Deciphering the role of hostplant miRNAs in the regulation of herbivore gene expression

A Thesis

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by

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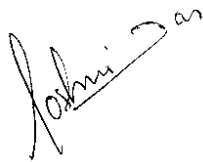
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April, 2020

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Certificate

This is to certify that this dissertation entitled **Deciphering the role of hostplant miRNAs in the regulation of herbivore gene expression** towards the partial fulfilment of the BS-MS dual degree program at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Roshni Das at Indian Institute of Science Education and Research under the supervision of Dr. Sagar Pandit, Assistant Professor, Department of Biology, during the academic year 2019-2020.
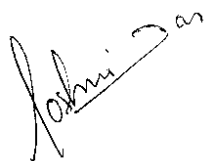
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Declaration

I hereby declare that the matter embodied in the report entitled **Deciphering the role of hostplant miRNAs in the regulation of herbivore gene expression** are the results of the work carried out by me at the Department of Biology, Indian Institute of Science Education and Research, Pune, under the supervision of Dr. Sagar Pandit and the same has not been submitted elsewhere for any other degree

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Table of Contents

List of Tables

List of Figures

Abstract

Plants and their insect herbivores have coexisted for hundreds of millions of years. Insects depend on plants as their primary source of nutrition. This dependence harms the plants immensely and plants produce various defense responses to deter the herbivores in the form of physical barriers and toxic chemicals. Insects, on the other hand, have evolved to counter these defense strategies like detoxification of the chemicals, avoidance, sequestration, etc. It has been shown that along with the various chemicals, plants also possess various sRNAs many of which also get upregulated following herbivory. Although the chemical responses have been studied extensively, the role of naturally occurring sRNAs has not been studied in the context of plant-insect interaction. This study focuses on the role of natural diet-derived *Arabidopsis thaliana* miRNAs (a class of sRNAs) in the interaction with the specialist pest *Plutella xylostella*. The miRNAs predicted to be targeting *P. xylostella GSS* by *in silico* prediction were selected for the study. GSS plays an important role in the interaction between *A. thaliana* and the insect herbivore by helping the insect in detoxification of the toxin Glucosinolates. A miRNA free artificial diet was standardized for the specialist insect. Both precursor and miRNA/miRNA* duplex forms of the candidate miRNAs were shown to be stable at room temperatures for up to 24 hours. The candidate miRNAs were *in vitro* synthesized and fed to the insect via artificial diet. This study reveals that the candidate miRNA Ath-miR824 is induced upon herbivory.

Acknowledgments

Introduction

Plants and their insect herbivores have existed in a close association for at least 350 million years (Gatehouse, 2002). Insect herbivores depend upon plants for their nutrition and energy. For plants, such heterotrophic organisms are a form of biotic stress (Bonnet et al., 2017). In response to insect attack, there are a plethora of defense responses that are induced in plants. Plant defense against herbivore attack is mainly of two types, indirect and direct. The indirect defense is where the plant attracts the natural enemies of the insect, it includes the production of volatile compounds by the plant which acts as an attractant for the predator or parasite of the herbivore. In direct defense, plants can employ morphological features that act as mechanical barriers against the insect or produce secondary metabolites that affect the insect directly (Mithöfer and Boland, 2012). The very first bite by the herbivore causes damage to the plant cells which leads to a change in membrane potential which causes the cytosolic Ca^{2+} levels to increase and H_2O_2 are produced. These changes lead to the activation of cascades of MAP kinases. This eventually causes the activation of the Jasmonic acid (JA) pathway (Schmelz et al., 2003). JA is a plant phytohormone known to act as a major defense signalling molecule (Okada et al., 2015). This leads to the activation of various transcription factors and induction of defense-related genes (Bodenhausen and Reymond, 2007; McConn et al., 1997). This is followed by the production of various secondary metabolites that deter the herbivore from feeding on the plant (Fürstenberg-Hägg et al., 2013).

Insects, on the other hand, have also evolved multiple strategies to counter the defense elicited by plants. Insects can sense the toxic chemicals and avoid feeding on plants or plant parts that contain such harmful chemicals. Insects can also carry out rapid excretion of the ingested plant toxins upon ingestion. Another strategy of the insects is to sequester the toxins in specialized organs. Insects can also use these sequestered toxins to protect itself against its predators(Kumar et al., 2016). Another strategy evolved by the insect to counter the plant defense is called metabolic resistance. Insects can modify the ingested toxin to make it harmless with the help of detoxification enzymes. Insects can also modify the plant metabolites to prevent the formation of toxic chemicals (Després et al., 2007).

Along with the induction of defense pathways in plants upon attack by the herbivore, it has been shown that small RNAs (sRNAs) are produced in plants in response to herbivory. (Brant & Budak, 2018). sRNAs have been shown to play a crucial role in the development of plants (Voinnet, 2009). sRNAs are also shown to have a role in defense response within the plant (Pandey et al., 2008, Li et al., 2011). When an insect is feeding on the plant, along with all the metabolites, it also ingests the nucleic acids present in the plant. Plants and insects have co-evolved for a very long time and hence the possible role of nucleic acids in the interaction cannot be overruled. Recently various studies have shown non-coding RNAs playing role in trans kingdom interactions between plant and pathogen (Hua et al., 2018), host and nematodes (Buck et al., 2014).

sRNAs are non-coding, single-stranded RNA molecules of length 20-30 nucleotides (Zeng et al., 2019). Though the secondary metabolites can take from hours to days to be produced, sRNAs are produced within seconds to minutes following the attack (Sattar et al., 2012). Recent studies show that artificial expression of siRNA producing dsRNAs in the host species can lead to the silencing of target mRNAs in the parasites and pathogen (Panwar et al., 2013). This phenomenon is known as host induced gene silencing (HIGS). HIGS has also been shown to be effective against insects. Here, dsRNA for insect specific gene is expressed in plants via virus-induced gene silencing (VIGS). When the insect feeds on the plant, the dsRNAs enter the insect and leads to silencing of the target mRNA (Kumar et al., 2016). This shows that artificially expressed sRNA molecules from plants can enter the insect gut and also carry out target level regulation. This suggests that naturally present sRNAs in a plant which are continuously ingested by insects might also play some role in the cross-kingdom interaction between plant and insect.

Micro-RNAs (miRNAs) are one such class of naturally occurring sRNAs (Winter et al., 2009). miRNAs are transcribed from their genes by RNA polymerase II and III. The resulting primary miRNA transcript (pri-miRNA) is then processed by an RNase III enzyme, Dicer like-1 (DCL1) and other partner proteins to form precursor miRNA (pre-miRNA). pre-miRNAs are long and have a hairpin structure. The pre-miRNA then undergoes another round of processing by DCL1 to form the functional mature miRNA duplex (miRNA/miRNA*). The mature miRNA duplex is composed of two complementary strands of RNA, the guide strand and the star strand. The

miRNA/miRNA* duplex then loads on Argonaute (AGO) protein to form the RNA induced silencing complex (RISC). One of the strands from the duplex is retained in the complex and is called the guide strand, while the other strand undergoes degradation and is known as star or passenger strand. The guide strand guides the RISC towards the target mRNA by base complementarity. Upon binding to the target mRNA, the target is either cleaved by AGO or undergoes translational repression. This process of post-transcriptional gene expression regulation by sRNAs is known as RNA interference (RNAi). The process of RNAi by sRNAs is a conserved process across eukaryotes (Zeng et al., 2019).

To study the role of diet-derived natural plant miRNAs in cross-kingdom target regulation in insects, the study system chosen was *Arabidopsis thaliana* and *Plutella xylostella. A. thaliana* is a model plant and also a member of the Brassicaceae family whereas *P. xylostella* is a lepidopteran specialist pest of the same family(Gur'ev, 2015; Talekar and Shelton, 1993). Brassicaceae family plants produce secondary metabolites known as glucosinolates and myrosinase enzymes as a primary defense against herbivores (Mithen, 2001). These two compounds are compartmentalized in different types of cells in the plant to prevent self-toxicity. When an insect feeds on the plants the compartments are broken and the two compounds come in contact with each other. Myrosinase acts on glucosinolates by removing the glucose moiety to form toxic compounds like isothiocyanates, nitriles, etc (Mithen, 2001). These toxic compounds deter the herbivores from feeding on the plant. *P. xylostella*, on the other hand, is a specialist pest and is known to feed especially on glucosinolate containing plants (Talekar and Shelton, 1993). The insect has evolved an enzyme known as glucosinolate sulfatase (*GSS*) as a counter defense against the plants. *P. xylostella* GSS has desulfatase activity. GSS acts by removing the sulfate group from glucosinolates hence producing desulfo-glucosinolates. Desulfo-glucosinolates are invisible to myrosinase and hence no toxic compounds are formed. Therefore, in *P. xylostella* majority of the glucosinolates are converted into desulfo-glucosinolates which are non-toxic to the insect instead of isothiocyanates (Ratzka et al., 2002)

To study the role of plant miRNAs in the above-explained interaction, the midgut transcriptome of *P. xylostella* was screened for potential targets of *A. thaliana* miRNAs. Three different algorithms namely, miRanda (Enright et al., 2003), RNA22 (Miranda et al., 2006) and RNAhybrid (Krüger and Rehmsmeier, 2006) were used for

the prediction. Among the several miRNA-mRNA target pairs predicted, two homologs of *P. xylostella* glucosinolate sulfatase, *PxGSS1* and *PxGSS2* were also found to be targeted by several miRNAs. As already described, GSS plays a very important role in the interaction between Brassicaceae family plants and *P. xylostella*. It prevents the formation of toxic compounds like isothiocyanates and hence enables the insect to feed on the plant. This makes *GSS* an interesting target to study.

Out of all the *A. thaliana* miRNAs predicted to be targeting *GSS* homologues, the ones which were commonly predicted by the three algorithms were chosen to be studied further (Fig. 1). Double-stranded RNA molecules have been shown to carry out RNAi as they can be taken up by cells, the double-stranded intermediate forms of candidate miRNAs, pre-miRNA and miRNA/miRNA* duplex were selected for the study. The miRNA/miRNA* duplex form was chosen as it is the functional form, and it gets loaded onto AGO to form the functional RISC. Pre-miRNAs were chosen to be studied as they are longer in length and have a hairpin structure. Studies have shown longer dsRNA molecules have higher efficiency of uptake (Chan and Snow, 2017). The hairpin structure can potentially make this form of miRNA more stable by preventing them from the action of exonucleases (Kevin Range, 2012).

The objective of this study was to understand the role of plant miRNAs in the interaction between plants and insects. To verify if the *A. thaliana* miRNAs can carry out *in vivo* regulation of their *in silico* predicted targets both dsRNA forms (pre and mature duplex) of the candidate miRNAs were *in vitro* synthesized and fed to the insect. A miRNA free artificial diet was standardized for the specialist pest *P. xylostella* to carry out the miRNA feeding assays. The temporal profile of the miRNAs of interest in the plants following herbivory was studied to understand the possible role of these miRNAs in plant defense against herbivores.
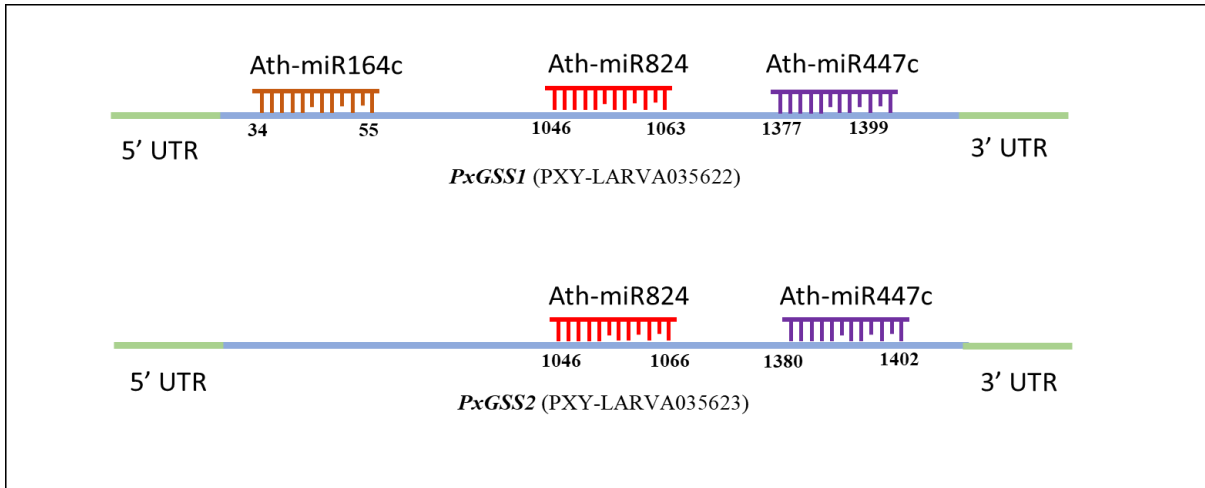
Fig. 1: Pictorial representation of binding sites of candidate *A. thaliana* miRNAs on *P. xylostella GSS1* and *GSS2* transcripts
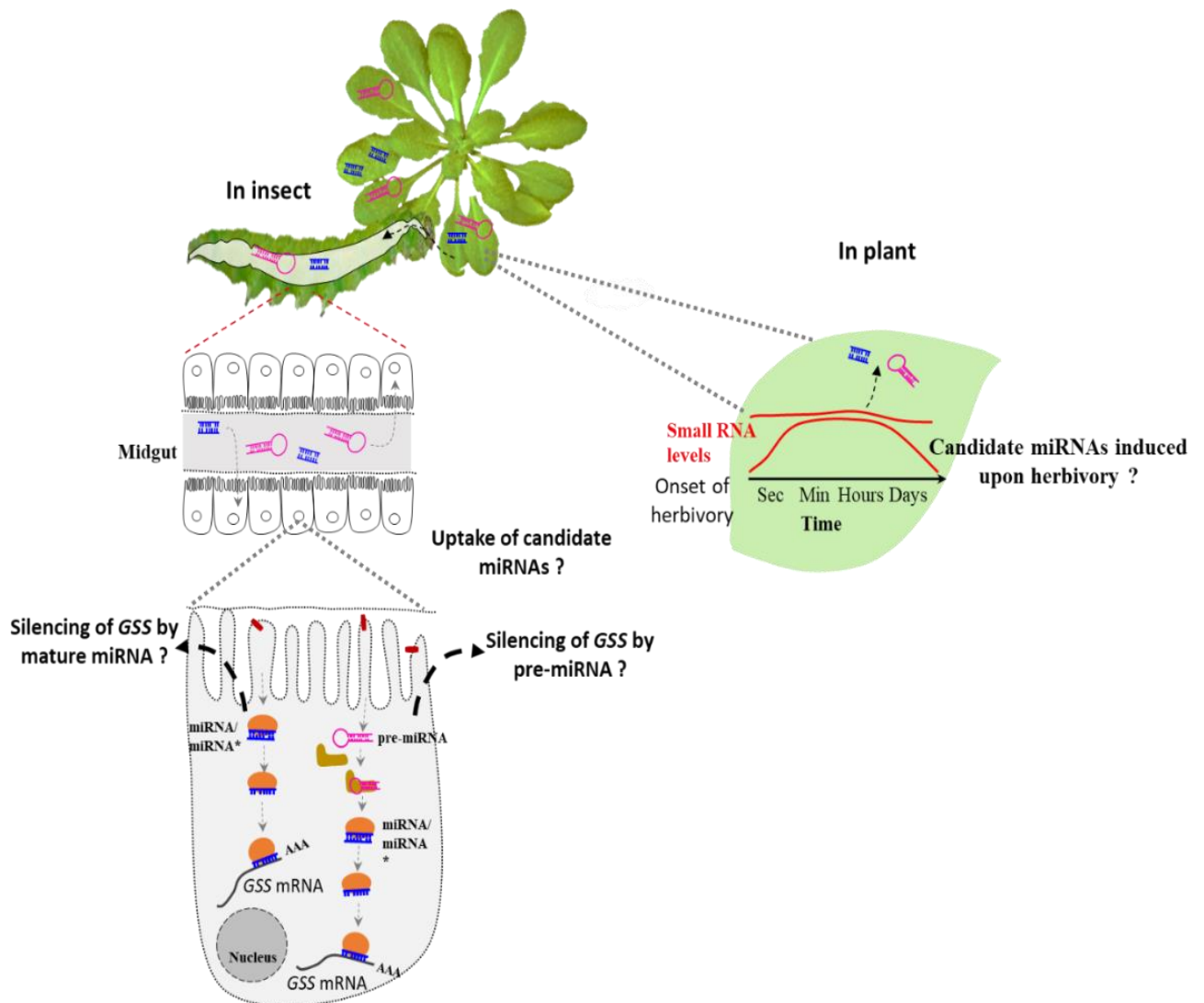
Fig. 2: Pictorial Representation of objectives of the project. The questions addressed were: Upon herbivory by *P. xylostella* do the candidate miRNA levels get induced in the plant? Upon ingestion of the candidate miRNAs do they survive the gut environment and get taken up by insect gut tissue? Can diet acquired plant miRNAs carry out silencing of insect mRNA (*GSS*) via RNAi.

Materials and Methods

Plant growth conditions

Wild type *A. thaliana* (Col-0) plants were grown in the HiPoint growth chamber at a temperature of 21°C with 10:14 hours light: dark photoperiod and 60% relative humidity.

Insect culture

P. xylostella cultures were maintained at 27°C with 14:10 hours light: dark photoperiod and 60% relative humidity. Larvae were reared on mustard seedlings and 10% honey solution-soaked cotton pads were provided to moths. For the experiments, eggs were collected on egg sheets. Egg sheets were made by soaking parafilm in cauliflower leaf juice and air-drying them. Eggs were collected every day and stored in petri plates for 2 days till neonates emerge. Larvae were reared on artificial diet from the neonate stage onwards.

Artificial diet

The soy B diet described by Carpenter et al., 2002, was modified for use in the experiments and for rearing *P. xylostella* larvae. Plant material was treated as described below to get rid of the miRNAs present before adding to the diet. Lyophilised *A. thaliana* leaf tissue was used instead of cabbage flour. The concentration of plant material used in the diet was reduced to half i.e., 1.5 g of lyophilized *A. thaliana* leaf tissue was used for a 100 ml diet. USDA vitamin pre-mix was used. All the dry ingredients were mixed and agar boiled in a microwave oven (for 3 minutes) was added to the dry mix. Vitamin pre-mix and antibiotic were added when the diet cooled down to the touch but still in a liquid state. Crude glucosinolates extract as described below was mixed with artificial diet and air-dried to evaporate the methanol.

Preparation of plant material for artificial diet

To get rid of the RNA from plant material that was to be used for the diet preparation following procedure done: Wild type *A. thaliana* leaf tissue was crushed at room

temperature using mortar and pestle. The crushed tissue was incubated at room temperature for 1 hour for the RNA to degrade. Post 1-hour incubation the tissue was washed with distilled water and centrifuged. The supernatant was discarded to get rid of the RNA. This process was repeated twice and the pulp was frozen in liquid nitrogen. The tissue was then lyophilized for long term storage.

Glucosinolate extraction

The presence of glucosinolates in the diet is required for the induction of Glucosinolate sulfatase in *P. xylostella* (Heidel-Fischer et al., 2019; Sun et al., 2019). Crude glucosinolates were extracted from wild type *A. thaliana* leaf tissue. Leaf tissue was crushed in liquid nitrogen using motor and pestle. The extraction buffer was prepared by adding Milli-Q water to HPLC grade 100% methanol to make 80% methanol. The pH of the buffer was adjusted to 3 with acetic acid. 3g of crushed *A. thaliana* leaf tissue was added in 35 ml of extraction buffer. The mixture was homogenized in a bead beater. The mixture was centrifuged at 4˚C at 10,000 rpm for 10 min. The supernatant was transferred in a new falcon and the spin was repeated. The supernatant was again transferred in a fresh tube and incubated at -20˚C overnight. After the incubation falcons were centrifuged at 4˚C at 10,000 rpm for 20 min and the supernatant was transferred in a fresh tube. The extract was used at physiological concentrations, for e.g. if original tissue was 3 gm, then extract from 3 gm leaves was mixed in 3 gm of artificial diet.

RNA isolation

A. thaliana leaf tissue was carried out by flash freezing the leaf tissue in liquid nitrogen and fine crushing them using motor and pestle in the presence of liquid nitrogen. The crushed leaf tissue was then stored -80°C till. Total RNA was isolated from leaf tissue using Takara TRIzol reagent, according to the manufacturer's protocol.

The midgut of third instar larvae were used for examining the change in *GSS1* and *GSS2* transcript level upon feeding *A. thaliana* miRNA. Midgut tissue from 5 larvae were pooled in TRIzol. Tissue was crushed by using glass beads and putting them in bead beater for homogenizing the sample. Total RNA was isolated from the midguts

of the third instar *P. xylostella* larvae using Takara TRIzol, according to the manufacturer's protocol.

cDNA synthesis

250ng of total RNA isolated from the tissues was used for cDNA synthesis. Random hexamers and gene-specific primers and Takara PrimeScript 1st strand cDNA Synthesis Kit was used for cDNA synthesis for quantification of pre-miRNAs in the samples.

For the quantification of mature miRNA/miRNA* duplex, cDNA was synthesized from 250ng of total RNA with Takara PrimeScript 1st strand cDNA Synthesis Kit. Two-tailed hemiprobe (primer) were used for cDNA synthesis as described by Androvic *et al.* (Androvic et al., 2017)*.* The cDNA synthesis reaction was multiplexed by using a mix of two-tailed primers for the candidate miRNAs and reference gene.

Table 1: Sequences of two-tailed hemi-probes used for cDNA synthesis of mature miRNAs

	cDNA synthesis of mature miRNAs
miRNA	Two-tailed hemiprobe/primer sequence
Ath-miR447c-3p	TCCCCAACTAATGTCCTCCAGGTACAGTTGGTACCTGTAACTCACTCAACAA
Ath-miR164c-5p	CTTCTCCACTATGCTCTCCAGGTACAGTTGGTACCTGTCTCCACTTCGCACG
Ath-miR824p	AGAAGGACACACACCAGGTACAGTTGGTACCTGACTCCACGCTCTAGACCA
5s rRNA	AGTCGTTTTCTATGTCCTCCAGGTACAGTTGGTACCTGTCTGTCTATCGAT

qRT-PCR

To study the induction of miRNAs in plants upon herbivory, RT-qPCR was done using cDNA synthesized from total RNA isolated as described above. Takara qRT-

PCR SYBR green master mix was used for RT-qPCRs. 5s-rRNA was used as an internal control for the analysis of mature plant miRNAs. Actin elongation factor was used as the internal control for analysis of plant pre-miRNAs.

All the primer sequences, gene and miRNA accession numbers are listed in table 2.

Table 2: List of primers used for qRT-PCR of miRNAs

		qPCR primers for pre-miRNAs
Pre-miRNA		Sequence
Ath-miR447c-3p	FP	CATTAAGCCCCTTACAATGTCG
Ath-miR447c-3p	RP	GATGTCGTCCCCAAATACCG
Ath-miR164c-5p	FP	TAACACTTGATGGAGAAGCAGGG
Ath-miR164c-5p	RP	AGACACGTGTTGGAGTAGTAGAACAC
Ath-miR824p	FP	CGAACTAGTCCAAGCCGATTCTC
Ath-miR824p	RP	CAAACATCTCCCCACTCCCC
		qPCR primers for mat-miRNAs
Mature miRNA		Sequence
Ath-miR447c-3p	FP	TCCCCAACTAATGTCCTCCAG
Ath-miR447c-3p	RP	TGGGGACGACATCTTTTGTT
Ath-miR824	FP	GAAGGACACACACCAGGTACAG
Ath-miR824	RP	TTCTCATCGATGGTCTAGATGG
Ath-miR164c-5p	FP	AGCTTCTCCACTATGCTCTCCAG
Ath-miR164c-5p	RP	ATTGGAGAAGCAGGGCAC
		Others
5s rRNA	FP	TCGTTTTCTATGTCCTCCAGGT
	RP	AAACGACTCTCGGCAACG

		qPCR primers for pre-miRNAs
Pre-miRNA		Sequence
Ath-miR447c-3p	FP	CATTAAGCCCCTTACAATGTCG
Ath-miR447c-3p	RP	GATGTCGTCCCCAAATACCG
Ath-miR164c-5p	FP	TAACACTTGATGGAGAAGCAGGG
Ath-miR164c-5p	RP	AGACACGTGTTGGAGTAGTAGAACAC

Ath-miR824p	FP	CGAACTAGTCCAAGCCGATTCTC
Ath-miR824p	RP	CAAACATCTCCCCACTCCCC
		qPCR primers for mat-miRNAs
Mature miRNA		Sequence
Ath-miR447c-3p	FP	TCCCCAACTAATGTCCTCCAG
Ath-miR447c-3p	RP	TGGGGACGACATCTTTTGTT
Ath-miR824	FP	GAAGGACACACACCAGGTACAG
Ath-miR824	RP	TTCTCATCGATGGTCTAGATGG
Ath-miR164c-5p	FP	AGCTTCTCCACTATGCTCTCCAG
Ath-miR164c-5p	RP	ATTGGAGAAGCAGGGCAC

Synthesis of candidate miRNAs

miRNA/miRNA* duplex

mature miRNA duplex consists of two complementary strands of RNA, miRNA the guide strand and miRNA* the star or passenger strand. For synthesis of miRNA/miRNA* duplex both the strands were synthesized separately by IVT. The template for IVT was made by annealing the T7 promoter sequence to the DNA oligos of the miRNA strand. IVT was done by incubating the template with T7 polymerase, rNTPs, DTT and buffer at 37°C for 4 hours. The IVT products were purified using NaCl and 100% ethanol.

Both the star and guide strands of miRNAs were annealed together by gradually cooled from 65°C to 25°C, decreasing the temperature by 0.5°C per minute, to get the final miRNA/miRNA* duplex. Another round of purification was done and miRNA was dissolved in nuclease-free water.

Precursor miRNAs (pre-miRNAs)

For the synthesis of pre-miRNAs longer than 100 bases, plasmids were to be used as template. Ath-miR447c and Ath-miR824 have lengths of 198 bases and 689 bases respectively whereas Ath-miR164c is only 100 bases long. Therefore Ath-miR447c and Ath-miR824 were cloned into pGEM®-T Easy Vector and Ath-miR164c was *in vitro* synthesized using DNA oligo.

Amplification of *A. thaliana* pre-miRNAs

Amplification of pre-miRNAs was done from the cDNA prepared using total RNA of *A. thaliana* leaf tissue. Total RNA of *A. thaliana* plants subjected to herbivory for time ranging from 1 h to 72 h were pooled. Pooled total RNA was used to make cDNA using random hexamers and gene-specific primers. This cDNA was used for PCR amplification of the miRNAs of interest using high fidelity Takara Ex Taq polymerase.

Cloning of pre-miRNAs

The amplified product was used as template to carry out a second round of PCR with the forward primer having the T7 promoter region to include the T7 promoter sequence in the insert. Amplified products were purified using HiPurA PCR Purification Kit. Purified products were ligated into pGEM®-T Easy Vector and transformed into *E. coli*. Colony PCR was used to confirm positive colonies using insert specific primers. Positive colonies were sent for plasmid sequencing to confirm the insertion of miRNA with the correct sequence.

In vitro transcription (IVT) of pre-miRNA

Template for IVT of Ath-miR447c and Ath-miR824 were amplified using purified plasmid from the confirmed positive clones. Forwards primer with added T7 promoter region was used for the PCR amplification of the templates. The amplified product was PCR purified product and used as template for IVT. Ath-miR164c is 100bp long and hence, DNA oligo was used as template for IVT. IVT was done same as for mature miRNA/miRNA* duplex. The IVT product was purified using ammonium acetate and 100% ethanol. The purified product was then ramp cooled in the presence of DTT and Tris-HCl to fold the miRNA get the characteristic hairpin structure. Another round of purification was done using sodium acetate and Isopropanol, and RNA was dissolved in nuclease-free water.

Table 3: List of templates used for IVT of mature(m) and star(s) strands of miRNA/miRNA* duplex and primers used for amplification and cloning of pre-miRNAs

		Template for IVT of miRNA/miRNA*(m/s)
miRNA	Sequence	
Ath-miR447c-3p_m	CAACAAAGATGTCGTCCCCAATATAGTGAGTCGTATTA	
Ath-miR447c-3p_s	ATTAGCGGACACATGCTTTGTTATAGTGAGTCGTATTA	
Ath-miR164c-5p_m	CGCACGTGCCCTGCTTCTCCATATAGTGAGTCGTATTA	
Ath-miR164c-5p_s	GTTGGAGTAGTAGAACACGTGTATAGTGAGTCGTATTA	
Ath-miR824-m	TCCCTTCTCACAAATGGTCTACTATAGTGAGTCGTATTA	
Ath-miR824-s	TCTAGACCATCGATGAGAAGGCTATAGTGAGTCGTATTA	
	Primers for amplification of pre-miRNAs	
miRNA		Sequence
Ath-miR164c-5p	FP	TAACACTTGATGGAGAAGCAGGG
Ath-miR164c-5p	RP	AGACACGTGTTGGAGTAGTAGAACAC
Ath-miR164c-5p	FP+T7	TAATACGACTCACTATAGGGGATAACACTTGATGGAGAAGCAGGG
Ath-miRAt447c-3p	FP	CATTCTTAATATACAATACTTCTTTTTCATG
Ath-miRAt447c-3p	RP	CATTCTTGATATATAATACTACAATTTCTTC

Ath-miRAt447c-3p	FP+T7	TAATACGACTCACTATAGGGCATTCTTAATATACAAT ACTTCTTTTTCATG
Ath-miR824	FP	ATCACCATTGTTCTTTACCTCCATG
Ath-miR824	RP	TATCACCATTTGTACTTGAGTTGTCTCTC
Ath-miR824	FP+T7	TAATACGACTCACTATAGGTATCACCATTTGTACTTG AGTTGTCTCTC

miRNA induction by Methyl-Jasmonate

Ath-miR824 and Ath-miR447c could not be amplified from the cDNA of *A. thaliana* leaf tissue which was not subjected to herbivory. To verify if these miRNAs are induced upon herbivory, methyl-jasmonate (MeJA) was used to induce herbivory in *A. thaliana* plants. MeJA is a methyl ester of JA. MeJA is hydrolyzed to form JA (Świ\katek et al., 2004). As, JA is the major defense priming molecule in plants against insect herbivores, application of MeJA is routinely used for herbivory induction in plants (Baldwin, 1998; McConn et al., 1997). 12.2µl of MeJA was dissolved in 1 ml of Vaseline. 50 µl of this mix was applied at the leaf petiole junction. The leaves were collected in liquid N_2 after 1 hour. cDNA was prepared from total RNA of these leaves and PCR amplification of the three miRNAs of interest was done as described above.

miRNA stability

The miRNA feeding assays were to be carried out at room temperature for a time ranging from 12 to 72 hours. To ensure that the miRNAs added to the diet are stable at room temperature their stability was checked at 12 hours and 24 hours after addition. The three *in vitro* synthesized candidate pre-miRNAs, Ath-miR447c, Ath-miR824, and Ath-miR164c were pooled together in equal concentration. The pooled miRNAs were mixed in 2.4 g of artificial diet so as get a concentration of 11µg/g (Whyard et al., 2009). The diet mixed with miRNAs was then divided into 3 equal

parts of 0.8 g each for 3-time points: 0h, 12h, and 24 h. The .8 g diet was further divided into 100mg portions and transferred into 8 separate 2ml Eppendorf tubes. The 0 h tubes were immediately frozen in liquid N_2. Diet mixed with miRNAs was kept for 12 and 24 hours at room temperature. After each time point diet was frozen in liquid N_2. Diet mixed with an equal volume of nuclease-free water was also kept for each time point as control. The same was done for *in vitro* synthesized mature miRNA/miRNA* duplex form of the candidate miRNAs.

miRNA feeding assay

To validate the targeting of *GSS1* and *GSS2* by each miRNA, both the miRNA forms of each miRNA were fed to the insects separately. miRNA was mixed in artificial diet and the larvae were allowed to feed on the diet for different time intervals. A concentration of 11μg/gram (Whyard et al., 2009) of miRNA was used for the assay. Diet was changed every 24 hours for time intervals exceeding 24 hours.

RNAi has been shown to be successful in *P. xylostella* at 24 hours post dsRNA feeding (Bautista et al., 2009). The time points chosen for the study were: 0 h, 12h, 24h, 48h, 72h.To understand the effect of continuous feeding on miRNA containing diet, the larvae were allowed to feed continuously on the diet. After each time point, the larvae were dissected and the mid-gut was collected in TRIzol. For 0-hour treatment the larvae were dissected without feeding on miRNA containing diet. Each time interval had a control where larvae were fed on a diet without any added miRNA.

1 Replicate: 5 larvae, 200 mg Diet + miRNA (concentration= 11µg/g)

Treatment= Diet + miRNA   Control= Diet + Nuclease free water

Time points: 0 h, 12h , 24h, 48h, 72h

Larvae dissected – midgut collected in TRIzol
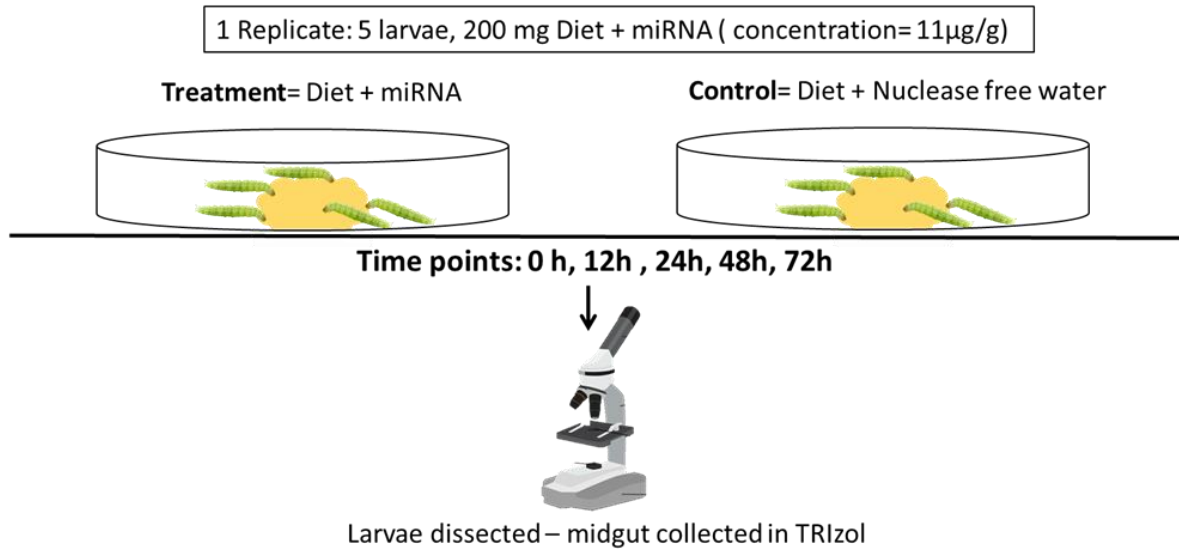
Fig. 3: miRNA feeding assay design. Both pre and mature forms of each candidate miRNAs were separately by mixing in artificial diet. The concentration of miRNAs was adjusted to be 11µg/g. Each replicate consisted of 5 larvae per treatment and a water control (negative control) where diet mixed with nuclease free water was used. The time points used were: 0h, 12h, 24h, 48h, 72h.

Temporal kinetics assay

To understand the temporal profile of miRNAs in plants upon herbivory wild type *A. thaliana* plants were subjected to herbivory for different time intervals. A single, 10-15 leaf stage *A. thaliana* plant was kept with 8 larvae for each time point, per replicate. The larvae were allowed to feed for the specific time period after which leaves with bite marks were collected and frozen in liquid nitrogen. Leaves from the plant with no feeding were also collected at each time point as no herbivory control.

Treatment : Herbivory by *P. xylostella*   **Control** : No herbivory

Leaf tissue collected after
various time points

Time points: 0h, 1h, 6h, 12h, 24h, 48h, 72h

Fig. 4: Experimental design for miRNA temporal kinetics assay. Time points used for the experiment were: 0h, 1h, 6h, 12h, 24h, 48h, 72h. Eight larvae were allowed to feed on a single plant for each replicate and the experiment consisted of 6 replicates.

Statistical analysis

One-way ANOVA was done for statistical analysis of RT-qPCR results. Tukey's pairwise test was used to determine statistical significance (*p≤0.05).* Past3 was used for the statistical analysis

Results

In vitro synthesis of *A. thaliana* pre-miRNA and mature miRNA duplex

To verify if diet acquired plant origin miRNAs can regulate target mRNA levels in insects, the miRNAs were synthesized. Both precursor and mature forms of miRNAs of interest were synthesized by *in vitro* transcription.

IVT of mature miRNA/miRNA* duplex

Both strands of mature miRNA/miRNA* duplex were synthesized by *in vitro* transcription using DNA oligos of the respective strand. Both the complementary strands were then annealed to get the final miRNA duplex (Fig. 5, a)

pre-miRNA synthesis

Pre-miRNAs (Ath-miR447c-198 bases and Ath-miR824-689 bases) are long in length and hence plasmids serve as better template for *in vitro* transcription than single-stranded oligos. Ath-164c- 100 bases was synthesized using DNA oligo by IVT.

Ath-miR447c could not be amplified from cDNA of control *A. thaliana* leaf tissue- no herbivory. Amplification was also not successful from the cDNA of *A. thaliana* leaf tissue in which herbivory was induced using MeJA. Whereas Ath-miR164c could be amplified from both the treatments. Amplification of Ath-miR447c was successfully done from the cDNA of *A. thaliana* tissue which was subjected to herbivory by *P. xylostella*.

Therefore, cDNA made from total RNA of post herbivory *A. thaliana* leaf tissue was used for amplification of the pre-miRNAs (Ath-miR447c, Ath-miR824) (Fig 5, b). The amplified product was then cloned into the pGEM-t easy vector. Positive clones were identified by colony PCR (Fig. 5, c) and the sequence was verified by plasmid sequencing.

The pre-miRNA along with the T7 promoter region was successfully cloned. These positive clones were used to carry out *in vitro* transcription of the pre-miRNAs of interest (Fig. 5, d).
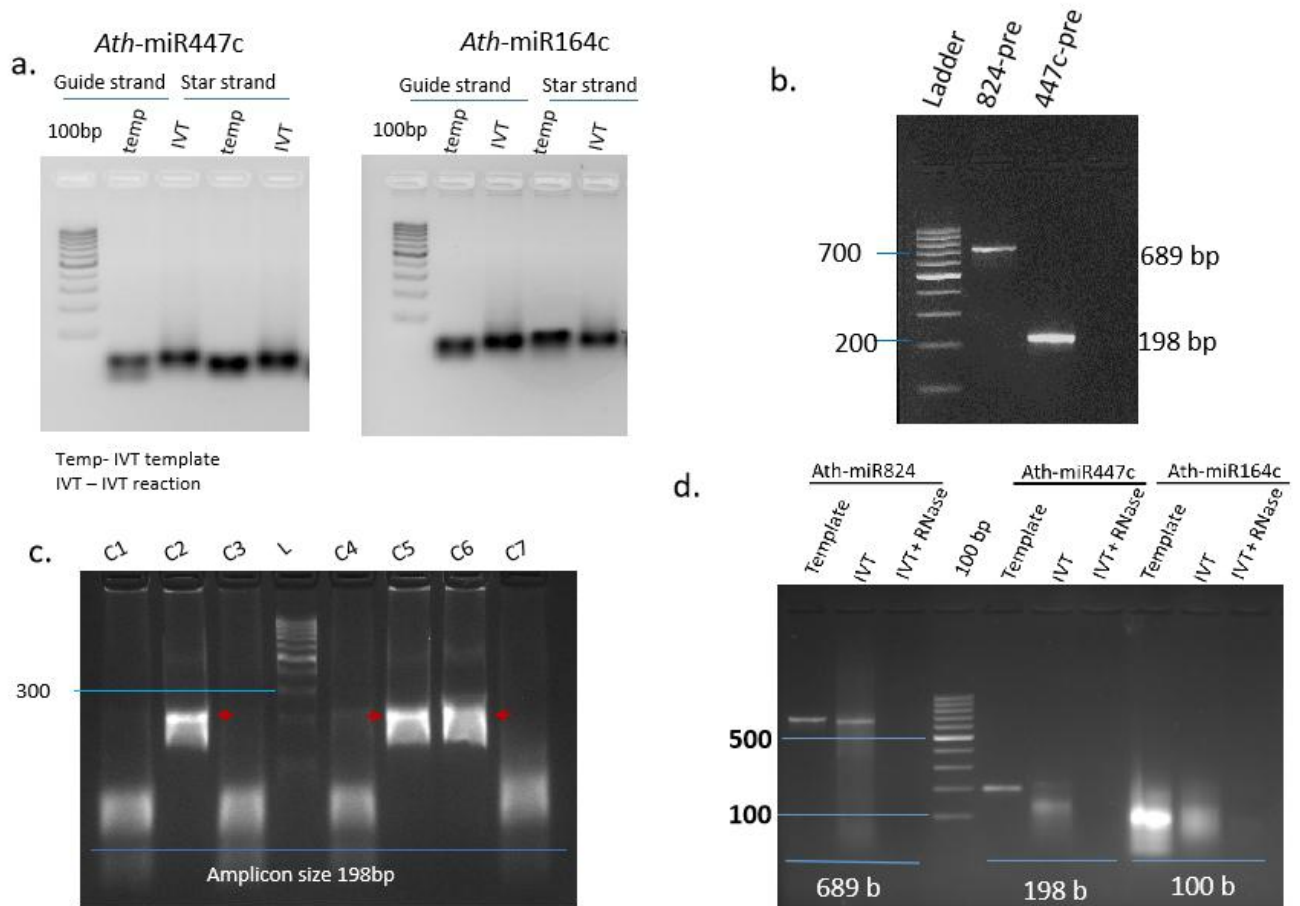
Fig. 5: 3% Agarose gel images for synthesis of candidate plant miRNAs. IVT- *in vitro* transcription product, Temp- template used for IVT(a)Gel image for IVT of mature and guide/star strand of miRNA/miRNA* duplex. (b) Gel image for amplification product of candidate miRNAs from *A. thaliana* cDNA. (c) Gel image for colony PCR for colony screening of 447c, positive colonies. Red arrow indicates positive colonies verified via plasmid sequencing later. (d) Gel image for IVY of pre-miRNAs. IVT+ RNase lane shows that the band seen in IVT lane was of successful IVT of candidate miRNAs

Standardization of artificial diet for *P. xylostella*

To verify if the miRNAs of interest play any role in the regulation of *GSS1* and *GSS2* levels in insects, a plant free-feeding medium was required. Since *P. xylostella* is a specialist pest it didn't feed on most published artificial diets that were plant material free. It was observed that the *P. xylostella* larvae do feed on diets containing plant material. Since a miRNA free background was needed the miRNAs were degraded by incubating at room temperature for 1 hour followed by 2 rounds of wash with distilled water. The level of miRNA was checked in treated tissue that was to be added to the diet. The levels of miRNAs are significantly lower in *A. thaliana* tissue that was given the above-described treatment compared to the control (Fig. 6).
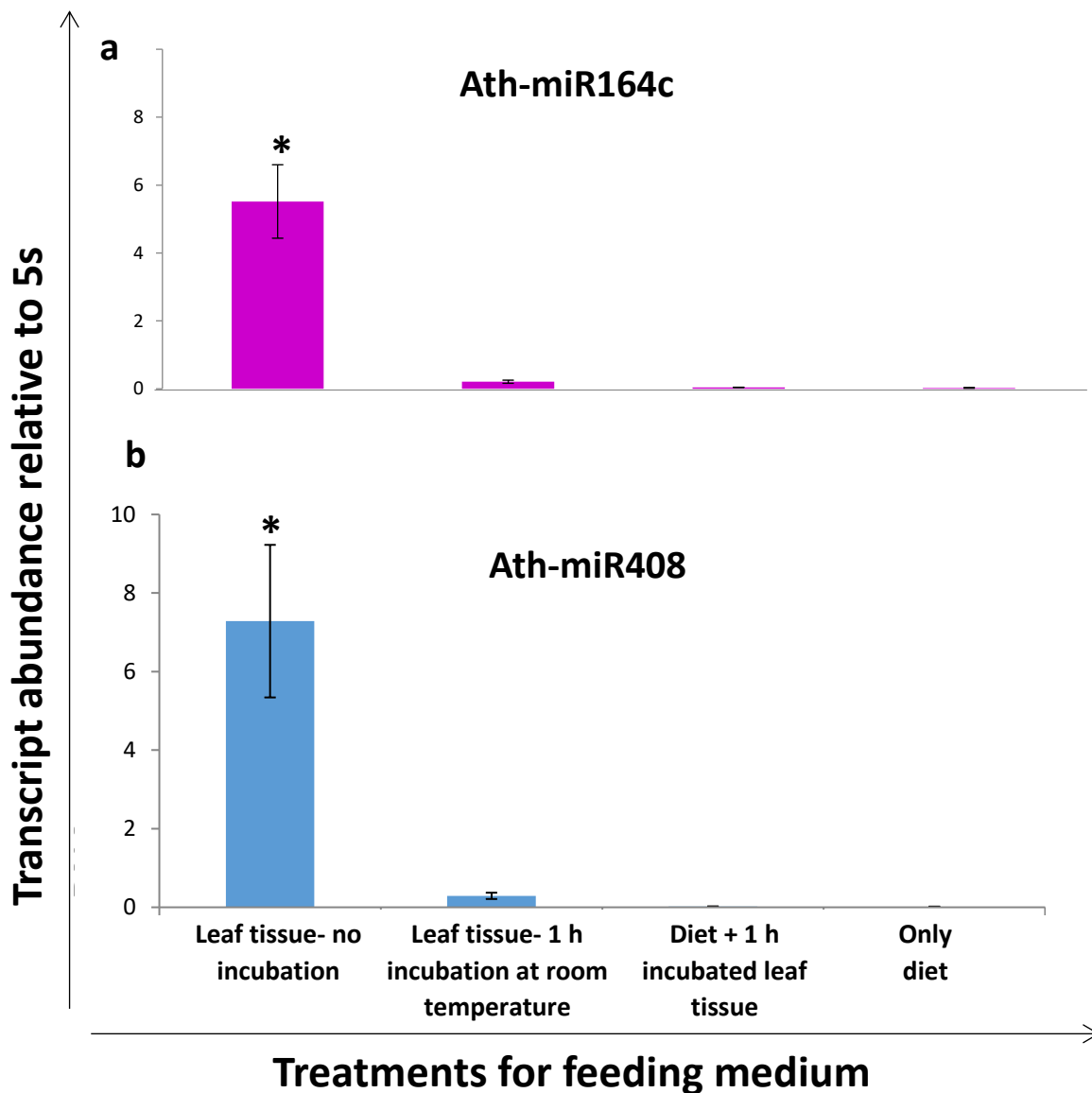
Fig. 6: qPCR results to check for the presence of miRNAs in the treated *A. thaliana* tissue used in diet. One-way ANOVA, n=6, Star indicates significant difference from other treatments *(p≤0.05).* Levels of miRNAs is shown to be significantly reduced in the treated tissue i.e. tissue incubated at room temperature followed by 2 round of washes using distilled water. miRNA levels are negligible in only diet which doesn't have any plant material added and diet with treated plant tissue.

miRNA stability at room temperature

The stability of both precursor and mature miRNA/miRNA* duplex forms of candidate miRNAs were checked at room temperature. The stability was checked at 12h and 24h after addition. Both forms of miRNAs were stable at room temperature for 24 hours (Fig. 7). No significant difference was seen in the level of pre-miRNAs and mature miRNAs at both 12 and 24 hours compared to 0-hour control for Ath-miR164c and AthmiR824. The level of Ath-miR447c pre form at both 12 hour and 24 hour is not significantly different from 0 hour. The level of mature duplex form is seen to be significantly reduced at 12 hour and 24 hour compared to 0 h. No significant reduction is seen after 12 hour to 24 hour.
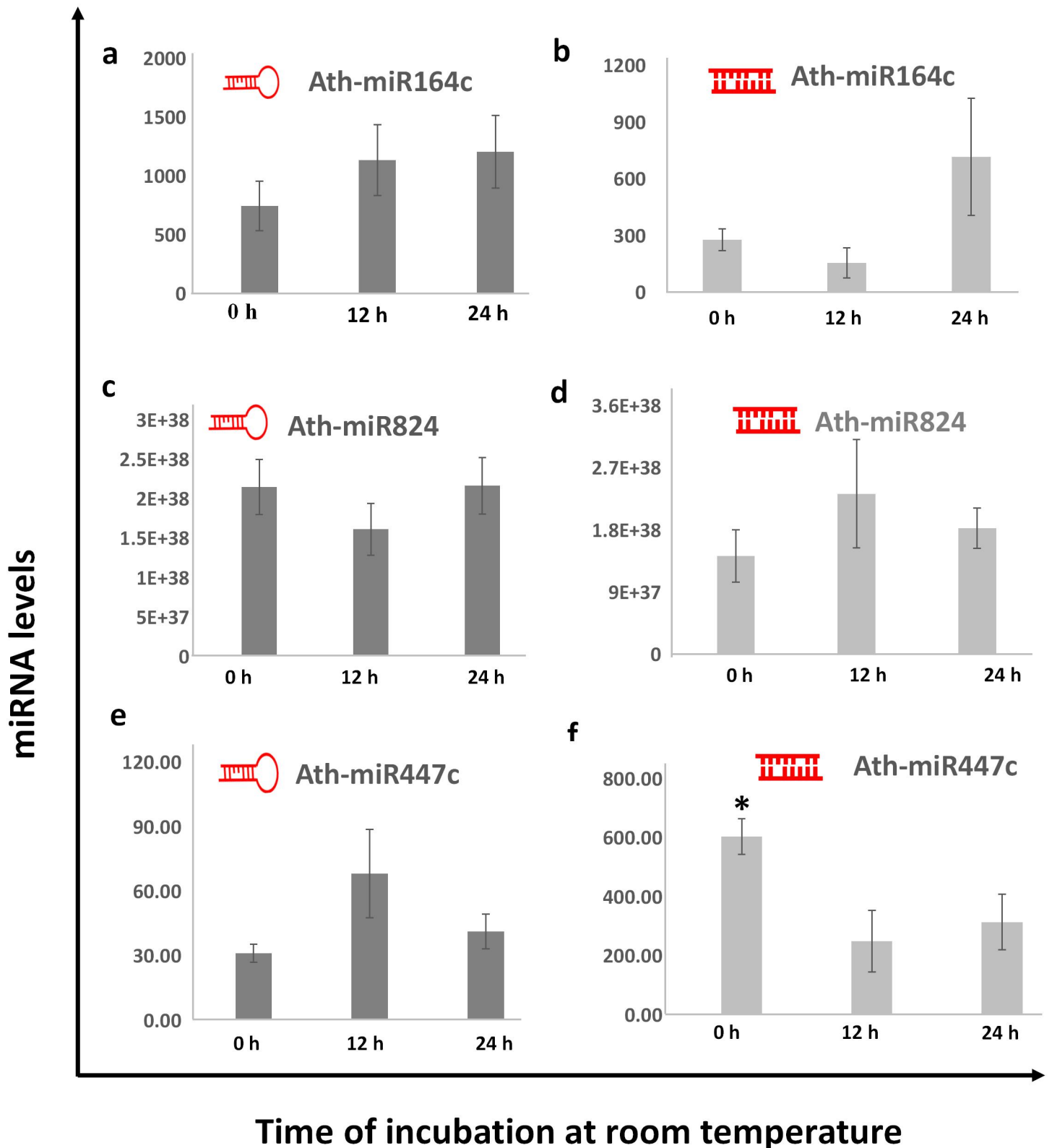
Fig. 7: RT-qPCR results for miRNA stability assay. One-way ANOVA, n=8, Star indicates significant difference from other treatments *(p≤0.05)*. No significant difference is seen between the levels of miRNAs at 0h, 12h and 24h after incubation at room temperature for both forms of candidate miRNAs. Pre and mature form are indicated with the help of symbols. The hairpin structure stands for pre-miRNA and the duplex structure for miRNA/miRNA* duplex.

Temporal kinetics of miRNA in plants upon herbivory and the transcript levels of their targets in insects

To understand the temporal profile of the miRNA of interest in plant upon herbivory, the miRNA levels were quantified at different time points following herbivory. The target mRNA levels in the insect were also quantified at each time point. The miRNAs are seen to be induced upon herbivory. The levels of pre-miRNA form of Ath-miR824 increased significantly at 6 hour post herbivory compared to no herbivory control. The mature-miRNA form of Ath-miR824 is induced at 48 hours following herbivory (Fig.9).
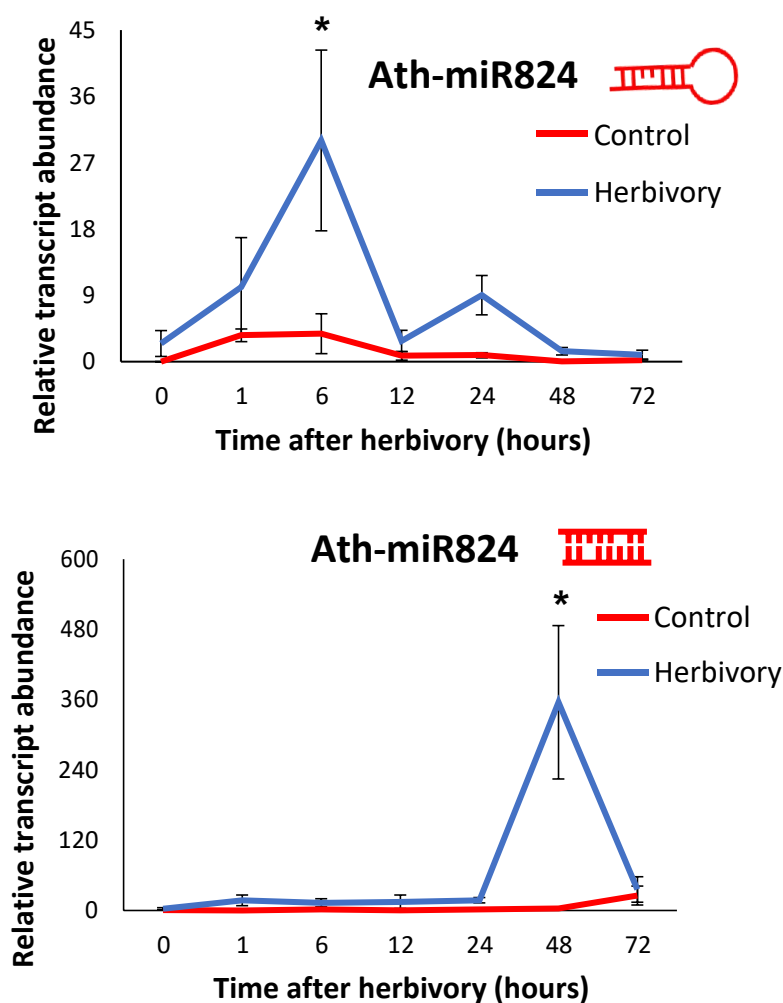
Fig. 8: Temporal kinetics of both forms of miRNAs following herbivory by *P. xylostella*. One-way ANOVA, n=6, star indicates significant difference *(p≤0.05).* Both pre-miRNA and mature duplex forms of Ath-miR824 shows significant difference in levels compared to control tissue- no herbivory. The blue line indicates transcript levels in wild type *A. thaliana* plants which were subjected to herbivory by *P. xylostella*. The red line is for transcript levels in control tissue were the plant was not subjected to

Discussion

Micro-RNAs predicted to be targeting *PxGSS1* and *PxGSS2* commonly by three different algorithms viz, MiRanda, RNAhybrid, RNA22 were chosen to be studied to understand their role in cross-kingdom interaction between *A. thaliana* and *P. xylostella*. Ath-miR447c and Ath-miR824 were predicted to be targeting both homologues of *P. xylostella* GSS transcripts i.e.; *GSS1* and *GSS2* whereas, Ath-miR164c was predicted to target *GSS1*. miRNAs are small (21-25 nucleotides), non-coding, endogenous RNA molecules. miRNAs were first discovered in 1993 in *Caenorhabditis elegans.* (Lee et al., 1993) and since have been shown to play various important and regulatory roles within the organism (Fu et al., 2013). miRNAs are shown to be essential for development and knockouts result in serious developmental defects (Bernstein et al., 2003; Park et al., 2010; Wang et al., 2007). miRNAs are known to play important roles in plant development and regulate various stress-related responses of the plant. Ath-miR447c has been shown to play role in abiotic stress in *A. thaliana* plants specifically they get downregulated in drought condition (Hajdarpašić and Ruggenthaler, 2012) Ath-miR824 is involved in the flowering response of the plant under stress (Hu et al., 2014) and also plays role in stomatal development (Kutter et al., 2007). Ath-miR164c was shown to be upregulated in response to Sulphur deficiencies (Liang et al., 2015) and also involved in dark-induced senescence (Huo et al., 2015). miRNAs are also released in the extracellular environment and have been shown to influence gene expression regulation in other cells and tissues (Hergenreider et al., 2012; Mittelbrunn et al., 2011). Although the role of miRNAs within the organism has been extensively studied, their role in cross-kingdom interactions is not that well understood. In this study, it was observed that both Ath-miR824 and Ath-447c were successfully amplified from herbivory induced tissue only and not from the control tissue with no herbivory. This further suggests that these miRNAs might play some role in herbivory related response of the plant. Further Ath-miR447c could only be amplified from plants which were subjected to herbivory by *P. xylostella* and not from Plants which were treated with MeJA. JA is known to be involved in the defense response of the plant against herbivores (McConn et al., 1997) and recently it has also been shown

that some miRNAs are upregulated in response to JA (Neller et al., 2018). On the contrary Ath-miR447c was successfully amplified from *A. thaliana* plants after herbivory by *P. xylostella*, this further points towards Ath-miR447c playing role in the interaction between *p. xylostella* and *A. thaliana*.

To understand the role of diet acquired plant miRNAs in the cross-kingdom interaction, especially for target validation it is essential to have a miRNA free-feeding medium. *P. xylostella* is a specialist pest that feeds specifically on plants of Brassicaceae family. It is especially difficult to rear such specialists and feral insects on artificial diet and *P. xylostella* is notorious for this (Carpenter and Bloem, 2002). The insects used in this study were originally collected from brassica crop fields near Pune. The cultures were maintained on mustard seedlings in cages. Various published artificial diets for *P. xylostella* without any plant materials were tested (Guanghong, Ying, Xiaoling, Linbai, & Dongrui, 1996; Hou, 1986). None of these diets were suitable for the insects as very little to no feeding was observed. The diet described by Htwe *et al*., 2009 contains plant materials, and larvae were observed to be feeding on this diet. Upon feeding on this diet for extended periods of time high larval mortality was observed. Soy B diet as described by Carpenter and Bloem, 2002 (Carpenter and Bloem, 2002) was successfully modified for use.
Plant material used in the Soy B diet was changed from cabbage flour to *A. thaliana* leaf tissue. The Diet was modified to contain only half of the concentration of plant material prescribed in the protocol. The miRNA present in the plant material was successfully degraded and a miRNA free pulp was obtained by giving multiple washes with distilled water. The miRNA degradation in the treatment of the *A. thaliana* leaf tissue and diet was confirmed by RT-qPCR. The levels of both the miRNAs (Ath-miR164c and Ath-miR408) checked were significantly reduced in the treated tissue and negligible levels were detected in the artificial diet without any plant material and diet mixed with treated plant material.

The miRNA feeding assay was to be carried out at room temperature and the time period used in the experiment ranged from 12h to 72 hours. The miRNA stability at room temperature was checked for 12 hour and 24 hour time points. Both the pre and mature duplex forms of the miRNAs were observed to be stable at room temperature for up to 24 hours. Ath-miR447c miRNA/miRNA* duplex form levels

were significantly reduced after 12 hours by about 50%. No significant difference was observed between 12 hours and 24 hours for the same. Therefore, the diet mixed with miRNA was changed every 24 hours to ensure the constant ingestion of miRNA by the feeding insect throughout the experiment. miRNAs are shown to be stable in the extracellular environment and also under a range of temperatures and pH, within the organism (EM and Pogosova-Agadjanyan, 2008). This study shows that miRNAs are stable at room temperature for an extended period of time. This makes it easier to carry out feeding experiments at ambient temperatures for long durations.

Understanding the temporal kinetics of the candidate miRNAs post herbivory can help us understand their role in the interaction better. The pre-miRNA form of Ath-miR824 was seen to be induced at 6 hour following herbivory by *P. xylostella*. The mature miRNA form levels increased significantly at 48 hours post herbivory. *P. xylostella* is a voracious eater and early induction of pre-miRNA form might result in a larger pool of pre-miRNA entering the feeding insect.

Work remaining

The dissections for miRNA feeding have been completed for both pre-miRNA and miRNA/miRNA* duplex forms of the candidate miRNAs. The Transcript levels of both *PxGSS1* and *PxGSS2* will be quantified in larvae fed on miRNA containing diet and compared with transcript levels in larvae fed on control diet (no miRNA). The RT-qPCRs for confirmation of regulation of target level by the candidate miRNAs is yet to be completed, due to the sudden termination of lab activities caused by the pandemic.

Conclusion and future directions

miRNAs predicted to be targeting *P. xylostella GSS* transcript by three algorithms were shortlisted. Precursor miRNA form of Ath-miR447c and Ath-miR824 were successfully cloned and the sequence was verified by plasmid sequencing. Pre-miRNA form and miRNA/miRNA* duplex forms of the three candidate miRNAs were synthesized by *in vitro* transcription (IVT). A miRNA free artificial diet was successfully standardized for the specialist insect *P. xylostella*.

The stability of miRNA added to the artificial diet at room temperature was confirmed. Both the forms of the candidate miRNAs were shown to be stable at room temperature for up to 72 hours. The temporal kinetics of the candidate miRNAs post herbivory was checked in the plant. The levels pre-miRNA form of Ath-miR824 was shown significantly increase at 6-hour post herbivory. The levels of mature miRNA form were shown to increase significantly 48 hours after herbivory. The miRNA feeding experiment was completed for 3 replicates.

After validation of targeting of *GSS* transcripts by candidate miRNAs, the efficiency of targeting by both forms can be compared. This comparison will reveal more about the dynamics of the miRNA mediated interaction in nature. The temporal kinetics of the *GSS* transcript post herbivory on *A. thaliana* should be studied to get a better understanding of the target level regulation by the candidate miRNAs. Upon validation of the targeting, looking at the physiological effect of rearing the insect on a miRNA silenced plant would be interesting. It will reveal the ecological importance of these plant miRNAs in this interaction.

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