide (PAA) gel and probed for DIRS-1 siRNA. tRNA was used as a purity and U6 RNA as a loading control. Cell were lysed for 3 min (*nuclear RNA preparation 1*) and 6 min (*nuclear RNA preparation 2*). *M*, RNA marker.

would be detected in both fractions. The Northern blot showed presence of DIRS-1 siRNA just in the total RNA sample, indicating that this siRNA is probably present only in the cytoplasm. Probing with DIRS-1 siRNA probably also labeled DIRS-1 mRNAs present in the nucleus, as can be seen by signal in most upper part of the membrane in fig. 10.

This result indicates that siRNAs (at least one of them) are probably present just in the cytoplasm. Further studies of different siRNAs in *D. discoideum* are necessary to confirm this hypothesis.

The same membrane was stripped and probed for mica1198 miRNA, one out of five isolated putative miRNAs present in

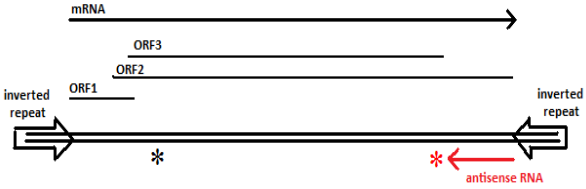


Fig. 11. The DIRS-1 retrotransposon in *D. discoideum* . The small black asterisk indicates the location of the DIRS-1 siRNA, for which was probed in this study and the small red asterisk indicates another DIRS-1 siRNA (Hinas et al., 2007), which this work refers to.

D. discoideum (Hinas et al., 2007). Even after two weeks of exposure, no signal was detected.

Enrichment for small RNAs

There is just approximately 0.1% of small RNAs in the total RNA from the whole cell, so it is very difficult to study these small RNAs in the total RNA sample. The small RNA enrichment should yield about 10-20% of the starting total RNA, discarding larger ribosomal RNAs and most of the mRNAs and result in enhanced sensitivity of small RNA detection. The procedure serves to enrich for small RNAs 200 bp or smaller (Ambion, Applied Biosystems).

Small RNAs were enriched from the total RNA preparations from growing *D. discoideum* cells. Agarose gel analyses (Fig. 12) and Northern blots (Fig. 13) were performed to see how effectively the method works. Based on the agarose gel analyses, the separation of large and small RNA fractions seemed to have worked well. The small RNA fractions did not contain any larger ribosomal (r)RNAs and did contain most of the transfer (t)RNAs (Fig. 12). When analyzed by Northern blot, there still seemed to be some small RNAs in the large RNA fraction (Fig. 13). By one enrichment reaction was gained 7 µg of small RNA fraction and loaded on a Northern blot together with 20 µg total RNA and large RNA fraction. This means that less than a half an amount of small RNA fraction was loaded on the Northern blot (Fig. 13) compared to

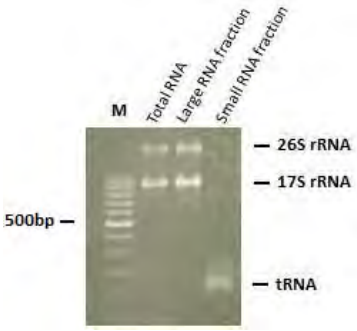


Fig. 12. An 0.8% agarose gel of enrichment for the small RNAs. 500 ng of each RNA fraction from separation was loaded on an agarose gel and compared with the total RNA. *M*, RNA marker.

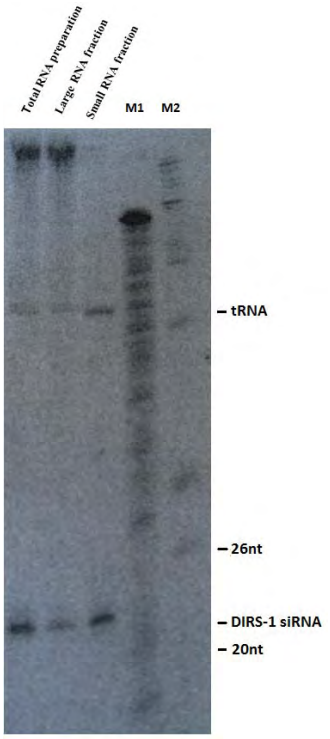


Fig. 13. Northern blot analysis of the small RNAs enrichments. Total RNA and large RNA fraction (both 20 μg) and small RNA fraction (7 μg) from enrichment was loaded on 10% polyacrylamide gel. The membrane was probed with DIRS-1 siRNA and tRNA as a control for proper small RNAs separation. *M1*, RNA marker, *M2*, DNA marker.

total and large RNA fractions and that must be considered when interpreting results.

tRNA signal was much higher in the small RNA fraction compared to the total RNA and large RNA fraction, suggesting a good enrichment for small RNAs in the small RNA fraction. On the other hand, DIRS-1 siRNA seemed to be present at approximately same levels in the total RNA and small RNA fraction. Since the amount of RNA loaded on a gel differed between those fractions substantially, the result indicates that the small RNAs were enriched.

To gain more of the small RNA fraction, more enrichment reactions were performed in parallel. From quantification data (not shown) the enrichment for the small RNAs yielded about 10% of the starting amount of total RNA, so the method seems to be working well. The usefulness of this method lies in the possibility of analyzing higher amounts of the small RNA fraction on Northern blots (since the RNAs >200 bp are lost by the enrichment) and therefore the ability to detect miRNAs in a sample should be much faster than it was possible so far (about 10-14 days). Therefore up to 30 μg of the enriched small RNA fraction could have been loaded on a Northern blot and compared to the pace of RNA detection in

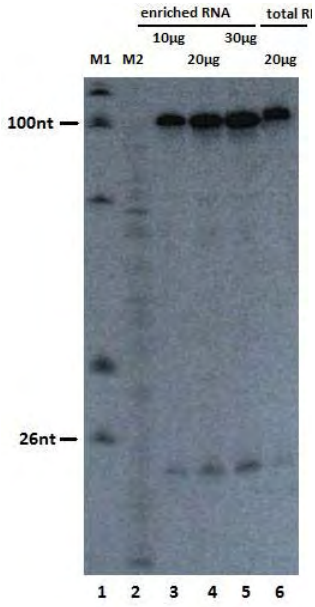


Fig. 14. The loading capacity of the polyacrylamide gel. Different amounts of enriched small RNA fractions (Lane 3, 10 μg; Lane 4, 20 μg; Lane 5, 30 μg) were loaded on 10% polyacrylamide gel and compared to total RNA (Lane 6, 20 μg). The membrane was probed for DIRS-1 siRNA and U6RNA; *M1*, DNA marker**,** *M2*, RNA marker**.**

the total RNA sample. (The amounts of RNA higher than 30 μg would not give good resolution : a smear rather than any band would be detected). From quantification of the results in the fig. 14 it seems as if loading of 10 μg of enriched small RNA fraction corresponds to approximately 20 μg of the total RNA (Fig. 14). The enrichment visibly increased the ability to detect small RNAs in the sample.

Biogenesis of microRNAs and small interfering RNAs - 5´end analysis

The composition of the mi- and siRNA ends differs between species and can suggest us the way of small RNAs biogenesis. It can be studied in different ways.

The identity of the 5´ends was first investigated by dephosphorylation reaction with alkaline phosphatase (FastAP) enzyme of total RNA samples from growing and developing cells (Fig. 15).

The RNA samples were firstly treated by FastAP enzyme and then analyzed by Northern blot. Alkaline phosphatase is an enzyme, which catalyzes the release of all phosphate groups from DNA and RNA molecules, nucleotides and proteins. If a molecule contains at least one phosphate at the 5´end, a shift on Northern blot should be seen when treated with this enzyme. Treated RNA (in lanes 5 and 7) is clearly shifted compared to untreated one (in lanes 3 and 6) (Fig. 15). This reaction, however, did not clearly show how many phosphates the RNA contains at the 5´end. To be able to elucidate that, five different reactions using four different enzymes were performed. After the treatments, all the samples were analyzed by Northern blot

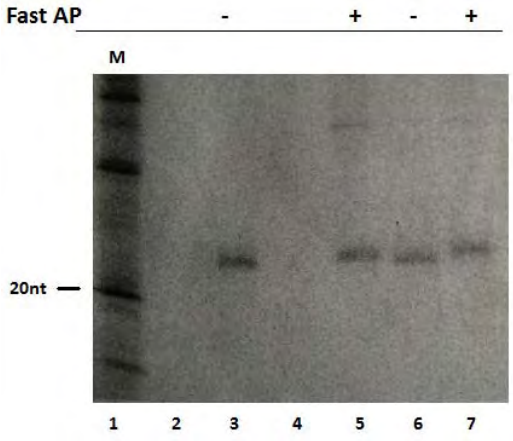


Fig. 15. Northern blot analysis of alkaline phosphatase (FastAP) treatment. 20 µg of total RNA from growing (Lane 3,5) and developing cells (Lane 6,7) was probed for DIRS-1 siRNA. RNA in lane 5 and 7 was treated with FastAP enzyme. Lanes 2 and 4 are empty. (15% polyacrylamide gel)

and compared to an untreated sample in lane 1 (Fig. 16). 15% acrylamide gel was used for better resolution of individual shifts in the gel. The membrane was first probed for DIRS-1 siRNA.

RNA in lane 2 was treated with Fast AP enzyme, as in the first reaction (Fig. 15), which should result in a shift in size, due to the loss of all the phosphates at the 5´end of the molecule. RNA in lane 3 was treated with FastAP and T4 Polynucleotide kinase (PNK) enzyme. PNK is an enzyme, which transfers γ-phosphate from ATP to the free 5´hydroxyl end of single- or double-stranded DNA or RNA molecules. This treatment results in molecules with one phosphate at the 5´end. DNA or RNA molecules treated first by FastAP and then by PNK enzyme should return back to the same position on Northern blot (as untreated sample in lane 1), in case the original molecule contains 5´-monophosphate. If the original molecule has three phosphates at the 5´end, the RNA is expected to end up in between the untreated and the FastAP treated RNAs (lanes 1 and 2, respectively). Already by looking at these lanes in fig. 16 it can be suggested that the DIRS-1 siRNA probably contains three phosphates on its 5´end, since the treated sample (lane 3) did not return to the same position as the untreated sample (lane 1). That indicates different number of phosphates between these molecules.

RNA in lane 5 was treated with Terminator 5´-Phosphate-Dependent Exonuclease (TE). TE is a processive 5´ to 3´ enzyme degrading RNAs, which have 5´-monophosphate. Hence, RNA with 5´-monophosphate should not be detected on Northern blot, but an RNA molecule with 5´-triphosphate should be visible. A weak

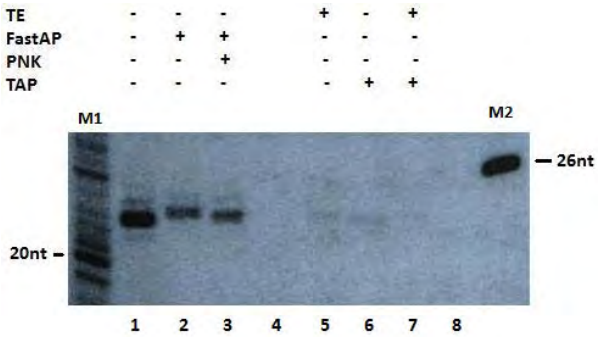


Fig. 16. Northern blot analysis of dephosphorylation assay. 10 µg (Lane 1-3) and 1µg (Lane 5-7) of enriched small RNA fraction was treated with different enzymes and probed for DIRS-1 siRNA. Lanes 4 and 8 are empty. *TE*, Terminal endonuclease, *FastAP*, alkaline phosphatase, *PNK*, polynucleotide kinase, *TAP*, Tobacco Acid Pyrophosphatase, *M1*, RNA marker, *M2*, DNA marker. (15% polyacrylamide gel)

signal in the lane 5 is visible suggesting that the siRNA might have a 5´-triphosphate. RNA in lane 6 was treated with Tobacco Acid Pyrophosphatase (TAP). TAP is an enzyme, which converts 5´-triphosphate into 5´-monophosphate bearing DNA or RNA molecules. In case of a molecule with 5´-triphosphate, we should see a shift in size, but if the treated molecule has just 5´-monophosphate, no size shift should be detected on Northern blot. The expected shift after TAP treatment could not be detected, which may indicate that the enzyme was not active under the experimental conditions (if our presumption from the previous treatments is correct). RNA in lane 7 was treated with TAP and then TE enzyme. After TAP treatment, all molecules regardless of their original 5´phosphorylation status should have 5´-monophosphate and when treated with TE, all should be degraded. This lane serves as a control for TE treatment and the RNA seems to be degraded. Since TAP and TE enzymes are quite expensive, only 1/10 amount of RNA was used for the last three reactions. The signals from lanes 5-7 are weak and therefore any conclusion can be drawn from the assay. Nevertheless, the results from this assay indicate that the siRNA has three phosphates at the 5´end (at least when comparing the shift in the lanes 1-3). However, the TAP treatment (lane 7) needs to be repeated and the incubation time of the whole assay should be increased as well.

The same membrane was also probed for siRNA derived from the second most abundant retrotransposon in *D. discoideum*, Skipper (Elchinger et al., 2005) and the predicted miRNA mi1198 (Hinas et al., 2007). However, even after a week of exposure, no signal could be detected.

Biogenesis of microRNAs and small interfering RNAs - 3´end analysis

Small RNAs of some species are modified at the 3´end on the 2´ hydroxyl group; methylation is the most common modification. Any modification of a hydroxyl group at the 3´end can be found by so called β-elimination assay. RNA molecule is sensitive to periodate and β-elimination treatment in case of no modification of the hydroxyl group at the 3´end. Samples without any modification undergoing this treatment will be shifted down on a gel by one base pair (Fig. 17). In case of modification of the hydroxyl group of RNA at the 3´end, no shift on the gel would be seen.

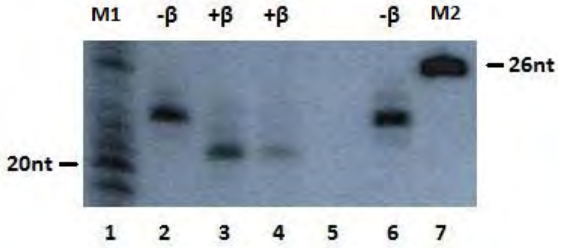


Fig. 17. Northern blot analysis of β-elimination assay. 10 μg of enriched small RNA fraction was treated by β-elimination assay according to two different protocols and loaded together with untreated RNA on 15% acrylamide gel. Treated samples (+β) in lanes 3 and 4 were compared to untreated ones (-β) in lanes 2 and 6. *M1,* RNA marker, *M2*, DNA marker.

10 μg of enriched small RNA were treated by β-elimination assay according to two different protocols (Akbergenov et al., 2006; Schoenberg, 2004) and compared to untreated samples (Fig. 17). The membrane was probed for the same siRNA as in fig. 15 and 16. A shift in size can be seen when comparing treated (lanes 3 and 4) and untreated (lane 2 and 6) RNA samples, indicating no modification of the 3´end of at least one of the siRNAs in *D. discoideum.*

The membrane was stripped and probed for miRNA mi1198 (Hinas et al., 2007), but no signal could be detected even after two weeks of exposure.

Discussion

Why study RNAi in Dictyostelium discoideum?

Small RNAs seem to have a huge influence on the gene expression in different organisms. More detailed study of small RNAs in *D. discoideum*, which gave the insight into the small silencing RNA issue in the amoebae, was performed quite recently (Hinas et al., 2007). Up to date, few siRNAs and putative miRNAs present in *D. discoideum* have been found and their biogenesis and function is being investigated.

D. discoideum has been an useful model organism already for many decades, but until quite recently it was not known that also small silencing RNAs can be studied in this amoebae. Since *D. discoideum* stands (from the evolutionary position) somewhere between plants and animals, it is very interesting to study the small RNAs in this organism, because it can give us a clue, how the evolution worked from plants to animals concerning the RNAi pathway (it differs in some points between these kingdoms). It might be easier to study these small RNAs in *D. discoideum*, since it grows in the form of sigle cells and just by applying starvation conditions can switch to multicellularity.

Post-transcriptional silencing in Dictyostelium discoideum

How miRNAs target mRNAs in *D.* discoideum is still an unaswered question. If a cleavage (mostly seen in plants) or translational silencing (predominantly in animals) of targeted mRNA happens, is still under investigation.

Since it was first thought that RNAi in *D. discoideum* might be more related to plants, I firstly started to investigate some of the predicted miRNA targets by 5´RACE method. Since no cleavage could be detected, our suspicion was that the targeted mRNAs are translationally silenced. Therefore it was decided to construct vectors with hairpin pri-miRNAs on one and with 3´UTRs on another plasmid. If translational silencing is how the mRNAs are regulated by the miRNAs, this method should provide positive results. The particular miRNA signals from the new strains with those miRNAs overexpressed seem to be much higher and could be detected already after one day of exposure (compared to almost two weeks in wt strain). This result was predicted, but was seen just in one of the newly constructed strains so far (AX2 mi2).

This approach looks promising, since the setup for cloning 3´UTRs to the other vector – pDM326 (Veltman, 2009a; Avesson, Reimegård and Söderbom, unpublished data) seems to be working as well. The timespan of the project did not allow me to proceed further with these experiments. Since there is no clear classification into plant and animal RNAi pathway, the posttranscriptional silencing in *D. discoideum* might be caused both by translational silencing and cleavage, even if the latter one has not been seen in any predicted target mRNAs investigated so far.

Localization studies

I have also been looking at the localization of the si- and miRNAs. Since mature mRNAs localize to the cytoplasm, we would expect the si- and miRNAs to be present also in the cytoplasm, if they associate with mRNAs. Another case would be, if the si- or miRNAs are involved in transcriptional silencing in the nucleus, causing formation of heterochromatin or other DNA rearrangements, even prior to transcription. From the data it seems that siRNAs (at least DIRS-1 siRNA) localize to the cytoplasm. This suggests that at least one

of the siRNAs associate with mRNA in the cytoplasm.

Biogenesis of small RNAs

The ends of small RNAs reflect the biogenesis of the molecules and in which pathway they are involved. Hence, it is very interesting to look at their composition. In general, small RNAs seem to have just one phosphate at their 5´ends, as a sign of processing by Dicer, but three phosphates have been seen as well, in *C. elegans* (Sijen et al., 2001). 5´end of the molecules can be studied by treatments with different enzymes and by comparing the shifts on a gel, the number of phosphates at the 5´end can be determined.

It is known that RNAs treated with alkaline phosphatase migrate approximately half a nucleotide slower than untreated (Sijen et al., 2007), but the exact number of phosphates cannot be judged by just this treatment. Therefore we also performed more extensive assay with different kinds of enzymatic treatments to be able to recognize between one and three phosphates. We succeeded in getting a good signal from DIRS-1 siRNA suggesting that it has three phosphates at the 5´end (Fig. 16). This indicates that at least one of the siRNAs in *D. discoideum* is not processed by Dicer (at least majority of DIRS-1 siRNAs seen on Northern blot). There might be still a minor population of siRNAs (known as primary siRNAs acting as an original trigger), which are processed by Dicer, but the majority of siRNAs (known as secondary siRNAs and serve as an additional small RNAs enhancing the signal to promote efficient silencing) are probably processed by RNA-dependent RNA polymerases (RdRPs). There are many different opinions on how the RdRPs actually work. There seems to be two different ways of secondary siRNA production, unprimed synthesis resulting in cleavage of long double-stranded RNA by Dicer (in plants) and primed synthesis (by primary siRNAs) resulting in either longer strand cut by a different endonuclease (since the secondary siRNAs have 5´-triphosphate) or synthesis of just short stretches of ~20nt, where cleavage is not necessary (in *C. elegans)* (reviewed in Sijen, T. et al., 2007). Since the DIRS-1 siRNA seems to contain three phosphates at the 5´end, it probably has the same machinery as *C. elegans*. From my experiments concerning the 5´end it seems that the silencing is more like in animals, because just siRNAs in *C. elegans* were seen to have three phosphates when synthesized by RdRPs (Sijen et al., 2001).

The particular DIRS-1 siRNA that was studied, was found to be downregulated in an rrpC$^-$ (RdRP knockout) and the levels were not affected in a drnB$^-$ (Dicer knockout) strain (Avesson and Söderbom, unpublished data). The miRNA mica1198 was on the other hand found upregulated in a rrpC$^-$ and downregulated in a drnB$^-$ strain (Hinas et al., 2007). There is a little bit inconsistence in the data though, another DIRS-1 siRNA, coming from a loop region of the DIRS-1 mRNA (Fig. 11 and Hinas et al., 2007), was found to have probably just one phosphate and the levels were without change in rrpC$^-$ and drnB$^-$ strains (Hinas et al., 2007). This indicates that the two siRNAs studied so far and derived from the same retrotransposon (Fig. 11), are probably generated by different pathways.

The composition of 3´ends differs. The 3´end was found to be 2´-O-methylated in some small RNAs in *Drosophila* and *Arabidopsis*) (Yang et al., 2006; Horwich et al., 2007; Pelisson et al., 2007). To investigate if the 3´ends of the small RNAs are modified in *D. discoideum*, so called β-elimination assay was performed. From the results it seems that siRNAs are not modified at their 3´ends, at least not the DIRS-1 siRNA as a shift on a gel was seen after the β-elimination treatment (Fig. 17).

This is also the case of animal small RNAs. Further investigation of both ends of small RNAs in *D. discoideum* is necessary to be able to understand the biogenesis of small RNAs in *D. discoideum* in detail.

The importance of this study

In conclusion, both biogenesis and function of small RNAs in *D. discoideum* seem to be more similar to animals than plants. That is supported by some results of this study. No cleavage of targeted mRNAs by miRNAs has been seen and the 5´ and 3´ends of at least one of the siRNAs in *D. discoideum* were found to correspond to animal siRNAs.

D. discoideum was found to be a good model to study small RNAs and RNAi pathway, because it is easy to construct knockouts and to isolate RNA from growing cells as well as from various stages of development. This is the first time most of these techniques have been performed in *D. discoideum* and during my stay in the laboratory I established them for use in this model organism.

Materials and Methods

Description of strains & plasmids

D.discoideum AX2 strain (Watts and Ashworth, 1970) was used for all the experiments.

Plasmids for cloning experiments were ordered from dictyBase (www.dictybase.org) : pDM304, pDM326 (Veltman et al., 2009a) and pDM310 (Veltman et al., 2009b).

Oligonucleotides used

See Table S1.

How to handle RNA

Gloves should be always worn, when handling with RNAs and RNase-free H_2O used for resuspention of RNA. Before loading on a gel, RNA should be always denaturated at 95°C for 5 min and then chilled on ice for 2-3 min. The concentration is measured on Nanodrop 3300 (Thermo Scientific) and the quality of RNA sample can be analyzed on an agarose gel, when loaded 500 ng.

Electrophoresis

For both DNA and RNA samples 0,8% (if not stated otherwise) RNase-free agarose (Invitrogen) was used diluted in 0.5x TBE. 5 µl EtBr/100ml was added before gel solidification.

Phenol extraction

The solution was scaled up to 200 µl with H_2O and mixed 1:1 with phenol (different ones used for DNA and RNA), vortexed 15 sec and centrifuged 5 min at 16000 x g at RT. Then upper phase was transferred to a new eppendorf tube, H_2O up to 200 µl was added and mixed 1:1 with chloroform. The mixture was vortexed for 15 sec, centrifuged for 5 min at 16000 x g at RT and the upper phase transferred to a new eppendorf tube.

Ethanol precipitation

RNA solution was mixed with 0.1 vol. 3 M NaOAc pH 5.2 and 3 vol. ice-cold 99% EtOH, 1 µl of glycogen (Ambion, 5µg/µl) was added when handling with small amounts of RNA and incubated at -20°C overnight. The mixture was then centrifuged for 30 min at 16000 x g, supernatant was removed and pellet was washed 2x with 70% EtOH. In between it was centrifuged for 5 min at 16000 x g at RT. Supernatant was carefully removed, the pellet was air dried at RT and resuspended in 10-100 µl of H_2O. Concentration was measured on NanoDrop 3300 (Thermo Scientific).

Solutions used

Church buffer : 0,5 M $NaPO_4$ pH 7.2, 7% SDS, 1 mM EDTA, 10g/l BSA

Healing solution : 100 mM $CaCl_2$, 100 mM $MgCl_2$

1L HL5 media : 10 g peptic peptone, 5 g yeast extract, 2.5 mM Na_2HPO_4, 2.5 mM KH_2PO_4, adjusted to pH 6.4 with orto-phosphoric acid; 0.4% glucose added after autoclaving

1L LB media : 10 g tryptone, 5 g yeast extract, 0.125 M NaCl, (20 g agar)

1ml 2x loading dye : 916 µl formamide, 34 µl 0.5 M EDTA, 25 µl 1% bromophenol blue, 25 µl 1% xylene cyanol

1ml 6x loading dye : 650 µl H_2O, 300 µl glycerol, 25 µl 1% bromophenol blue, 25 µl 1% xylene cyanol

Lysis Buffer : 50 mM KCl, 10 mM Tris pH 8.3, 2.5 mM $MgCl_2$, 0.45% NP40, 0.45% Tween 20

Nuclei buffer : 40 mM Tris-Cl pH 7.8, 1.5% sucrose, 0.1 mM EDTA pH 8.0, 6 mM $MgCl_2$, 50 mM KCl, 5 mM DTT and 0.4% NP-40, sterile filter

PDF buffer : 20 mM KCl, 5 mM $MgCl_2$, 20 mM KPO_4, pH 6.2, sterile filter

PEG/NaCl precipitation solution : 20% PEG 8000, 2 M NaCl

1L Polyacrylamide (PAA) gel : 25% acrylamide : 420 g urea, 100 ml 10xTEB and 0.75 l 40% acrylamide, sterile filter, degased and diluted in urea (7 M urea in 1x TEB)

20% SDS : 200 g/l, pH 7.2

20x SSC : 3 M NaCl, 0.34 M NaCitrate, pH 7.0, autoclaved

1L of 5xTBE : 54 g Tris base, 27.5 g Boric acid, 20 ml 0.5M EDTA, pH 8

ZAP buffer : 10 mM Na_2HPO_4, 50 mM sucrose, sterile filter

Total RNA preparations

Approximately 10^8 cells ($1\text{-}3\times10^6$ cells/ml) were centrifuged at 300 x g at 4°C for 5 min in swing-out rotor. Then they were resuspended in cold PDF buffer (the same amount as the media was) and centrifuged as above. Cells were resuspended in 1 ml Trizol reagent (Invitrogen), vortexed and incubated at room temperature (RT) for 5 min. 200 µl of chloroform was then added, vortexed and incubated at RT for 3 min and subsequently centrifuged for 15 min at 16000 x g. The upper (aqueous) phase was trasferred to a new fresh tube and 500 µl of isopropanol was added followed by incubation for 10 min at RT and centrifugation for 10 min at 16000 x g. Washing of the pellet was done twice, each time with 1 ml of 70% ethanol and centrifuged for 5 min at 16000 x g. Then ethanol was removed, the pellet was air dried briefly and dissolved in 10-100 µl of RNase-free H_2O (depending on the expected concentration).

Northern blots

10-12% polyacrylamide (PAA) gel mixed with 1% APS and 0.1% TEMED was prepared for usual Northern blot (15% when needed for a better resolution). The gel was polymerized for an hour and then prerun at 20-22 W for about another hour. 20 µg total RNA/lane was mixed 1:1 with 2x loading dye. 0.2 µg end-labeled pUC19/MspI (Fermentas) and 0.025 µg end-labeled Decade marker (Applied Biosystems) was used as a DNA ladder and RNA marker, respectively (end labeling described below). All RNA samples (together with the RNA marker) were denatured for 5 min at 95°C, then chilled on ice. The gel was run until the lower tracking dye was at the bottom of the gel (approx. 1,5 h) and the gel was then trimmed if necessary. Two Whatman papers (cut to fit the gel and soaked in 1xTEB) were put on the top of the gel and a Hybond N+ membrane (Amersham, GE Healthcare), soaked in 1xTEB, was put on the other side of the gel. Two additional Whatman papers were put on the membrane. Everything was placed in between two pads in the blotting device (BioRad TransBlot Cell) and electrobloted in 1xTEB at 20 V for 16 h (overnight) at 4°C.

The membrane was UV crosslinked (UVC500 Crosslinker, GE Healthcare, 150 kJ) the following day. The oligonucleotide used as a probe for the hybridization was labeled (8 pmol of the oligonucleotide, 50 µCi γ-ATP, 2 µl buffer A (Fermentas), 1 µl T4 PNK (Fermentas, 10U/µl) and add H_2O up to 20 µl, incubate 30 min at 37°C and followed by removal of unincorporated nucleotides on G-50 column (illustra ProbeQuant G-50 MicroColumns, GE Healthcare). The membrane was prehybridized in a hybridization tube with 20-30 ml of Church buffer in a

hybridization oven at 42°C for 1 h. The labeled probe was denatured for 5 min at 95°C and then chilled on ice. After one hour, 20-30 ml of fresh prewarmed Church buffer was poured into the hybridization tube with the membrane, the labeled probe was added and the membrane hybridized at 42°C overnight.

The following day the membrane was subsequentially washed with 20-30 ml prewarmed (42°C) washing solutions at 42°C : rinsed briefly with 2xSSC/0.1% SDS buffer, washed 2x 5 min with 2xSSC/0.1% SDS buffer, 2x 10 min with 1xSSC/0.1% SDS buffer and 2x 5 min with 0.5xSSC/0.1% SDS buffer, then placed into a plastic hybridization bag, sealed, and exposed for few hours to two weeks (depending on the intensity of the signal) before analysing the result with PhosphoImager 400S (Molecular Dynamics).

Note : In case of reprobing, the membrane was stripped by boiling in 0.1xSSC/0.1% SDS 2x 45 min before hybridization with another probe.

Labeling of pUC19/MspI marker : 2 µl pUC19/MspI (Fermentas, 1µg/µl), 2 µl 10x buffer A (Fermentas), 1 µl T4 PNK (Fermentas, 10U/µl), 10 µCi γ-ATP and 15 µl H_2O was mixed and incubate at 37°C for 30-60 min. The probe was purified with G-50 column (GE), 1:1 of 2x loading dye was added and the probe was stored at -20°C.

Labeling of RNA decade marker : 1 µl Decade Marker (Applied Biosystems), 10 µCi γ-ATP, 1 µl 10x T4 PNK Reaction Buffer (Ambion), 1 µl T4 PNK (Ambion) and 6 µl H_2O was mixed together and incubated for at 37°C 30-60 min. Then it was mixed with 8 µl H_2O and 2 µl 10x cleavage reaction, incubated 5 min at room temperature (RT) and finally 20 µl 2x loading dye was added.

5´RACE

1 µg of mRNA was treated 2x with DNase (5 µg of mRNA, 10 µl DNase I buffer, H_2O up to 95 µl and 5 µl DNase (50u) was mixed and incubated at 37°C for 10 min). The solution was then phenol extracted and ethanol precipitated. The pellet was then mixed with 5 µl 10x TAP buffer (Epicentre), 2.5 µl TAP (Epicentre, 10u/µl), 1 µl RNase inhibitor and 41.5 µl H_2O. The mixture was incubated for 60 min at 37°C followed by phenol extraction and ethanol precipitated. The pellet was resuspended in 10 µl of H_2O and 1µl of denaturated GeneRacer RNA oligo (500 pmol), 5 µl 10x T4 RNA ligase buffer (BioLabs), 5 µl DMSO, 2.5 µl T4 RNA ligase 1 (BioLabs, 20u/µl), 1 µl RiboLock RNase Inhibitor (Fermentas, 40u/µl) and 25.5 µl of H_2O was added. The ligation mixture was then incubated for 12 h at 17°C and stopped by phenol extraction and ethanol precipitation. The pellet was dissolved in 15 µl of H_2O and 5 µl of the RNA was taken for the following reactions.

The RNA was mixed with 1 µl of gene specific primer (see Appendix; 20 pmol), 2 µl of 10 mM dNTP mix (10 mM each of dATP, dGTP, dCTP and dTTP) and 4 µl of H_2O, incubated at 65°C for 5 min and placed on ice. After addition of 4 µl 5x cDNA synthesis buffer (Invitrogen), 1 µl 0.1 M DTT, 1 µl Thermoscript RTase (GibcoBRL, 10u/µl), and 1 µl RiboLock RNase Inhibitor (Fermentas, 40u/µl), l µl H_2O, the mixture was incubated for 20 min at 55°C, 20 min at 60°C, 20 min at 65°C and 5 min at 85°C, then chilled on ice and spun down. Then 1 µl of RNase H (Fermentas, 5u/µl) was added and mixture incubated at 37°C for 20 min. 1 µl was taken as a template for further reactions : mixed with 2.5 µl 10x PCR buffer, 2.5 µl 25 mM $MgCl_2$, 10 mM dNTPs, 0.5 µl 5´specific primer (see Appendix; 10 pmol), 0.5 µl 3´specific primer (see Appendix; 10

pmol), 0.125 µl AmpliTaq Gold (hotstart) (Applied Biosystems, 5u/µl) and 17.875 µl of H_2O. Cycling conditions were : 95°C for 9 min; 5 cycles of 95°C for 30 sec, 60-55°C for 40 sec, 72°C for 40 sec; 35 cycles of 95°C for 30 sec, 55°C for 40 sec, 72°C for 40 sec, followed by 72°C for 10 min. 5 µl of the PCR product was run on an 0.8% agarose gel. Nested PCR was performed with 1 µl of this PCR product, the solution was mixed in the same way, but with different primers. Cycling conditions were 95°C for 9 min; 20 cycles of 94°C for 30 sec, 54°C for 40 sec, 72°C for 1 min; followed by 72°C for 10 min. 5 µl of the product was run on 0.8% agarose gel.

Cloning

Template for cloning was prepared by quick DNA extraction : 25µl cells from growing culture was mixed with 25µl Lysis Buffer and 1µl Proteinase K (20µg/µl). Proteinase K was then inactivated for 10min at 95°C.

Four different predicted miRNA precursors (pre-miRNA; mipolB, mi1, mi2, mi1129) were amplified by PCR : 25µl of 2xPCR mix (Fermentas), 1µl forward primer, 1µl reverse primer, 1µl template, 22µl H_2O. Cycling conditions were : 95°C for 5min; 30cycles of 95°C for 30sec, 50°C for 30sec, 60°C for 1min, 60°C for 10min. 5µl of PCR products was run on 1,2% agarose gel and the rest digested with BglII and SpeI restriction enzymes. pDM304 and pDM310 plasmids were digested in the same way, but additionally treated with Fast AP (Fermentas, 1u/µl) to prevent self-ligation and purified from agarose gel (Gene JET Gel Extraction Kit, Fermentas, #K0692). Then PCR products were ligated into the vectors in a total volume 20µl (100ng vector DNA, 5:1 molar ratio of insert DNA over the vector, 2µl 10xT4 Ligase buffer (Fermentas), 1µl T4 DNA ligase (Fermentas, 5u/µl) at 22°C for 10min. Ligation was inactivated at 65°C for 10min and 5µl of the mixture was used for transformation.

Heat-shock transformation of bacteria

Frozen 50µl DH5α competent E.coli cells were thawed on ice and 0.1µg plasmid DNA was added. Tubes were placed on ice for 5min, then heat-shocked at 42 °C for 30s and return to ice again for 2min. 10µl was mixed with 300µl LB media together with appropriate antibiotics and spread evenly on an Ampicilin (Amp) plate. The rest of the mixture was processed in the same way and spread on another plate. The plates were incubated at 37°C overnight. Plasmids were isolated from bacteria (Gene JET plasmid Miniprep kit, Fermentas, #K0503) and checked by digestion.

Transformation of Dictyostelium discoideum (electroporation)

First, competent cells were prepared. $2x10^7$ cells per transformation was taken, chilled on ice for 15min and spun down at 4°C for 4min at 300xg. Supernatant was removed and the pellet resuspended in 10ml ice-cold PDF buffer. The cells were then pelleted in the same way again and resuspended in 10ml ice-cold ZAP buffer. After third centrifugation, the cells were resuspended in 1ml ice-cold ZAP buffer and kept on ice.

5-10µg plasmid was gently mixed with 700µl competent cells in a cold sterile cuvette and placed on ice. Electroporator (Gene Pulser, Bio-Rad) was set at 3mF, 1KV (2,5KV/cm), the cells were zapped, transferred to a Petri dish on top of 8µl of healing solution, mixed by swirling and incubated at RT for 15min. Then 10 ml HL5 media with Penicillin and Streptomycin (10000 µg/ml Penicillin, 10000 µg/ml Streptomycin; Invitrogen) was added to the plate and cells were mixed by swirling again. Cells were let to recover at RT overnight.

After 24h the media was changed to HL5 + 10µg/µl Geneticin G418 Sulphate (Invitrogen) and exchanged for fresh media after each third day until the plate was covered with cells. Then the cells were transferred into a shaking culture and total RNA was prepared from them.

Nuclear RNA preparations

About 10^8 cells was pelleted at 4°C for 5min at 300xg. The supernatant was discarded, cells gently resuspended on ice in 1ml ice-cold nuclei buffer by swirling and transferred to an eppendorf tube. The cells were lysed for 3-6min and nuclei were pelleted at 4°C for 5min at 500xg. The supernatant was carefully removed, the pellet gently resuspended in 1ml nuclei buffer and incubated for 2-3min on ice. The nuclei were centrifuged at 4°C for 5min at 500xg and after careful removal of the supernatant, the pellet was resuspended in 1ml Trizol (Invitrogen) and then total RNA was prepared. Resulting RNA samples were loaded on an agarose gel, together with total RNA preparation to check for the purity of the separation. According to calculations done previously (Hinas, A., unpublished data), 3.7µg of nuclear and 20µg of total RNA preparations were loaded on a Northern blot.

Enrichment for the small RNAs

RNA (up to 120 µg of total RNA) was dissolved in 200 µl H_2O and 200 µl of PEG/NaCl precipitation solution was added, mixed and kept on ice for at least 30 min. The mixture was centrifuged at 16000 x g for 15 min and the supernatant transferred to a new tube (as small RNA fraction). The pellet was diluted in 15 µl H_2O (as large RNA fraction). The small RNA fraction was precipitated by addition of 2.5 volumes of cold 99% ethanol (EtOH). The solution was mixed and incubated at -20°C overnight followed by centrifugation at 16000 x g for 15 min, washed twice with 70% EtOH (with centrifugation in between). The pellet was air dried and disolved in 10 µl H_2O.

Dephosphorylation assay

One ´starter´ tube was made with 70 µg small RNAs (~33pmol/µl) and 0.5 pmol of OmrA RNA (a generous gift from E.Holmqvist) and 0.5 pmol AS DdR21 RNA (a generous gift from L.Avesson) were added as a control with 1P and 3P, respectively. Six different reactions were made :

1) *Control tube :* 10 µl from the „starter“ tube was phenol extracted and ethanol precipitated.

2) *Treatment with Terminator Exonuclease :* To 1 µl from the ´starter´ tube was added 2 µl 10x Terminator reaction buffer (Epicentre), 0.5 µl RiboLock RNase Inhibitor (Fermentas, 40u/µl), 2 µl Terminator Exonuclease (Epicentre, 1u/µl) and 14.5 µl H_2O, mixed thoroughly and centrifuged briefly and the mixture was incubated for 30 min at 37°C. Then the mixture was centrifuged down, 180 µl of H_2O was added and it was phenol extracted and ethanol precipitated.

3) *Treatment with Alkaline phosphatase (FastAP) :* 10 µl from the ´starter´ tube was mixed thoroughly with 10 µl 10x FastAP buffer (Fermentas), 10 µl FastAP (Fermentas, 1u/µl), 0.5 µl RiboLock RNase Inhibitor (Fermentas, 40u/µl) and 69.5 µl H_2O, centrifuged briefly and incubated for 30 min at 37°C. The reaction was then heated for 5 min at 75°C and spun down again. After that 100 µl of H_2O was added and RNA was phenol extracted and ethanol precipitated.

4) *Treatment with FastAP and Polynucleotide kinase (PNK) :* 10 µl RNA from ´starter´ tube was firstly treated in the same way as in the reaction before (3), but the RNA pellet from precipitation was resuspended in 10 µl of RNase-free H_2O and mixed with 2.5 µl of 10mM ATP, 5 µl

10x Reaction buffer A (Fermentas), 5 µl T4 PNK (Fermentas. 30u/µl), 0.5 µl RiboLock RNase Inhibitor (Fermentas, 40u/µl) and 27 µl of H_2O. The reaction was incubated for 30 min at 37°C, 2.5 µl of 0.5M EDTA pH 8.0 was added to stop the reaction and the solution was ethanol precipitated.

5) *Treatment with Tobacco Acid Pyrophosphatase (TAP) :* 1 µl RNA from the ´starter´ tube was mixed with 5 µl 10x TAP reaction buffer (Epicentre), 2 µl TAP (Epicentre, 10u/µl), 0.5 µl RiboLock RNase Inhibitor (Fermentas, 40u/µl) and 41.5 µl H_2O, incubated 2 h at 37°C and centrifuged down. 150 µl H_2O was added and the solution was phenol extracted and ethanol precipitated.

6) *Treatment with TAP and Terminator Exonuclease:* 1 µl RNA from the ´starter´ tube was mixed with 5 µl 10x TAP reaction buffer (Epicentre), 2 µl TAP (Epicentre, 10u/µl), 0.5 µl RiboGuard and 41.5 µl H_2O, incubated 2 h at 37°C, centrifuged down, added 150 µl H_2O, phenol extracted and ethanol precipitated. RNA was resuspended in 10 µl of H_2O and 2 µl 10x Terminator reaction buffer (Epicentre), 2 µl Terminator Exonuclease (Epicentre, 1u/µl), 0.5 µl RiboLock RNase Inhibitor (Fermentas, 40u/µl) and 5.5 µl of H_2O was added. The mixture was incubated for 30 min at 37°C, 1 µl of 100 mM EDTA pH 8.0 was added and the solution was ethanol precipitated.

All the samples were loaded on a 15% acrylamide gel.

β-elimination assay

Samples for the β-elimination assay were taken from a ´starter´ tube with 40 µg (1µg/µl) of enriched small RNA. The ´starter´ tube was divided into 4 different reactions. One of the samples was loaded on a gel directly without any treatment, the second one was just precipitated and the two remaining samples (each 10 µg) were used for the β-elimination assays :

1) 10 µg of enriched RNA fraction was dissolved in 13.5 µl H_2O. Then 4 µl 5x 300 mM borax/boric acid buffer pH 8.6 (150 mM borax (a generous gift from ICM/Micro department, BMC), 150 mM boric acid) and 2.5 µl 200 mM $NaIO_4$ (a generous gift from Bioorganic Chemistry department, BMC) was added. The mixture was incubated for 10 min at RT in dark, then 2 µl of glycerol was added to quench unreacted $NaIO_4$ and the solution was dried under vacuum. The pellet was dissolved in 50 µl 1x borax/boric acid buffer pH 9.5 (5x borax/boric acid buffer pH 8.6 was diluted in H_2O and pH adjusted with 10 M NaOH) and solution was incubated 90 min at 45°C. After that 3 µl 5 M NaCl, 150 µl 99% EtOH and 1 µl glycogen (Ambion, 5µg/µl) was added and the reaction was incubated on ice for 2 h. The pellet was collected by centrifugation (16000 x g for 30 min), washed with 80% EtOH twice, air dried and dissolved in 10 µl H_2O. 10 µl 2x loading dye was added and the sample was run on a Northern blot (Schoenberg, 2004).

2) 10 µg of enriched RNA fraction was dissolved in 17.5 µl borax buffer pH 8.6 (4.375 mM borax, 50 mM boric acid) and 2.5 µl 0.2 M $NaIO_4$. The mixture was incubated for 10 min at RT in dark and after addition of 2 µl glycerol incubated another 10 min at the same conditions. The mixture was dried under vacuum, dissolved in 50 µl borax buffer pH 9.5 (33.75 mM borax, 50 mM boric acid, pH adjusted by 10 M NaOH) and the solution was incubated for 90 min at 45°C. Then 150 µl 99% ice-cold EtOH, 5 µl 3 M NaAc pH 5.2 and 1 µl glycogen was added and precipitated overnight at -20°C. The following day the pellet was collected by centrifugation (30 min at 16000 x g), washed twice in 70% EtOH and dissolved in 10 µl H_2O. 10 µl 2x loading dye was added before loading on a Northern blot (Akbergenov et al., 2006).

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Appendix

Table S1. Oligonucleotides (in 5´ to 3´ order) used as probes for Northern blots (NB) and as primers for PCR reactions (PCR) and 5´RACE.

Oligo name	Target/description	Comment	Sequence
17	cDNA from Gene Racer oligo	5´RACE	CGACTGGAGCACGAGGACACTGA
102	U6 RNA probe	NB	GGATGCCTGCCGGTTGCCCGGAGG
176	Skipper small RNA, loop	NB	AGCTAAAACCATTGATGCTTT
311	mi1	NB	GCTTTCCTTGATAAAAATTGG
312	mica1198	NB	GAACCATTAAACCCTAACTGG
356	mica1198, Nested 5'-RACE	5´RACE	GACACTGACATGGACTGAAGGAGTA
465	siRNA DIRS-1	NB	GGCCATTACTCCCACTACTGG
626	mipolB, internal forward primer	5´RACE	GGCAAAATTACATATCCATTATCA
627	mipolB, nested 5´RACE primer	5´RACE	GAAGATATTGGATGTTTATAACCTCT
628	mipolB	5´RACE	GGAATTGCATCATGGTCCATTGGA
629	mipolB, internal reverse primer	5´RACE	GCTAAACATTTTACAGAGGTTGGT
632	med12, RT primer/5'RACE	5´RACE	CAATGGTAAGTCTGTTGATGGTT
633	med12, 5'RACE nested	5´RACE	CTTTCCCAACCTCTATTCTCTTC
635	med12, internal forward primer	5´RACE	ATTGCAACTTGGTTTAGGTCCA
636	med12, nested primer	5´RACE	CTTGATAATGGATATGTAATTTTGCCA
639	mipolB forward,	PCR	ctaAgatctCATGTGAGAATCTACCATTGGT
640	mipolB reverse	PCR	ctaActagtATATGAGAATCTAGTATTGATG

641	mi1 forward	PCR	ctaAgatctGTTAATGACGATTTTCAAGTGA
642	mi1 reverse	PCR	ctaActagtGCCTTTCGTCAATCAAGAAATT
643	mi2 forward	PCR	ctaAgatctGGAATGGTTACAAATCTTATAA
644	mi2 reverse	PCR	ctaActagtGTAACCATAATGTAGTCATCAA
645	mi1129 forward	PCR	ctaAgatctCTAGATTAAAAGGAGAAATTC A
646	mi1129 reverse	PCR	ctaActagtCAAATTTGTCCGAGTCAATAT
Gene Racer RNA oligo		5´RACE	CGACUGGAGCACGAGGACACUGACAU GGACUGAAGGAGUAGAAA
AH-$tRNA^{Arg}$	$tRNA^{Arg}$	NB	TACCGGTTCGTAGCCGGTC
291	DdR21 AS	NB	GTTGACCTTACAGCAAACCC
Eho- 406	OmrA	NB	CCACTCTAATAAGCCATGCGAGAAGCAT GGGACAGAG

Figure S1. One of the putative miRNAs, PolB miRNA (mi*polB*). The sequence of PolB pri-miRNA and its structure. Own pri-miRNA sequence is indicated in green, primers for this sequence in yellow. 300nt upstream and downstream of this pri-miRNA sequence are shown as well.

UUUAAUGAGAUUGAACAAAAAGUACCAAGAAAUGAAAUUGAACAAUUUGAAG
AAUUAGUUAGACAAGCAUUAUUAAAAAUUGAUAAAAAAAAUUAUUUAUGAAAC
AUGUGGUAGUUAGUAUGUAUUUUCUUUUUCUUUUUUUUUUUUUUUUUUUUU
UUUUGAAAUAAUUUAUUAUUACUAUAAUCUCAAAUAAUAAUUAAUAAUAAUA
AUAAUUAAUAAUAAUAAAUUAUAAAUAACAUGUGAGAAUCUACCAUUGGUGU
UUAUUUUAUUUAAUUCAUCUUUAUUAUUAUUGUUGAACUCUUGUCUUAAUUC
CUUGAUAGCUUUAUAGCUCGUAGCAUUUUCUUCGUCAUUUGUUUAUUGGAUA
UUCCUAAAGAUGAAUUAUCAAGAAAAUGUUACGAACUAUAAAUCUAUCAAAG
AAGUAAGAUAAGAGUUCAACAAUCUCAUUAGAGAUGAAUUAAAUAAAAUAAA
CAUCAAUACUAGAUUCUCAUAUAAUAAUAAUAAUAAUAAUAAUAAUAAUAAU
AAUAAUAAUAAUAAUAAUAAUAAUAAUAAUAAUAAUAAUAAUAAUAAUAAUA
AUAAUAAUAAUAAUAAUAAAUUCCAGGUAAAUAAAUAACUGAUAUUUGU

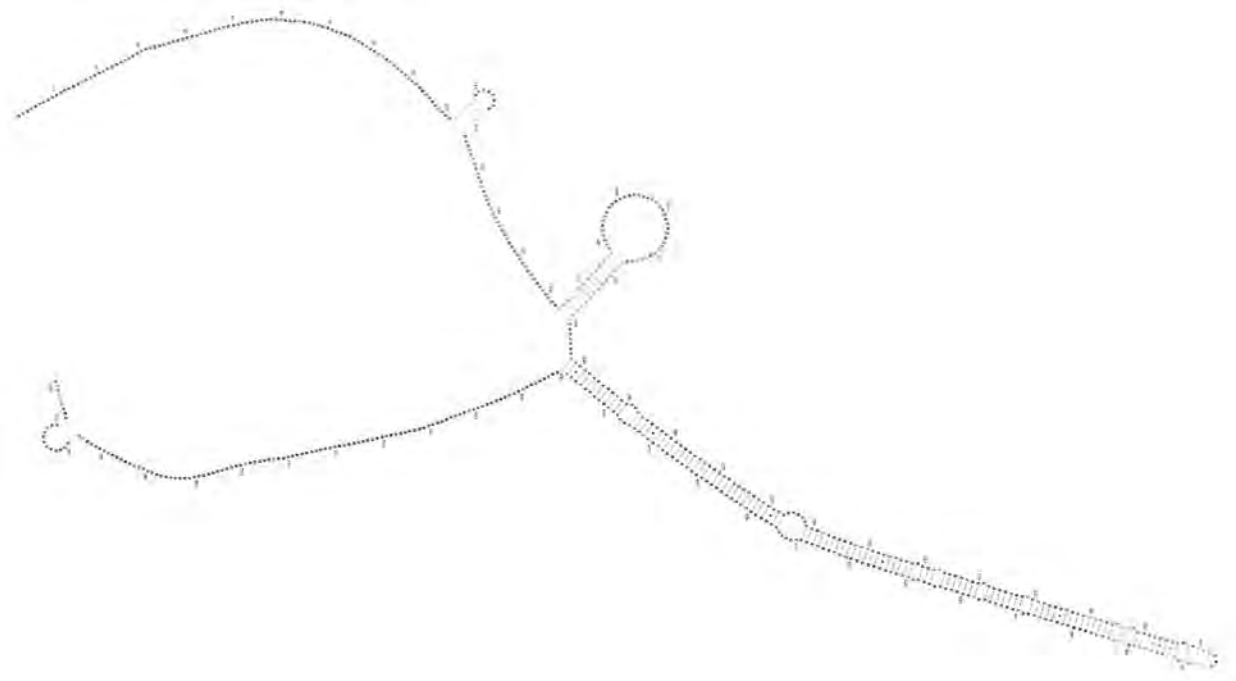

Figure S2. One of the putative miRNAs, miRNA 1 (mi*1*). The sequence of pri-miRNA 1 and its structure. Own pri-miRNA sequence is indicated in green, primers for this sequence in yellow. 300nt upstream and downstream of this pri-miRNA sequence are shown as well.

UUUUUUUUUUUUUUUUUUGCGACACAAUAAGUUUUCAUUUUAAAAAAUAAA
UAAAAAAAAAUAUUUUUAAAUAUCUAAAGAUUCUAAUCUCUUUUAUUAGUUUU
UUAAAUUUUAUUAGAUAAACUAACACCAAUGGUCAAACAAAAUGUUUUUGCU
GAAAUUAAUAAUGGUUUUUUCAAACUCAUUCAGUUUUAACUUCGAAUGGUUU
AAUCUAUGGCGCAAAGUUUAGAAAUGUUAAUGACGAUUUUCAAGUGAAACAG
GAAUUUGAAAAAAACCACUUCCCAAAGUCGUAUCAGGUGGCCAAUUUUUAUCA
AGGAAAGCUGUAUCAUCAAGUGCCUCCCUCUGUCUCUUGAUGAUUCAGCCUUC
CUUGACAAAAAUUGCCCACCUGAUACGACUGGGAAGUUGGUUUUUCAAAUUCA
AUCUGUAGAACAAUUUCUUGAUUGACGAAAGGCAUAUAUUAUAUAUUCACCCA
AUAUUUUGAUUCUUCUCUGAUCGAGUGAAUAUAUAAUAUAUAAAUUUUAAAA
UAAAAAAUAUACUCACCCAUCAAACCAAUUUUUUUAUUUUUUUUUUUUUUUU
UUUUCAUAUCUAUUUUUUUUAUUAUAAAUGUCAUUUUUUGUAUUU

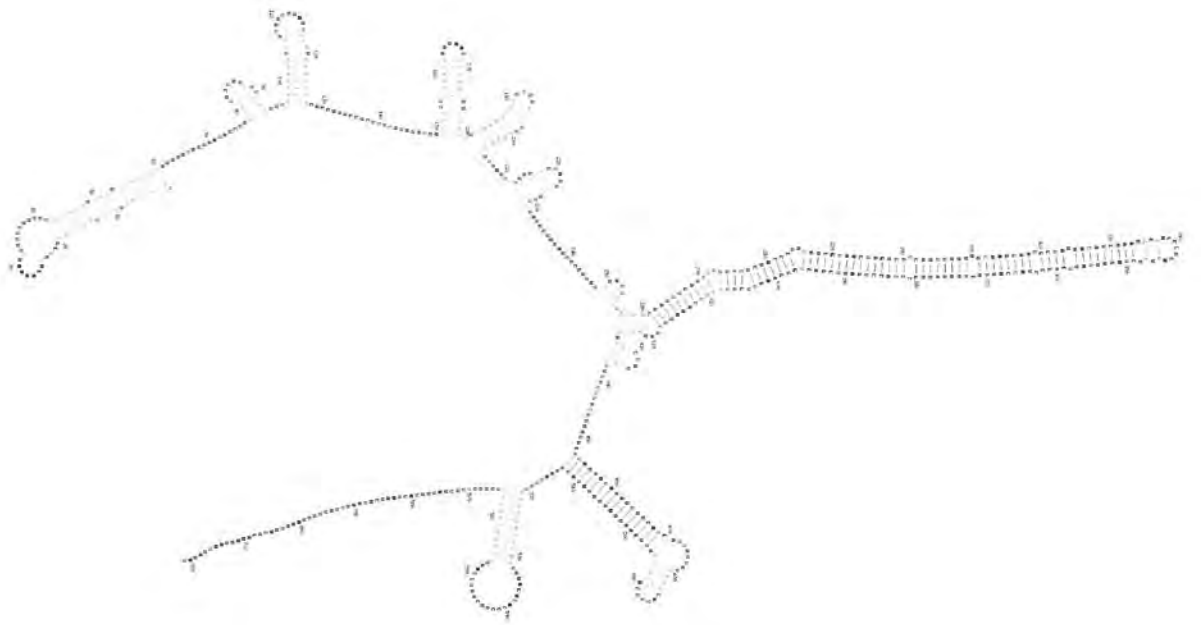

Figure S3. One of the putative miRNAs, miRNA 2 (mi*2*). The sequence of pri-miRNA 2 and its structure. Own pri-miRNA sequence is indicated in green, primers for this sequence in yellow. 300nt upstream and downstream of this pri-miRNA sequence are shown as well.

UUUUAUUUUUUUUUUUUUAUUUUUUAUUUUUUUUUUAUCUUUUUUUUUUUUUGU
UUUUACAUUUUUUUUUUUUUAUUUUAUUUUUUUUUAAUUUUUUUUUUUUUUUUU
UUUUUUUUUUUUAAUUUUUUUUUUAAUUUUUUUAAUUUUUUUUUUAAUUUUUUUUU
AAUUUUUUUUUUUUUUUUUUUUUUUUUUUGGAAUGGUUACAAAUCUUAUAAAU
AGCUCAUUGGAAUUCAAAAAAAAUAAAAAAAAAAUAAAAAUAAAAAAACUAAA
AAUAGUAGUUAUUCAAUUUGUUAUCACCAAAUAAACUAAACCAGUUAGGGUU
UAAUGGUUCUAUCUCAAUUGUUUUUUUUGGAUCCCAAAUUUAAUCUAUUUU
AAUUUAAAAAAUGAUUAUUUGUUAUCCAGGAAAAAUAACUGGAAAAGAACCG
UUGAGCCCUUUCUGAUUUAUUAAUUAUUUGAUGACUACAUUAUGGUUACUAU
UAUUUUUAAUAUUAUUUAUUAUCUCC

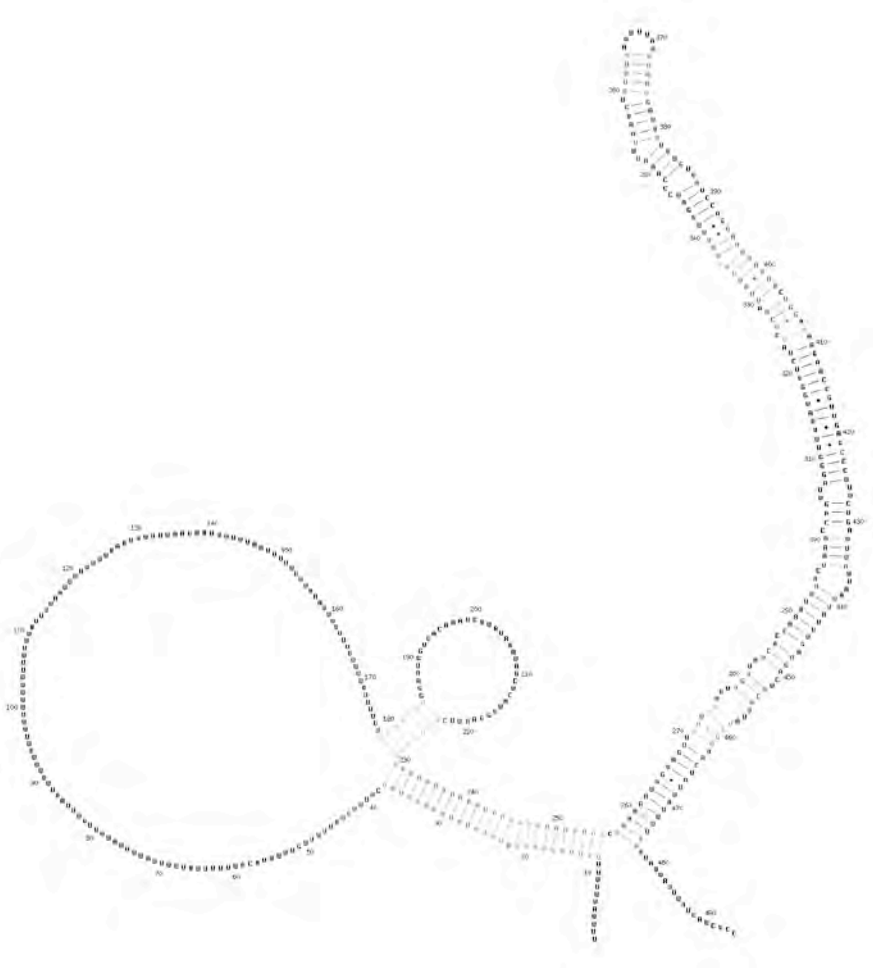

Figure S4. One of the putative miRNAs, miRNA mi1129 (mi*1129*). The sequence of pri-miRNA mi1129 and its structure. Own pri-miRNA sequence is indicated in green, primers for this sequence in yellow. 300nt upstream and downstream of this pri-miRNA sequence are shown as well.

AAAAAGGUAAUUUUUUUUUUUUUUUUUUUUUUUAAUAAUUGUAUAAUAAUUA
AAUUUUAAUGUUUUUAAAUUUAUUAUUAAAGUGAAGUAGAAAGAAUUAAUUC
UGAAAAGGAAAAAAAAAAAAAAAAAAAAUCAAAUCUAGAUUAAAAGGAGAAAU
UCAAAGAUAUAUUUUAGAAACUCAAAUAUUCAUUUUUUUUCCAUUUUUAACA
GAUACAAAACAAAAUAAUAGUAGAGGUUUAAUUAAACCUAAAAUUCAGAAUA
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AUUGGAUAAAAAAAUGAUUUUUUUUAGUUUGUAAAAUAUAUUUUGAUAUUGAC
UCGGACAAAUUUGUCAUUUUUGGUAUCUUUUUUUUCUUUUUUUUUUUUUUUGAU
UUUCAAAAAUAAAAAAAAAUAAAAUAAUCAAUAUUGAUUUAUUAUAAUAAAUA
AUGAAAAAAAAAAAGAAUUAAUUCUAUUUUUAACCCCUUUUUUUUUUUAUCAUU
UUUUUUUAUUUGUUAUUAAUUUAUGAUUUUUUUUUAUUUUUUUUUUAAUUGUUU
UUCUAAACAAAUUUUUUUUAUAAAUUGUUUCCCAUUUUGAGUGCACUAC