Identification of differentially expressed microRNAs under imidacloprid exposure in *Sitobion avenae* Fabricius

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Research article

Keywords: MicroRNAs (miRNAs), *Sitobion avenae*, imidacloprid, resistance

Posted Date: October 24th, 2019

DOI: <https://doi.org/10.21203/rs.2.9874/v2>

Abstract

Background MicroRNAs (miRNAs), which are short single-stranded non-coding RNAs, regulate the expression of target genes, especially those involved in the regulation or metabolism of endogenous or xenobiotic compounds. **Results** De novo assemblies of the transcriptome of *Sitobion avenae* Fabricius under control conditions and under imidacloprid treatment were obtained using Illumina short-read sequencing technology. Fifty-seven miRNAs, of which 36 were known and 21 were novel, were identified. Quantitative analysis of miRNA levels showed that five miRNAs were significantly up-regulated, and 11 miRNAs were significantly down-regulated in the nymphs of *S. avenae* treated with imidacloprid in comparison with those of the control. Analysis of the candidate target genes in *S. avenae* that could be regulated by these miRNAs were also carried out. The functions of the miRNAs, which could potentially regulate target genes that participate in metabolism, regulatory or detoxification pathways in *S. avenae*, were clarified based on Gene Ontology and KEGG pathway analysis. The effects of the miRNAs api-miR-1000, api-miR-316, and api-miR-iab-4 on susceptibility of *S. avenae* to imidacloprid was determined. Modulation of the abundances of api-miR-1000, api-miR-316, and api-miR-iab-4 by the addition of the corresponding inhibitors to the artificial diet significantly changed the susceptibility of *S. avenae* to imidacloprid, which further demonstrated the effect of these miRNAs on the regulation or metabolism of insecticides. **Conclusion** The results of this study suggested that miRNAs differentially expressed in response to imidacloprid could play a critical regulatory role in the resistance of *S. avenae* to imidacloprid.

Background

MicroRNAs (miRNAs) are between 18 and 23 nucleotides in length and have been shown to be responsible for the post-transcriptional regulation of mRNAs. miRNAs can silence expression of target genes by complementarily binding to 5'/3' untranslated regions (UTRs) or coding sequences.^{1–5} UTRs of target mRNAs are mainly bound through imperfect complementary base pairing.⁶ The sequence of the target is an exact match to the miRNA.⁷

miRNAs have crucial roles in development in many organisms and regulate most mammalian protein-coding genes,⁸ which makes them critical mediators in a series of physiological and developmental pathways,^{9–12} Such as apoptosis, cell proliferation, morphogenesis, and the metabolism of compounds.^{5, 13}

MiRNAs can mediate the detoxification/metabolism of xenobiotics through regulation of the expression of detoxification genes, including nuclear receptors and xenobiotic-metabolizing enzymes.⁵ For example, human UDP-glucuronosyltransferase (UGT) 1A, which metabolizes raloxifene metabolites, was shown to be regulated post-transcriptionally by mir-491-3p,¹⁴ and human P450 CYP1A1, which is associated with the metabolism of carcinogenic and metabolites, can be regulated post-transcriptionally by miR-892a.¹⁵

Previous studies conducted in *Plutella xylostella* indicated that miRNAs could be involved in chlorantraniliprole resistance by regulating the expression levels of UGT genes.¹⁶ *UGT2B7* and *UGT2B15* were shown to be regulated post-transcriptionally by miRNAs;¹⁷ *Culex pipiens* miRNAs are involved in pyrethroid resistance by regulating the expression levels of P450 genes.^{18, 19} There are many miRNAs known to be involved in regulating enzymes that metabolize xenobiotics in animals, while the effects of miRNAs on the regulation of xenobiotic detoxification/metabolism in wheat aphids is less understood.

The grain aphid *Sitobion avenae* is the main pest of wheat and is distributed worldwide. *S. avenae* not only harms crops by sucking juice from plants but also spreads yellow dwarf virus disease, leading to serious economic losses. The control of this aphid has depended largely on the use of chemically synthesized pesticides,²⁰ such as imidacloprid, which is a neonicotinoid that targets sucking pests, such as aphids and the green leaf bug.²¹ It has been widely applied in wheat fields to control wheat aphid in China. As a result, *S. avenae* has developed serious resistance to imidacloprid.²²

Here, we performed high-throughput sequencing of short RNA libraries of control and imidacloprid-treated *S. avenae* nymphs to detect differentially expressed microRNAs, and determine the functions of miRNAs in regulating genes involved in the metabolism/detoxification of xenobiotics. The results of this study will help increase our understanding of the roles of miRNAs in the regulation of resistance to imidacloprid in insects.

Methods

2.1 Culture conditions

The susceptible strain of *S. avenae* used was maintained in a greenhouse for more than 10 years and has never been exposed to any insecticides. The culture conditions used were described previously.²³

2.2 Imidacloprid treatments

To test the susceptibility of *S. avenae* to imidacloprid, the leaf-dipping method was conducted as previously described by Chen *et al.*²⁴ with minor modifications. Imidacloprid was dissolved in acetone, and then serially diluted with 0.05% (v/v) Triton X-100 in water. Wheat leaves were cut into pieces 20 mm long. The leaves with aphids were dipped into 0.05% (v/v) Triton X-100 in water with 0.5 mg/L imidacloprid (LC₁₀ concentration, Table S1) or without imidacloprid as a control. The leaves with treated aphids were put into a glass tube (6 cm in length, 2 cm diameter) and the open end was covered with cotton to prevent insect escape. Surviving third instar nymphs (N = 5 for each treatment) were collected at 24 h after imidacloprid or mock challenge. Aphids were flash frozen in liquid nitrogen before being stored at -80°C for RNA extraction. The experiment was replicated three times.

2.3 RNA isolation, miRNA library construction and Illumina sequencing

Total RNA was extracted from control and imidacloprid-treated *S. avenae* using TRIzol reagent (Invitrogen, Shanghai, China) according to the manufacturer's protocol and resuspended in nuclease-free water. Electrophoresis on 1% agarose gels was used to determine if the extracted RNA was degraded or contaminated, and then purity, concentration and integrity of the RNA were measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE), a Qubit® RNA Assay Kit with a Qubit® 2.0 Fluorometer (Life Technologies, CA, USA), and a RNA Nano 6000 Assay Kit with the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA), respectively. Approximately 3 µg of total RNA from each sample was used as input material for constructing the small RNA library, and the platform used for sequencing was conducted in Novogene, Beijing, China as previously described.²⁵

2.4 Bioinformatics analysis, miRNA prediction, and miRNA target prediction.

S. avenae miRNAs, were confirmed using miRDeep2 software.²⁶ Raw reads from the four libraries (imidacloprid treatment and control, two biological replicates each) were used as input for miRDeep2, and the data from each library were separately analyzed. The default options and settings were used to perform the miRDeep2 analysis. The raw read sequences with polyA tails and miRNAs with lengths ranging from 18 to 30 nt were selected for further analysis after trimming adaptor sequences and removing snRNA, rRNA, snoRNA and tRNA sequences. The sequences that mapped to the predicted mature *Acythosiphon pisum* miRNAs annotated in miRBase were identified as known mature miRNAs. The clean reads from the *S. avenae* transcriptome have been deposited in the NCBI/SRA database under accession number SRP182781. The Miranda, RNAhybrid, and Target Scan programs were used to predict the target gene of the miRNAs according to the miRNA-binding sites.²⁷⁻²⁹ Then only the binding sites commonly identified by the three tools were selected for subsequent analysis. The putative miRNA targets were used as queries in searches against the *S. avenae* transcriptome sequences.

The predicted miRNA target genes were selected for further analysis. The predicted target genes were aligned using the NCBI BLASTX program, and mapping and annotation of the gene sequences were conducted using BLAST2GO.³⁰ The Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases were used to further analyze the putative target genes of the miRNAs.

2.5 miRNAs differentially expressed between control and imidacloprid-treated *S. avenae*

In order to identify the aphid miRNAs that were affected by imidacloprid, the differences in the expression levels of miRNAs, as assessed by miRDeep2, between control and imidacloprid-treated *S. avenae* were determined. The edgeR software package (3.10.2) obtained from Bioconductor version 3.1 was used to analyze the read counts of the identified miRNAs

(<http://www.bioconductor.org/packages/release/bioc/html/edgeR.html>).³¹ edgeR is a Bioconductor software package for analyzing the differential expression of digital

gene expression data. The P-values were calculated using the Benjamini-Hochberg method.³² A corrected P-value of 0.05 was set as the default threshold for significance. Normalization of miRNA counts

between libraries of the controls and treatments was executed according to the total number of reads across libraries. Normalized expression = Actual miRNA count/Total count of clean reads $\times 10^6$. A fold change in miRNA expression ≥ 2 with a false discovery rate (FDR) < 0.05 was deemed to be significant.

2.6 Quantitative real-time PCR (qPCR) validation

To validate the miRNA data obtained by deep sequencing, the expression levels of three miRNAs were confirmed by qPCR. First strand cDNA was synthesized from 2 μg of total RNA using the miScript II RT kit (Qiagen) following the manufacturer's instructions. The SYBR Green Master Mix (miScript SYBR Green PCR Kit, Qiagen) was used for miRNA expression assays, and qPCR was performed

as previously described.³³ Relative expression was calculated using the $2^{-\Delta\Delta C_t}$ method.³⁴ U6 snRNA was used to normalize the expression levels of miRNAs as an endogenous control. The primers used are shown in Table 1.

2.7. MiRNA inhibitor feeding *in vitro*, and the subsequent impacts on imidacloprid susceptibility

The rearing method and the artificial diet used were the same as previously described.³⁵ A sterilized transparent glass tube with both ends open was used as the feeding device (4 cm in length, 2.5 cm diameter). The artificial diet, 25% sucrose, was put between the two layers of parafilm were sealed or was the parafilm containing the artificial diet used to seal one end of the feeding device, and then healthy apterous adults were transferred to the device; the ends were covered with mesh to prevent the insects from escaping. Each sample contained three replications.

To evaluate the inhibitory effect of the miRNAs on *CYP6A14*, 50 healthy apterous aphids were fed an artificial diet containing miRNA inhibitors at a final concentration of 2.5 mM/L. The NC-inhibitor (a negative control) was used as the control. Following feeding for 24 h, the surviving aphids were collected for RNA extraction. There were three replicates for each sample.

To evaluate the effects of miRNAs on the sensitivity of *S. avenae* to imidacloprid, 50 healthy apterous adults were fed an artificial diet that contained imidacloprid (at the LC_{50} concentration, 1.5 mg/L) with an miRNA inhibitor (2.5 mM/L). The NC-inhibitor was used as the control. Three replicates were carried out, and mortality was scored at 48 h. The miRNA inhibitors used were provided by Sangon Biotech Co., Ltd (Shanghai, China).

Results

3.1 Illumina sequencing data analysis

A total of 14,841,584 raw reads were obtained from sequencing the control library and 13,964,533 raw reads were obtained from the imidacloprid treatment library (Table 2). The lengths of the *S. avenae* sRNAs ranged between 18 nt and 35 nt, comprising 5.28% of the imidacloprid reads and 5.08% of the control reads were 22 nt in size (Fig. 1A). After discarding low quality reads and adapter sequences,

6,992,451 (47.11% of total) and 6,349,363 (45.47%) unique mappable reads remained from the control and imidacloprid treatment libraries, respectively (Table 2). The length distribution of these mappable reads indicated that 3.75% of the total reads for the control and imidacloprid treatment, respectively, were 22 nt in length and 5.18% and 6.23% of the total reads, respectively, were 23 nt in length. The maximum lengths of reads from the imidacloprid and control libraries were 32 nt (8.59% of all reads) and 34 nt (9.76%), respectively, these reads are likely piRNA-like sRNAs in *S. avenae*. A few sRNAs had over a thousand reads; however, most had fewer than 10 copies (Fig. 1C).

The dominance of uracil (U) at the first position of the 5' terminus is considered to be a conserved feature of mature miRNAs. Given that the first base of the 5' end of miRNAs is known to play very important roles in the interaction between miRNAs and Argonaute complexes, the position-specific occurrence of nucleotides in the candidate miRNA sequences was analyzed. *S. avenae* miRNAs showed a bias towards U at the first nucleotide position; sRNAs showed a significant bias for U at the 5' (96.99%, and 97.51%, respectively) and 3' ends (53.38%, and 55.32%, respectively) in both libraries. The nucleotide U was the most abundant nucleotide at most of the positions. This higher abundance of U was significant at positions 1, 6, 9, 12, 16, and 22 based on analysis of the base composition of the miRNAs at each position.

3.2 Sequence and prediction of miRNAs

According to the distribution of read lengths, 22-nt miRNAs accounted for 43.86% of all species of miRNAs (Fig. 1D). The putative miRNAs in *S. avenae* were used as queries in searches against the miRNAs from the *A. pisum* genome in miRBase, and 36 conserved sequences were identified (Table S2). In addition, using the filtered *S. avenae* reads as queries in searches against mature miRNAs identified 26 miRNA sequence families from the miRBase database (Fig. 3A). It is predicted that the miR-10 family has the largest number of miRNAs ($n = 415$), followed by let-7 ($n = 401$), and miR-2 ($n = 180$). Insects were shared with these miRNAs, with the majority of miRNAs shared with the known *A. pisum* miRNAs, while a fewer extent miRNAs trended for other insect orthologs (Fig. 3B).

A total of 21 potentially novel miRNAs were identified; these were named with the prefix 'PC' (predicted candidate) based on the adopted nomenclature (Table S3). These novel miRNAs could be mapped to *S. avenae* transcriptome sequences. Mfold predictions revealed stem-loop hairpin secondary structures (<18 kcal/mole³⁶) that were consistent with miRNA precursor sequences. Some of the predicted structures are shown in Fig. S1.

Differential expression of miRNAs between the imidacloprid treatments and controls was also determined according to normalized differences between Illumina read counts. Of the 57 *S. avenae* miRNAs, 16 (28.1%) were differentially expressed, 11 of which were down-regulated and 5 of which were up-regulated (Table 3).

The PCR amplification products of api-miR-1000, api-miR-316, and api-miR-iab-4 were single bands with the expected sizes between 60 and 100 bp. Subsequent qPCR analysis confirmed that the expression

level of api-miR-1000 was 5.5-fold higher in the imidacloprid treatment samples than in the controls. The expression levels of api-miR-316, and api-miR-iab-4 were 91%, and 88% lower, respectively, in the imidacloprid treatment samples compared with the controls (Fig. S2). Overall, the results of qPCR analysis were similar to those of RNA-seq.

3.3 Prediction of miRNA targets.

The hypothetical miRNA target sequences within the UTRs of transcripts from *S. avenae* (including the targets of the 16 differentially expressed miRNAs) were predicted using Target Scan software (Table S4). To identify the possible functions of these hypothetical target genes, GO enrichment analysis was performed; target genes were assigned molecular function, biological process and cellular component terms (Table S5). The predicted miRNA target genes were annotated to 58 GO level 2 terms (Fig. 4A), like some of these terms are related to metabolic processes.

In KEGG enrichment analysis miRNA target genes were assigned to 71 metabolic pathways, indicating that these pathways might be targeted by miRNA regulation (Fig. 4B). The orthologs of some miRNA targets are involved in biological pathways such as xenobiotic metabolism, indicating that miRNAs might be involved in the posttranscriptional regulation of these pathways (Table S6).

To confirm the potential function of miRNAs in the resistance of *S. avenae* to imidacloprid, the predicted target genes of the miRNAs that might participate in xenobiotic metabolism were further focused on. Interestingly, target genes for some miRNAs, such as those encoding cytochrome P450s, UDP-glucuronosyltransferase and glutathione S-transferase, have been found to play critical roles in insect responses to xenobiotics (Table 4). Some miRNAs have many putative target genes, and many putative target genes are potentially regulated by multiple miRNAs (Table S3).

3.4 Modulation of miRNAs impacts the expression of predicted target genes and the susceptibility of *S. avenae* to imidacloprid

The expression levels of miRNAs miR-1000, miR-316, and miR-ab-4 were significantly lower in *S. avenae* adults fed an artificial diet containing the corresponding miRNA inhibitors than in adults fed the DEPC water control or NC control. The depression efficiencies of miR-1000, miR-316, and miR-ab-4 reached 85.0%, 89.1%, and 88.2%, respectively (Fig. 5A). The transcriptional levels of *CYP6A14* were dramatically increased by 2.48-fold in the miR-306-knockdown aphids. However, inhibition of the other two miRNAs had no significant effect on *CYP6A14* transcript levels (Fig. 5B). Under imidacloprid stress, mortality decreased by 21.73% in the aphids fed miR-1000 inhibitors compared with those fed the controls. In contrast, mortality increased by 26.75% and 21.86% in the aphids fed miR-316 and miR-ab-4 inhibitors, respectively, compared with those fed the controls (Fig. 5C).

Discussion

It is predicted miRNAs can regulate majority of protein-coding genes.³⁷⁻³⁹ It has also been demonstrated that miRNAs are involved in the regulation of various biological processes. In this study, miRNAs in *S. avenae* that might play regulatory roles in target gene responses to imidacloprid were identified and assessed. The identification and functional analysis of miRNAs differentially expressed in imidacloprid-treated *S. avenae* were conducted to increase our understanding of the regulation/detoxification of imidacloprid in insects. So far, high-throughput sequencing of small RNAs has been widely used to obtain miRNAs from a series of organisms, including *S. avenae*. Li et al.⁴⁰ identified 345 miRNAs from winged and wingless *S. avenae*, whereas we only identified 57 miRNAs in control and imidacloprid-treated *S. avenae* as we only selected *Acyrtosiphon pisum* as a reference. However, our sequencing data greatly enlarges the range of available information about the involvement of miRNAs in the regulation of metabolism genes and provide a critical basis for further studying miRNAs related to insecticide resistance in *S. avenae*.

Our analysis of read counts indicated that 16 miRNAs were differentially expressed between imidacloprid-treated and control *S. avenae*, indicating that imidacloprid could modulate the expression of miRNAs and suggesting a probable regulatory role of these miRNAs in the detoxication/metabolism of insecticides in *S. avenae*. Several miRNAs up/down-regulated by imidacloprid treatment, e.g., api-miR-1000, api-miR-316, and api-miR-iab-4, could be involved in the regulation of imidacloprid metabolism. The activity-dependent expression of *Drosophila melanogaster* miR-1000 could indicate a mechanism for allowing neuronal activity to fine-tune the strength of excitatory synaptic transmission, and regulate the expression level of vesicular glutamate transporters, and load the glutamate into synaptic vesicles.⁴¹ A target site of *D. melanogaster* dme-miR-1000 is located in the intron of musashi,⁴² which encodes an RNA-binding protein, that regulates gene translation by prior expression in the nervous system.⁴³ Honeybee miR-1000 is also thought to be involved in musashi function in the nervous system.⁴⁴ The first miR-1000 identified in insects, and is expressed in a highly selective manner in insect brains. One cytochrome P450 and two glutathione S-transferase genes could be recognized by mse-miR-316.⁴⁵ The accumulation of endogenous Ubx protein could be attenuated by ectopic expression of miR-iab-4-5p, and a classical homeotic mutant phenotype could also be induced (the transformation of halteres into wings).⁴⁶ *Drosophila* dme-miR-iab-4 and dme-miR-iab-8 are miRNAs targeting bithorax-complex (BX-C) HOX bunches and participate in the regulation of reproduction and neural patterning through suppression of HOX gene targets.⁴⁷

In order to further explore the role of miRNAs in the regulation of imidacloprid metabolism, the hypothetical target genes for the miRNAs identified were predicted, and many of them were annotated to biological processes. The target genes of several miRNAs from different families were previously shown to be involved in xenobiotic detoxication/metabolism.⁴⁸⁻⁵¹ By combining differential analysis with target gene prediction, we also found that several of the miRNAs targeting genes putatively involved in xenobiotic detoxication/metabolism were expressed differentially between the imidacloprid-treated and control *S. avenae*. *S. avenae* CYP6A14 could be regulated by api-miR-316 in our present study. Furthermore, the mortality of *S. avenae* exposed to imidacloprid significantly decreased after being fed an

api-miR-1000 inhibitor. In contrast, the mortality of *S. avenae* exposed to imidacloprid significantly increased after being fed an api-miR-316 or api-miR-iab-4 inhibitor. The differential expression of let-7 and miR-100 might be attributable to the influence of insecticides. A similar phenomenon was also observed in the host adaption of *Myzus persicae* to nicotianae, where let-7 and miR-100 participated in the post-transcriptional regulation of the expression of *CYP6CY3*. Our results show shows that differentially expressed miRNAs may participate in the metabolism of imidacloprid by modulating the expression of genes related to xenobiotic metabolism.

In conclusion, of 57 miRNAs identified in *S. avenae*, the transcript levels of 16 were differentially regulated by imidacloprid treatment. The prediction of target genes related to xenobiotic metabolism showed that differentially expressed miRNAs could participate in the detoxication/metabolism of imidacloprid. The data presented here represent a crucial new genomics resource for further study of small RNAs in *S. avenae*.

Abbreviations

miRNAs: MicroRNAs; qPCR: Quantitative real-time PCR; BLASTX: Similarity search of the NCBI protein database using a translated nucleotide query; GO: Gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; nr: NCBI non-redundant protein sequences database; nt: NCBI nucleotide collection; UTRs: untranslated regions; SRA: Sequence Read Archive.

Declarations

Acknowledgements

This work is supported by the National Key Research and Development Program of China (2017YFD0201700), the Key Science and Technology Program of Henan (Agriculture) (182102110053), and Project of Plant Protection Key Discipline of Henan Province (1070202190011005).

Funding

Not applicable

Availability of data and materials

The transcriptome clean reads and computationally assembled sequences from this study were submitted to the NCBI/SRA database under accession number: SRX374716. The file for edgeR is the file 1.

Author contributions statement

X.G and X.C conceived and designed the experiments, B.Z., and J.L. performed the experiments, B.Z., X.S., S.Z., and X.G. analyzed the data and wrote the manuscript. J.L., and L.L. participated in the data analysis.

All authors reviewed the manuscript.

Competing interest

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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Tables

Table 1. Primers used in the study

Primer name	Sequence (5'-3')	Size (bp)	Application
api-mir-1000	F:AGAGCACGATATTGTCCTGTCACA	73	qPCR
	R:TATGGTTGTTTCACGACTGGTTCAC		qPCR
api-mir-316	F:TTTGGTGTCTTTTTCCGCTTTG	69	qPCR
	R:GTGCAGGGTCCGAGGT		qPCR
api-mir-iab-4	F:AGGCGAAGCGACGTATACTAAATG	68	qPCR
	R:GTGCAGGGTCCGAGGT		qPCR
U6	F:CAGCACATATACTAAAATTGGAACG	76	qPCR
	R:ACGAATTTGCGTGTTCATCC		qPCR
MicroRNA inhibitor N.C	CAGUACUUUUGUGUAGUACAA	21	miRNA function
api-mir-1000 inhibitor	UACUGCUGUGACAGGACAAUUAU	22	miRNA function
api-mir-316 inhibitor	CGGCAGCAAAGCGGAAAAAGACA	23	miRNA function
api-mir-iab-4 inhibitor	UCAGGAUACAUUUAGUAUACGU	22	miRNA function
<i>CYP6A14</i>	F: TGTGCACCAAACAGTTCAGG	107	qPCR
	R:CGGATAGTACTGCGGGTCATG		qPCR
<i>Actin</i>	F:GATGCACCTGGTCACAGAGA		qPCR
	R:CCATCTTGTTACACCAACG		qPCR

Table 2. Distribution of miRNA reads from control and imidacloprid-treated *Sitobion avenae*

Parameter	Control	Imidacloprid treatment
Raw reads	14,841,584	13,964,533
Clean reads	14,224,837	13,492,898
3ADT and length filter	10,959,929	9,305,973
rRNA	253,777	247,747
tRNA	16	23
snoRNA	9,443	7,823
snRNA	25,993	24,246
Mappable reads	6,992,451	6,349,363

Note: 3ADT: reads that were removed because 3ADT was not found and length was less than 15 nt or greater than 35 nt. Mappable reads: the number of raw reads that passed through the many digital filters .

Table 3. The differentially expressed miRNAs in the small RNA libraries of *Sitobion avenae*

miR_Name	Imidacloprid treatment_FPKM	Control_FPKM	P- value	Log ₂ ^(Fold Change)	Difference
api-miR-998	4021.337154	14561.84926	0	-1.8564	Down
Novel-28	435.4314115	2094.17899	1.93E- 286	-2.2659	Down
api-miR-306	691.567536	2386.390012	1.54E- 246	-1.7869	Down
Novel-22	742.7947608	1801.967968	1.83E- 120	-1.2785	Down
Novel-9	717.1811484	1704.564294	1.79E- 110	-1.249	Down
api-miR-3047	332.9769618	779.2293917	3.01E- 50	-1.2266	Down
Novel-25	256.1361244	657.4747992	6.09E- 49	-1.36	Down
api-miR-1000	128.0680622	0	8.16E- 28	8.0008	Up
api-miR-316	0	48.70183698	2.37E- 13	-6.6059	Down
Novel-39	51.22722489	0	4.65E- 13	6.6788	Up
Nove-35	153.6816747	48.70183698	2.31E- 12	1.6579	Up
api-miR-263b	0	24.35091849	1.57E- 07	-5.6059	Down
api-miR-2796	0	24.35091849	1.57E- 07	-5.6059	Down
api-miR-iab-4	0	24.35091849	1.57E- 07	-5.6059	Down
api-miR- 3015a	25.61361244	0	2.52E- 07	5.6788	Up
api-miR-3042	76.84083733	24.35091849	7.05E-	1.6579	Up

Table 4 MiRNAs targeting putative genes related to xenobiotic metabolism in *Sitobion avenae*

miR_Name	Target genes related to xenobiotic metabolism
api-miR-998	S-(hydroxymethyl)glutathione dehydrogenase / alcohol dehydrogenase
novel-28	CYP3A19, glutathione S-transferase omega-1, glutathione S-transferase, C-terminal domain
api-miR-306	Microsomal epoxide hydrolase, glutathione S-transferase, C-terminal domain, P450 (GC vitamin D-binding protein), CYP4C1, CYP4V, CYP9Z4
novel-22	alcohol dehydrogenase 4, UDP-glucuronosyltransferase 2B10, UDP-glucuronosyltransferase 2B7, Cytochrome P450 (piezo-type mechanosensitive ion channel component 2), CYP4C1, Glutathione S-transferase (EEF1G elongation factor 1-gamma), CYP6A14
novel-9	UDP-glucuronosyltransferase 2C1, UDP-glucuronosyltransferase 1-7C UDP-glucuronosyltransferase 2B17, Glutathione S-transferase Mu 1, Glutathione S-transferase (phosphatidylinositol-binding clathrin assembly protein LAP), CYP4g15, CYP4C1
api-miR-3047	UDP-glucuronosyltransferase 2C1, carbonyl reductase 1, Microsomal glutathione S-transferase 1, UDP-glucuronosyltransferase 1-7
novel-25	UDP-glucuronosyltransferase 2C1, UDP-glucuronosyltransferase 1-7, CYP315A1, CYP2U1, P450 (piezo-type mechanosensitive ion channel component 2)
api-miR-1000	alcohol dehydrogenase class-3, S-(hydroxymethyl)glutathione dehydrogenase / alcohol dehydrogenase, glutathione S-transferase, C-terminal domain, CYP3A19,
api-miR-316	UDP-glucuronosyltransferase 2B7, UDP-glucuronosyltransferase 2B10, UDP-glucuronosyl transferase 351A3, Glutathione S-transferase (phosphatidylinositol-binding clathrin assembly protein LAP), Glutathione S-transferase (failed axon connections), Cytochrome P450 (piezo-type mechanosensitive ion channel component 2), CYP4C1, CYP6A2, CYP6A13
novel-39	UDP-glucuronosyltransferase 2B1, Cytochrome P450 (piezo-type mechanosensitive ion channel component 2), CYP4C1, CYP305a1, Cytochrome bd terminal oxidase
novel-35	Glutathione S-transferase GSTZ1
api-miR-263b	Glutathione S-transferase (phosphatidylinositol-binding clathrin assembly protein LAP), Cytochrome P450 (piezo-type mechanosensitive ion channel component 2), CYP49A1, CYP6A14, CYP315A1, CYP4C1, CYP6A13, CYP6J1
api-miR-2796	UDP-glucuronosyltransferase 2C1, UDP-glucuronosyltransferase 2A1, glutathione S-transferase C-terminal domain, CYP6A13, CYP4C1, CYP4V2, CYP49A1,
api-miR-iab-4	UDP-glucuronosyltransferase 2B18
api-miR-3015a	CYP314A1, CYP4C1, CYP6A13, CYP3A19
api-miR-3042	alcohol dehydrogenase class-3, UDP-glucuronosyltransferase 2A1dehydrogenase, glutathione S-transferase C-terminal domain, Glutathione S-transferase (phosphatidylinositol-binding clathrin assembly protein LAP), Cytochrome P450 (piezo-type mechanosensitive ion channel component 2), CYP3A19,
	Glutathione S-transferase (EEF1G elongation factor 1-gamma)

Additional Information

Table S1. Toxicity of imidacloprid on *Sitobion avenae*

Table S2. Summary of conserved miRNAs.

Table S3. Summary of predicted miRNAs.

Table S4. The predicted targets of miRNAs in *Sitobion avenae*.

Table S5. GO annotations for identified *Sitobion avenae* miRNA targets.

Table S6. KEGG analysis of targets of differentially expressed *Sitobion avenae* miRNAs.

Fig. S1. Predicted stem-loop structures for three novel miRNA precursors.

Fig. S2. qPCR validation of miRNAs potentially involved in resistance of *Sitobion avenae* to imidacloprid.

Figures

Figure 1

Characterization of miRNA sequences from *S. avenae*. A: Length distribution of raw reads, B: length distribution of mappable reads, C: frequency distribution of read counts, and D: length distribution of miRNAs.

Figure 2
Nucleotide bias of predicated miRNAs from *Sitobion avenae*.

Figure 3

MiRNAs characterization of *Sitobion avenae*.

Figure 5

Effects of miRNA modulation on CYP6A14 expression and imidacloprid susceptibility. (A) Orally mediated inhibition of miRNAs; (B) Effect of inhibition of the api-miR-1000, api-miR-316, and api-miR-iab-4 miRNAs on the expression of CYP6A14; (C) The effect of modulating the levels of api-miR-1000, api-miR-316, and api-miR-iab-4 using feeding inhibitors on imidacloprid tolerance in *S. avenae*. Mean mortality $\pm$ SE. Error bars indicate 95% confidence intervals. Different letters on the bars of the histogram

indicate significant differences based on ANOVA followed by Tukey's HSD multiple comparison test ($P < 0.05$).

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