High-throughput aequencing reveals the expression pattern of microRNA and xeno microRNA in alfalfa to daily cow serum

Xiaoyan Cai (caixy345@163.com)
Ningxia University
Jingying Jia
Ningxia University
Hongjuan Duan
Ningxia University
Bingzhe Fu
Ningxia University
Yun Ma
Ningxia University

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Abstract

The purpose of this study was to tendency the expression of microRNA and xenomiR in hay of two alfalfa varieties (*cv.Zhongmu No.1*, *cv.Xinyan No.52*), and analyze the expression level of miR-168c, miR-156a and miR-166e of alfalfa in cow serum, milk and colostrum. The miRs in fresh and dry samples of two alfalfa varieties were sequenced by high-throughput RNA sequencing technology, RT-qPCR was used to analyze the expression levels of miR-156a, miR-166e and miR-168c. In addition, the target gene of miR168c in dairy cows was verified by Double-Luciferase Report.The results showed that: (1) 771 miRs were detected, 113 and 84 miRs expression levels in dry samples were up- and down-regulated, 8855 target genes were predicted. (2) miR-156a, miR-166e and miR-168c expression levels in dry samples of two alfalfa varieties were opposite, which were down-regulated in *cv.Zhongmu 1* and up-regulated in *cv.Xinyan 52*. (3) The expression levels of miR-168c, miR-156a and miR-166e in high producing dairy cow serum were significantly higher than low producing dairy cow ($p < 0.05$), the expression levels of miR-168c in milk were higher than those in colostrum. (4)Alfalfa derived miR168c can target mRNA STARD7 in cattle. Our results prove that the miRs in alfalfa can enter into dairy cows and related to the production of dairy cows. These results lay the foundation for further exploration of the cross-kingdom effects of alfalfa xenomiRs on dairy cows.

Introduction

MicroRNA (miRNA, miR) is a kind of endogenous, non-coding small RNA which widely presents in organisms. MiRs enter and bind to the target mRNA (messenger RNA) through complementary base pairing, thus regulate the abundance and function of target mRNAs [1–3], and they play important roles in cell differentiation, proliferation and apoptosis[4, 5]. First found in *Caenorhabditis elegans*, miRs have been detected in a wide range of animals, plants and viruses[6, 7]. In 2002, miRs were first found in plants[8].

cv.Zhongmu 1 (*Medicago sativa* L.) is a salt-tolerant alfalfa variety developed by the Chinese Academy of Agricultural Sciences [9]. Meanwhile, *cv.Xinyan 52* is a new salt-tolerant alfalfa bred by the Agricultural School of Ningxia University. Current research of alfalfa miRs focuses on the regulation of alfalfa stem development, salt stress resistance, drought resistance, fall dormancy and biological yield improvement [10, 11]. However, there are a few reports about the cross-kingdom roles of miRs in alfalfa.

Some plant-derived miRs have been detected in human and animal sera, which were named exogenous miRNAs (xeno-miRNAs). One of the earliest mentions of xenomiRNA was reported by Zhang et al. [12]. Studies have found that proteins, amino acids, vitamins, flavonoids and other nutrients and chemical components, as well as unknown growth promoting factors, which can improve milk yield of dairy cows are riched in alfalfa[13, 14]. Furthermore, feeding alfalfa can affect the miRs expression profile of rumen, duodenum, liver and mammary gland, and change the expression patterns of 35 miRs, which are positively correlated with N utilisation efficiency, while 10 miRs are negatively correlated with miRs in breast tissue, with significant improvements in N conversion efficiency and protein yield ($P < 0.01$) [15].

miRs in mammary gland can affect N-conversion efficiency, such as miR-181a affacts milk protein content by targeting AA transport and phosphorylation gene[4]. In addition, xenomiR-168, miR-166 and miR-156 have detected in bovine blood.

Milk is rich in nutrients, which can be easily absorbed by human body. Milk is an important source of nutrition for mammalian infants, a large number of studies have shown that milk is rich in plant food-derived miRNAs. Anna Lukasik et al. detected five plant-derived miRNAs in whole milk samples of human milk of healthy volunteers by RT-qPCR, including common miR156, miR166 and miR168 with cross-border regulation[16]. And they detected 17 plant-derived miRNAs in pig breast milk, of which miR-168 had the highest expression[17]. Xi Chen et al. found through bioinformatics analysis that although the abundance of plant miR in breast milk is lower than that in saliva and urine, 151 miRNAs can still be detected, of which miR166 is the most common miRs in body fluid[18].

Based on these prior studies, it is evident that we pay attention to whether miRNAs in alfalfa, a common cow diet, can be detected in cow body fluid and play a cross-border regulatory role. Therefore, in this study, miRNAs in two alfalfa strains were sequenced and analyzed. Three alfalfa derived miRNAs were selected to detect their expression in cow body fluid. In addition, the target gene of miR168 with high expression in normal milk was verified. The data in this study are based on a large number of RNA sequencing data and quantitative verification, strongly support the existence and role of alfalfa derive miRNA in cross-kingdom regulatory action within dairy cows.

Materials And Methods

Experimental materials and sampling

Alfalfa *cv.Zhongmu 1* and *cv.Xinyan 52* were used in experiments. Fresh alfalfa were collected in October 2019 from Ningxia Modern Science and Technology Park, China, frozen in liquid nitrogen, and transported to the laboratory and stored at -80 ℃. Two entire alfalfa plant were air-dried, crushed and sieved through a 40-mesh screen comprised as dry sample. Three dry samples and fresh samples were processed for each variety. All samples were collected for subsequent experiments.

RNA extraction, transcriptome sequencing

The RNA samples were extracted with Trizol, and 1% agarose gelelectrophoresis were used to detect RNA whether degraded or contaminated. The alfalfa RNA sequencing were completed by Biomarker technologies (Beijing, China) using Illumina platform. The original image data files obtained from the sequencing were transformed into the raw reads by base calling.

RNA quality control

The RNA library was constructed by RNA-seq technology, and the original image data files were transformed into raw reads by base calling.

Low-quality reads, reads with unknown base contents of 10% or more, and reads without a 3 'joint sequence were removed. The 3' joint sequence was then removed. And reads were trimmed and cleaned by removing the sequences smaller than 18 nt or longer than 30 nt. At the same time, Q20, Q30, GC-content and sequence duplication level of the clean data were calculated. All the downstream analyses were based on clean data with high quality.

miRs identification, novel miRs prediction

Bowtie software was used to compare with the clean reads to sequences in the Silva, GtRNAdb, Rfam and RepBase database. Filtered the ncRNAs such as ribosomal RNA (rRNA), transfer RNA (tRNA), nuclear small RNA (snRNA) and repeated sequences and obtain unannotated reads containing miRs.

For the identification of known miRs, compared the mature sequences of the known miRNAs in the miRBase (V22) database with the reads of the reference genome and the range of 2nt upstream and 5nt downstream, and identified the known miRNAs. miRdeep2 software was used to predict the plant miRs among RNA sequences by adjusting the parameters and scoring system. Prediction of the new miR sequences was based on the distribution reads of the precursor sequences and the structural energy of the precursors, as determined by RNAfold. A bayesian model was used to score and finally predict the new miRs.

Analysis base preference of miRs

When Dicer and DCL enzymes recognise and cleave an miR precursor, the first base pair at the 5' terminus has a strong bias toward U. We analysed the base preference of miR. After base preference analysis, we then calculated the typical base ratio of miR.

Calculation of miRs expression

The sequencing reads of miRs in each sample were counted, and the expression was normalized by TPM (transcripts per million). TPM normalization formula is

$$\mathrm{TPM}=\frac{\mathrm{Re}\,adcount*1{,}000{,}000}{Mapped\,\mathrm{Re}\,ads}$$

Read count indicates the number of reads compared to a certain miRs, and mapped reads indicates the number of reads compared to all miRs.

Differential expression analysis

Differential expression analysis of two conditions/groups was performed using the DESeq2 R package (1.10.1). DESeq2 provide statistical routines for determining differential expression in digital miRNA expression data using a model based on the negative binomial distribution. The resulting P values were

adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate. miRNA with $|\log2(FC)| \geq 1.00$;FDR ≤ 0.01 found by DESeq2 were assigned as differentially expressed.

Target genes prediction and functional gene annptation of miRs

Target finder (http://targetfinder.org/index.php/findtargets) software was used to predict target genes. BLAST software was used to compare the predicted target gene sequences with sequences deposited in the NR, Swiss prot, go, COG, KEGG, KOG and Pfam databases. Results were used to annotate the target genes.

Detection of miR-168c, miR-156a and miR-166e in alfalfa by qPCR

miR-156a, miR-166e and miR-168c were screened from alfalfa, and verified by qPCR. The results of qPCR were divided into three groups for comparison. The qPCR results of 5s RNA as an internal reference.

The primer sequences of miR-168c, miR-156a and miR-166e for qPCR are shown in Table 1.

Table 1
miR-168c、miR-156a、miR-166e primer sequences

miR ID	Sequence
mtr-miR-168c	CTTGGTGCTGGTCGGGAAA
mtr-miR-156a	CTGACAGAAGAGAGAGAGCACAAA
mtr-miR-166e	GACCAGGCTTCATTCCCCA

Detection of Alfalfa miRs in dairy cows serum, colostrum and milk

To verify whether the miR in alfalfa can play a cross-kingdom regulatory role in dairy cows, three high producing dairy cows and three low producing dairy cows were selected to collect blood samples from the tail vein, the high producing dairy cows milk and colostrum also been selected. The miRNAs in serum and milk of dairy cows were extracted, and the oxidation experiment was carried out to verify whether they were plant miRNAs. RT-qPCR was used to determine whether the plant-derived miRNA in serum, colostrum and milk of high-yield dairy cows changed significantly.

Dual-Luciferase Reporter Assay

The binding sites of miR168c and candidate target genes were analyzed by targetscan database. The dual-luciferase reporter system was used to investigate whether StAR related lipid transfer domain containig 7 (STARD7) was the direct target gene of miRmiR168c. miR168c mimics and miR168c mimics NC were co-transfected into 293T cells with the constructed mutant and wild type vectors of candidate target genes, the dual-luciferase cativity was detected 48 hours later.

Statistical analysis

Date are reported as mean(± SD) and were analyzed by unpaired Student's t test. The significance level was prespecified as having a p-value < 0.05.

Results

RNAseq of dry Alfalfa reveals presence of miRs

In order to determine the type and quantity of miRs in alfalfa, we performed RNA sequencing on two alfalfa accessions. Sequencing results yielded 20,981,902 clean reads from dry samples and 18,619,197 from fresh samples of *Medicago sativa cv.* Zhongmu 1 (MsZM1). Similarly, there were obtained 24,378,571 and 22,020,880 clean reads, respectively, from dry and fresh samples of *Medicago sativa cv.* Xinyan No.52 (MsXY52). The total number of clean reads generated from each sample was greater than 15.42 M. The clean reads were then mapped to the reference genome (Medicago_truncatula.Mt4.0v1) (Table 2).

We next examined which specific reads could be identified as miRs and whether they were previously reported or novel. Analysis using the miRbase(v22) database revealed 771 miRs among the total clean reads from alfalfa samples, of which 548 were previously reported and 223 were newly predicted miRs (Table 2). These miRs belonged to 590 miR families. Supplementary Fig. 1 summarizes base preference for this set of miRs.

We also generated TPM density distribution maps based on the transcription levels of miRs in each sample. The results showed that similar peak heights between dry (MsZM1G) and fresh samples(MsZM1F) of MsZM1 (Fig. 1a), whereas miR transcript levels in dry MsXY52 (MsXY52G) were slightly higher than that in fresh samples(MsXY52F) (Fig. 1b). These results indicated that miRs could survive from the drying process in alfalfa.

Table 2
Expression profile of MsZm1vs MsXY52

Sample	Clean reads	Number of reads mapped to reference genome	Known-miRs	Novel-miRs	Total miRs
MsZm1F	18619197	3507055(25.44%)	421	222	643
MsZm1G	20981902	1835315(17.29%)	411	223	634
MsXY52F	22020880	3550429(26.27%)	461	207	669
MsXY52G	24378571	2502439(22.32%)	395	207	602
Total	-	-	548	223	771

miRs differentially expressed in dry and fresh Alfalfa

In order to investigate whether and how these miRs differed between dry and fresh samples and between cultivars, we next performed differential expression analysis for miR reads (Table 3). A heatmap generated by hierarchical clustering analysis revealed twelve major groups of miRs, which could be distinguished by differential upregulation or downregulation across samples (Fig. 2). In addition, miRs expression levels in dry samples generally showed higher than those in fresh samples.

We next examined differences in the presence of detectable known and novel miRs between dry and fresh samples of MsXY52 (Table 3). For convenience, combined highly homologous miRNAs with low expression (TPM ≥ 1000 reads per sample) and consistent trends in read abundance. We detect miRNA in fresh alfalfa as control, the results showed that 9 miRs were significantly differentially up-regulated, while 11 were down-regulated in dry samples.

Table 3
miRNA levels (for miRs with ≥ 1000 reads in dry samples) in MsXY52G (dry sample) and MsXY52F (fresh sample)

ID	MsXY52F miR average expression	MsXY52G miR average expression	MsXY52GvsMsXY52F miR differential expression level
mtr-miR396a	109086.2	39536.24	down
mtr-miR2643	20197.04	12632.93	down
mtr-miR162	6165.89	5561.32	down
mtr-miR156	2619.56	16817.31	up
mtr-miR166	1575.71	12465.36	up
mtr-miRmiR168c-3p	407.53	2782.55	up
mtr-miR390	451.78	2628.37	up
mtr-miR2592	98.53	1262.04	up
novel_miR_137	60291.63	37209.78	down
novel_miR_82	19887.09	9423.83	down
novel_miR_65	19887.09	9423.83	down
novel_miR_60	19887.09	9423.83	down
novel_miR_101	19887.09	9423.83	down
novel_miR_174	19887.09	9423.83	down
novel_miR_192	19633.37	9004.19	down
novel_miR_19	19633.37	9004.19	down
novel_miR_85	525	2632.6	up
novel_miR_49	363.43	1956.92	up
novel_miR_18	230.31	1111.89	up
novel_miR_146	161.83	1016.85	up

Then, miR transcript levels in fresh and dry samples of MsZM1 were compared (Table 4). In total, 9 up-regulated and 8 down-regulated miRs were identified in comparisons of dry vs. fresh MsZM1 alfalfa, which supported the finding of miRs transcript abundance in dry samples higher than those in fresh samples of MsXY52.

Collectively, these results showed that miRs differentially accumulated levels in dry and fresh sample, indicated their resistance to degradation and drying process.

Table 4
Expression levels of miRs in MsZm1G and MsZm1F

ID	MsZm1F miR average expression	MsZm1G miR average expression	MsZm1GvsMsZm1F miR differential expression level
mtr-miR396a-5p	94603.75	45973.84	down
mtr-miR166g-5p	860.91	8685.76	up
mtr-miRmiR168c-3p	502.52	3666	up
mtr-miR172d	723.45	3363.21	up
mtr-miR156	197.85	2466.86	up
mtr-miR398a-5p	155.15	1956.46	up
novel_miR_137	30408.67	20578.63	down
novel_miR_82	15671.26	6701.34	down
novel_miR_65	15671.26	6701.34	down
novel_miR_60	15671.26	6701.34	down
novel_miR_101	15671.26	6701.34	down
novel_miR_174	15671.26	6701.34	down
novel_miR_192	15571.74	6381.35	down
novel_miR_85	748.45	3516.34	up
novel_miR_49	276.36	1453.77	up
novel_miR_57	1633.99	1405.89	up
novel_miR_12	1603.87	1390.42	up

Differential expression of miRNAs in fresh and dry samples of cv.Zhongmu 1 and cv.Xinyan 52

We next validated the results of RNA-seq using RT-qPCR analysis to focus on the well-known xenomiRs miR-156a, miR-166e, and miR-168c, which confirmed the results of our above differential expression analysis between fresh and dry samples of each cultivar. In addition, the results of TPM normalization

and differential accumulation analysis showed significant down-regulation of these three xenomiRs in MsZM1 samples (Fig. 3a, 3b) *(P < 0.05).*

miR-156a, miR-166e, and miR-168c were up-regulated in dry samples of MsXY52, and the sequencing results were consistent with RT-qPCR results (Fig. 3c,3d) .

Expression levels of miRNA in dry samples of cv. Xinyan 52 and Zhongmu 1

We next compared the expression levels of these three xenomiRNAs across dry samples from alfalfa, and found that miR-166e and miR-168c expression levels in MsXY52 were significantly higher than those in MsZm1*(P < 0.05)* (Fig. 4) .

Target gene prediction and KEGG Analysis

In order to better understand the functions of the novel miRs we identified in alfalfa, we next performed target gene prediction analysis. Among the 548 known miR sequences, 535 putative target loci were identified, which contained a total of 6794 potential target genes, whereas among the 223 newly predicted miRs, 188 putative target loci were identified., which contained 2768 putative target genes. The total number of annotations for all miRs target genes and the number of annotations of miR target genes in different samples are shown in Table 5. The different combinations of miRs target genes in each database also annotated (Supplement Table.2).

Table 5
Target gene annotation results statistics

Anno-database	Annotation-Number	30 ≤ length < 1000	length > = 1000
COG-Annotation	3375	455	2912
GO-Annotation	6783	1739	4789
KEGG-Annotation	2533	502	2024
KOG-Annotation	4276	911	3316
Pfam-Annotation	6900	1501	5309
Swissprot-Annotation	6392	1407	4907
nr-Annotation	8832	2445	5804
All-Annotation	8833	2446	5804

Note: The column information in the tables is described below:
Anno-database: annotation detabase; Annotation-Number: the number of commrnts in each annotation database; length is the length of target mRNA.

GO enrichment analysis of target gene

Gene ontology enrichment analysis reveals that the target genes of differentially expressed miRNAs were significantly enriched in the following pathways: cellular component, molecular function, and biological process. At the level of cellular componen, membrane is the most abundant functional item. At the level of molecular function, binding and catalytic activity is the most abundant functional item. Metabolic process and cellular process are the most abundant functions in biological process (Fig. 5).

KOG enrichment analysis of target genes

All miRs were classified according to the sequence numbers used for KOG functional enrichment analysis. The following functions were the most commonly identified, in descending order: transcription, general function, signal transduction, carbohydrate transport and metabolism (Fig. 6).

Detection of alfalfa xenomiRNAs in cow serum, colostrum, and normal milk

Considered that miR-156a, miR-166e and miR-168c expression levels were up-regulated in dry samples of both *cv.Xinyan 52* and *cv.Zhongmu 1* (supplement Table 1). Moreover, it has been shown that miR-156a, miR-166e and miR-168c can be detected in animals. Therefore, this study carried out RT-qPCR analysis these miRs relative expression levels in bovine serum, colostrum and normal milk (Fig. 7). The expression levels of miR-156a, miR-166e and miR-168c in the serum of high producing dairy cows were significantly up-regulated *($p < 0.05$)*. miR156a*($p < 0.05$)* and miR166e expression levels in colostrum were higher than in normal milk, miR168c expression level is contrary*($p < 0.05$)*. This not only means that the miRs in alfalfa can indeed enter dairy cows, but also proves that high-producing dairy cows and alfalfa miRs are corelated. Moreover, miRs from alfalfa has stronger effect on colostrum than normal milk. It provides a basis for the cross-kingdom regulation based on miRNA in alfalfa. And also laid a foundation for the regulation of dairy cow milk quality by alfalfa miRs, because dairy cow colostrum is richer in nutrients.

Mtr-miR168c target STARD7 (StAR related lipid transfer domain containing7)

The luciferase activity of 293T cells co-transfected with STARD7-wt + mimics mtr-miR168c group were significantly lower than that transfected with STARD 7-wt + mimics NC mtr-miR168c internal reference group*($p < 0.05$)*. The results showed that mimics mtr-miR168c could inhibit the luciferase activity of wild-type STARD7 plasmid. However, there was no significant difference in luciferase activity between STARD7-mut + mimics mtr-miR168c co-transfected 293T cells and internal reference group, which further showed that mimics mtr-miR168c could not inhibit the luciferase activity of mutant STARD7 plasmid(Fig. 8). The above experimental results show that alfalfa derived miR168c can cross kingdom target the STARD7 gene in dairy cows, which lay a foundation for alfalfa miR to cross kingdom regulate the traits of dairy cows.

Discussion

Both *cv.Zhongmu 1* and *cv.Xinyan 52* have good salt and drought resistance, and many results have shown that drought resistance of alfalfa is related to miRs[19]. Therefore, we speculate that there is little difference in the types of miRs in alfalfa between the two salt and drought resistant varieties. According to our sequencing results, it can be found that there is little difference in the miRs between the two alfalfa strains, but there are more known types of miRs in the newly cultivated strain *cv.Xinyan 52*, which may be related to the better salt and drought resistance of *cv.Xinyan 52*.

In addition, different treatment methods of the same alfalfa strain will also cause the differential expression of miRs. There were 20 differentially expressed miRs in dry and fresh samples of *cv.Xinyan 52* and 17 differentially expressed miRs in *cv.Zhongmu 1*. Among them, the most common miR156, miR166 and miR168 with cross-border regulation were down-regulated in *cv.Zhongmu 1* dry sample, but up-regulated in *cv.Xinyan 52*. This alfalfa was commonly preserved as hay, we detected the presence of miRs in the hay of two alfalfa strains, which proved that they had chemical stability during the harvesting and drying process experienced under field conditions as well (narigenin and luteolin in dried lucerne samples suggest their chemical stability during the harvesting and drying process experienced under field conditions as well) [12, 20, 21]. The expression of verified cross-border regulatory miRs was up-regulated in *cv.Xinyan 52* dry samples, suggesting that miRs in this variety may have stronger cross-border regulatory ability. Braderick test found that compared with alfalfa silage, feeding alfalfa hay could significantly increase the daily gain (trial1: 3.07 vs 3.08; trial2: 2.94 vs 3.10) and milk protein content (trial1: 3.02 vs 3.08; trial2: 2.90 vs 3.01) [22], which is consistent with the research results of khadem AA and Zhang SZ teams, and alfalfa hay can increase the blood glucose of dairy cows[23, 24]. In addition, feeding fresh alfalfa samples will increase the concentration of phytoestrogen in heifers and destroy their reproductive function, but feeding hay feed will not, but this result needs to be further verified[25]. Therefore, we believe that alfalfa hay feeding cows has a better effect on the improvement of milk quality and reproductive system.

Fanlin Kong's research shows that alfalfa hay has a high proportion of acetate, acetic acid and propionic acid, which may promote the production of milk fat by providing acetate[26]. The regulation of alfalfa hay on milk fat of dairy cows must be multifaceted. Some studies have proved that adding alfalfa hay to the diet will reduce the concentration of milk fat, but will not affect the yield of milk fat[24, 27, 28]. These studies have proved that feeding alfalfa hay can affect milk fat content, but researchers have not reached a unified conclusion on how to regulate milk fat content. We further focused on whether miRs in alfalfa could regulate cow milk fat. Zhang L's team demonstrated first time that plant-derived miR168 can regulate low density lipoprotein receptor adapter protein 1 (LDLRAP1) mRNA in humans and mice, and then regulate lipids in liver tissue[12]. So whether alfalfa derived miR168 can regulate milk fat also has research significance. miR168 was detected in the cow's serum, colostrum, and normal milk, which laid a foundation for us to verify the conjecture. In addition, we verified that STARD7 was the target gene of

miR168. STARD7 is a lipid transporter, participate in the synthesis and secretion of steroids[29–33].Studies have shown that miR-206 is known to proliferate adipocytes by directly targeting STARD7[34], which proves that STARD7 has a regulatory effect not only on steroids, but also on lipids.

This study confirmed that alfalfa derived miR can be detected in serum, colostrum and normal milk of dairy cows, and proved that alfalfa derived miR has the potential of cross-border regulation. The target gene STARD7 of miR168 was further verified. Through previous studies, it was known that STARD7 has the effect of lipid transport, but whether it can regulate milk fat is worthy of further research and verification.

Conclusion

Summary, totally771 miRs in alfalfa were sequenced, there are 548 known miRs and 223 novel miRs of them. The expression patterns of miR-166e and miR-168c in the two varieties were opposite, down-regulated in *cv.Zhongmu 1* dry sample and up regulated in *cv.Xinyan 52*. The expression of miR-156a, miR-166e and miR-168c in *cv.Xinyan 52* were significantly higher than that in *cv.Zhongmu 1*. These three miRs in dry samples can cross-kingdom into the dairy cows, among which miR-166e is the highest expression in the serum of dairy cows. And mtr-miR168c was selected and its target gene STARD7 was verified by Dual-Luciferase Reporter Assay. The results lays the foundation and evidence for further study on alfalfa miRs in the dairy cows.

Declarations

Author Contributions

Jingying Jia responsible for experimental verification, data analysis and draft preparation, Hongjuan Duanhelped modify the article, Bingzhe Fu provides experimental samples,Yun Ma responsible for providing the experimental site and article modification ,Xiaoyan Cai is the designer of the whole experiment, helping with data processing and article revision.

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Competing Financial Interests

The author declare no competing financial interests.

Ethics approval and consent to participate

The Animal Ethics Committees of Ningxia University approved the experimental design and animal sample collection for the present study (permit number NXUC20200315). And animal experiments were conducted strictly followed the guidelines of the Regulations for the Administration of Affairs Concerning Experimental Animals (Ministry of Science and Technology, China, 2004).

Consent for publication

Not applicable.

Availability of data and materials

The raw data of the article will be accessible with the link of http://www.ncbi.nlm.nih.gov/bioproject/822492.

Conflicts of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figures

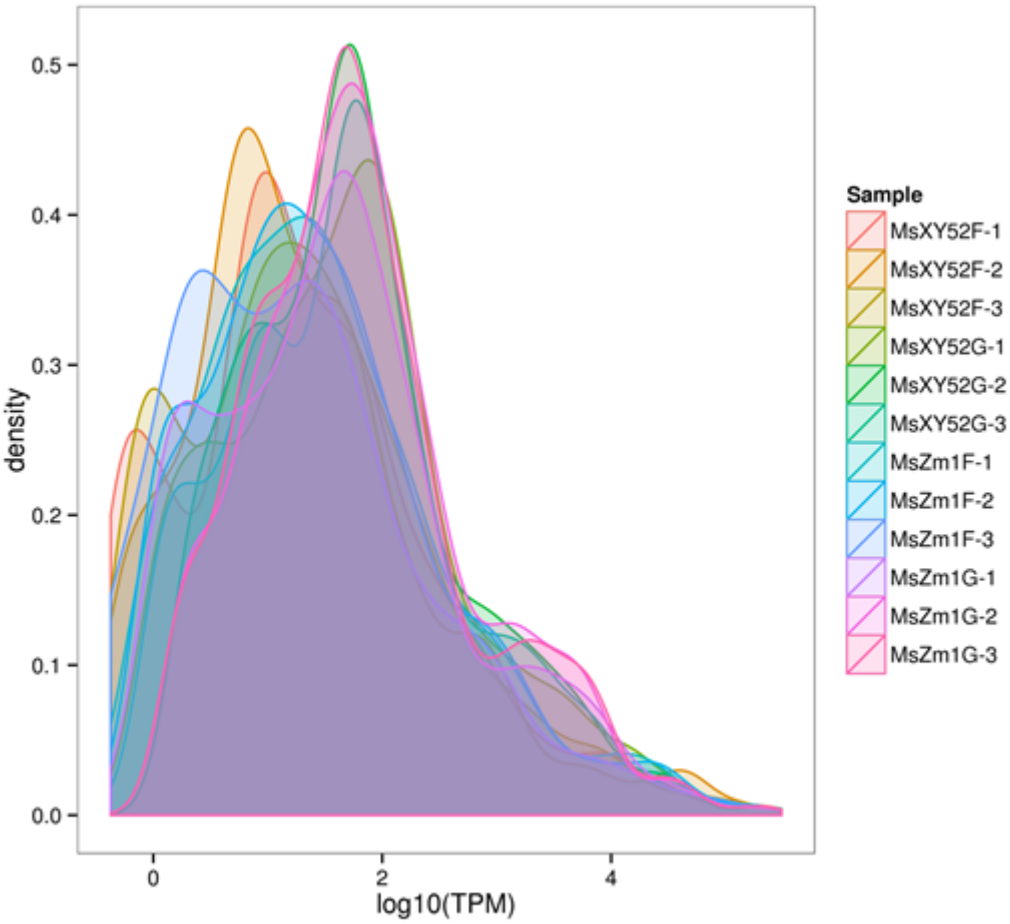

Figure 1

The TPM density distribution map showing the overall distribution of miRNAs in each fresh and dry samples of alalfa *cv.* Zhongmu 1 (MsZM1) and *cv.* Xinyan 52 (MsXY52). different colors represent different replicate samples; and the sum of the area in each peak equals 1. The peak height of the density curve represents the highest abundance (in Log10 transcripts per million) of each transcript in that sample.

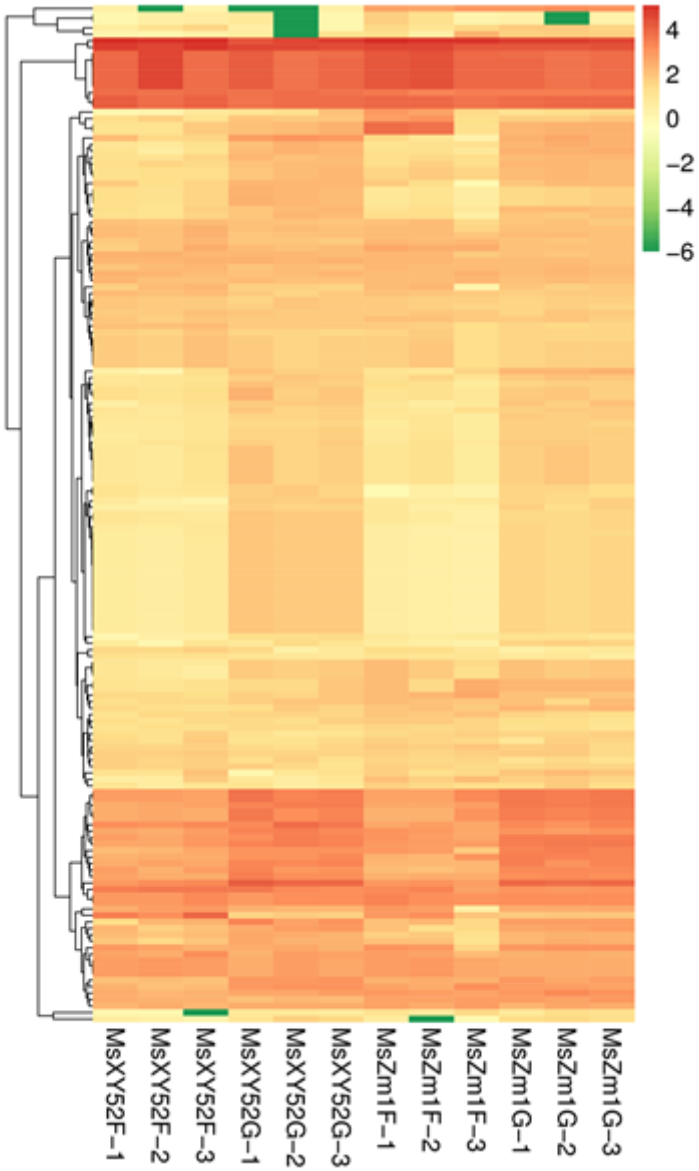

Figure 2

Hierarchical clustering analysis of differentially expressed miRs in dry and fresh MsZM1 and MsXY52 samples. miRs with similar patterns of expression and at cut-off of ≥1000 reads in dry samples were included in the analysis. Columns represent different samples; rows represent different miRs clustered by log10 (TPM+1e^{-6}) values. Red indicates high expression; green indicates low expression. Clustering patterns indicate twelve distinct categories of miRs shared across all samples.

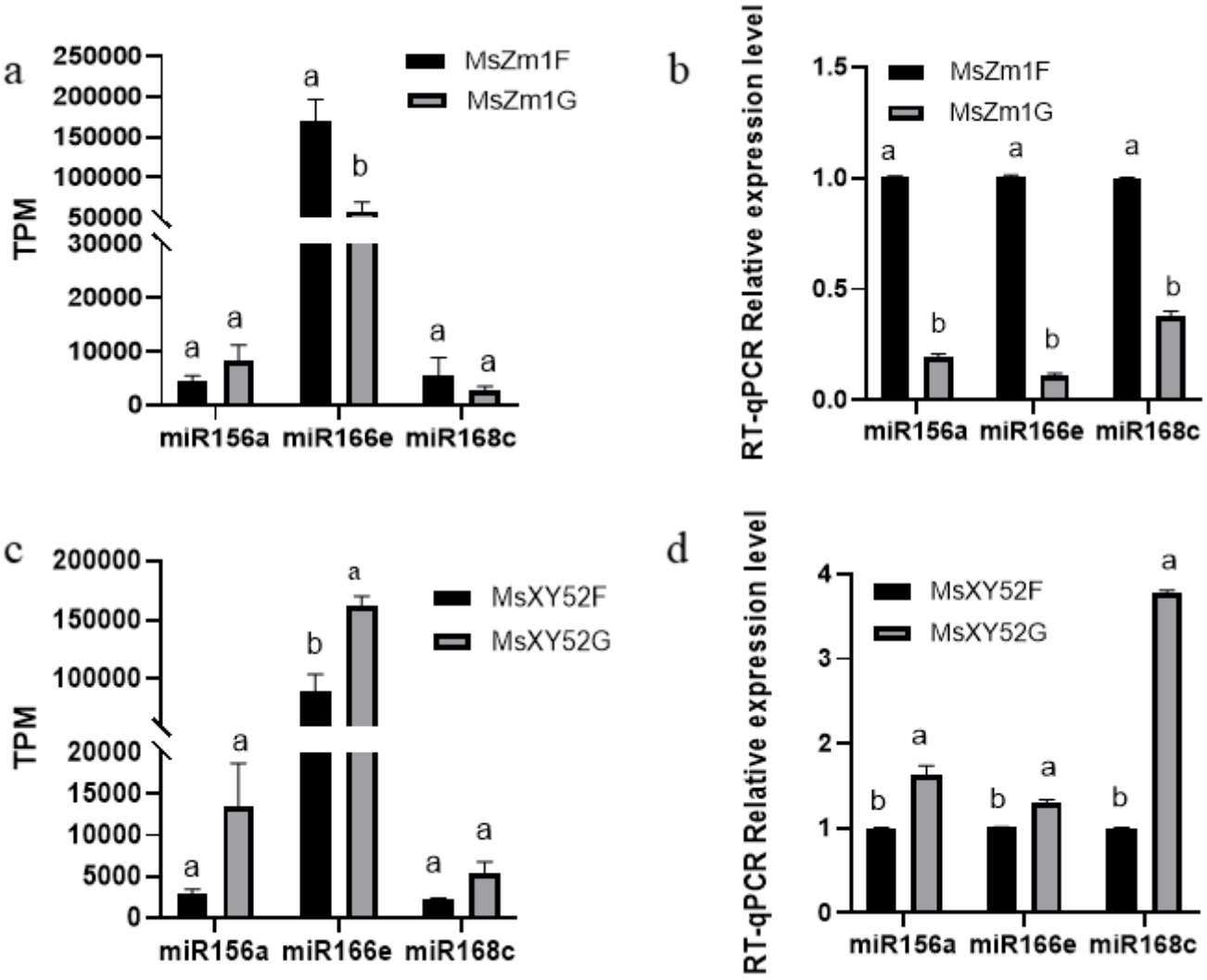

Figure 3

Expression level of miR-156a, miR-166e, and miR-168c a. sequencing of miR-156a, miR-166e, miR-168c in *cv.Zhongmu 1*; b. Expression level in *cv.Zhongmu 1*; c.Sequencing in *cv.Xinyan 52*; d. Expression level in *cv.Xinyan 52*.

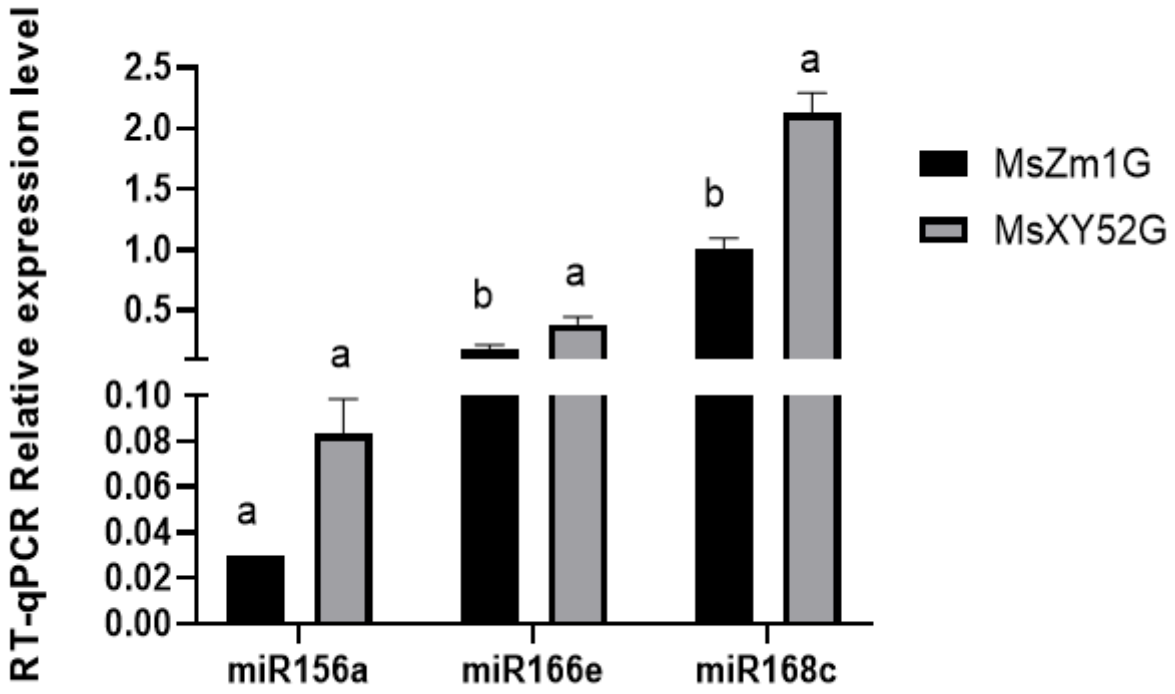

Figure 4

miR-168c, miR-156a, and miR-166e relative expression determined by RT-qPCR in dry alfalfa.

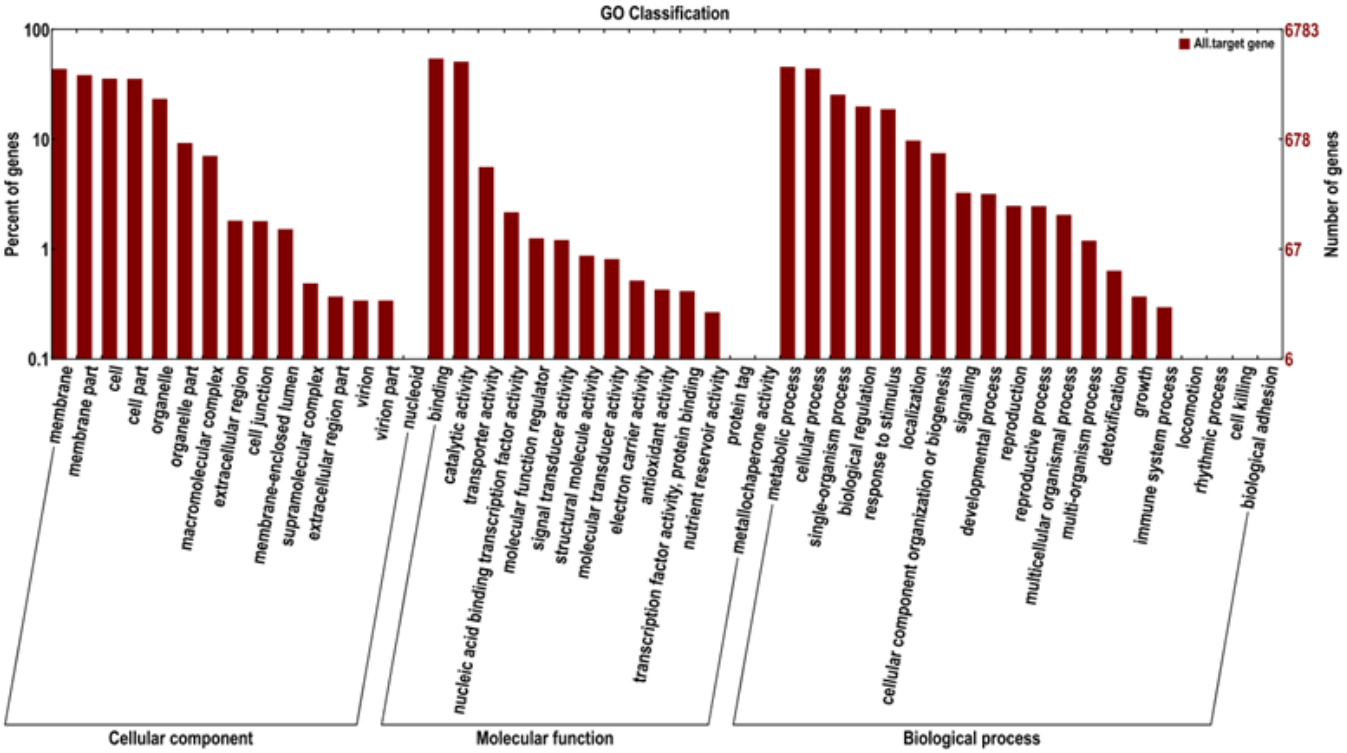

Figure 5

GO enrichment analysis of target genes for miR. The abscissa is the GO classification, ordinate left-hand side is the percentage of the number of genes, and the right-hand side is the number of genes.

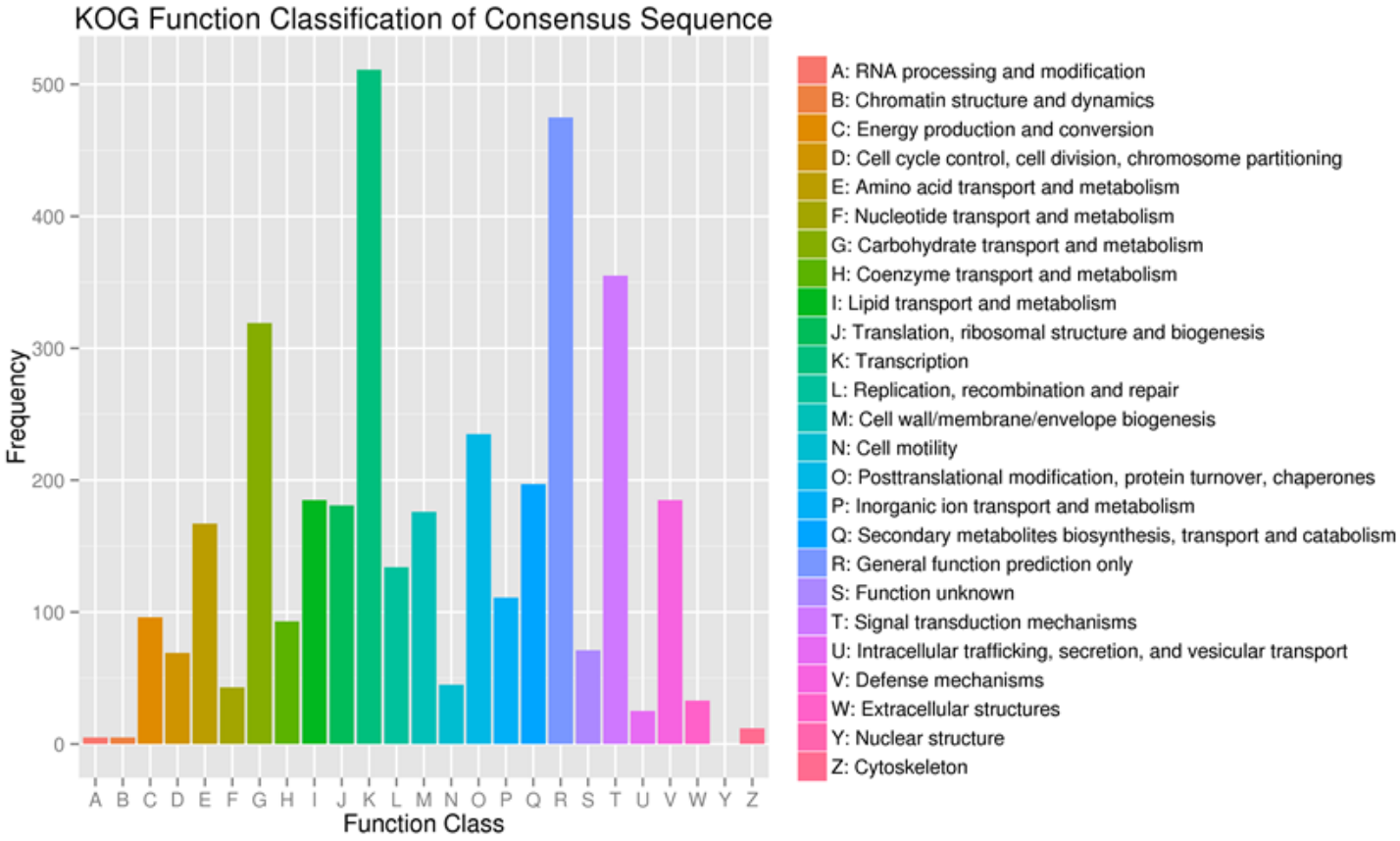

Figure 6

KOG enrichment analysis of target genes for miR

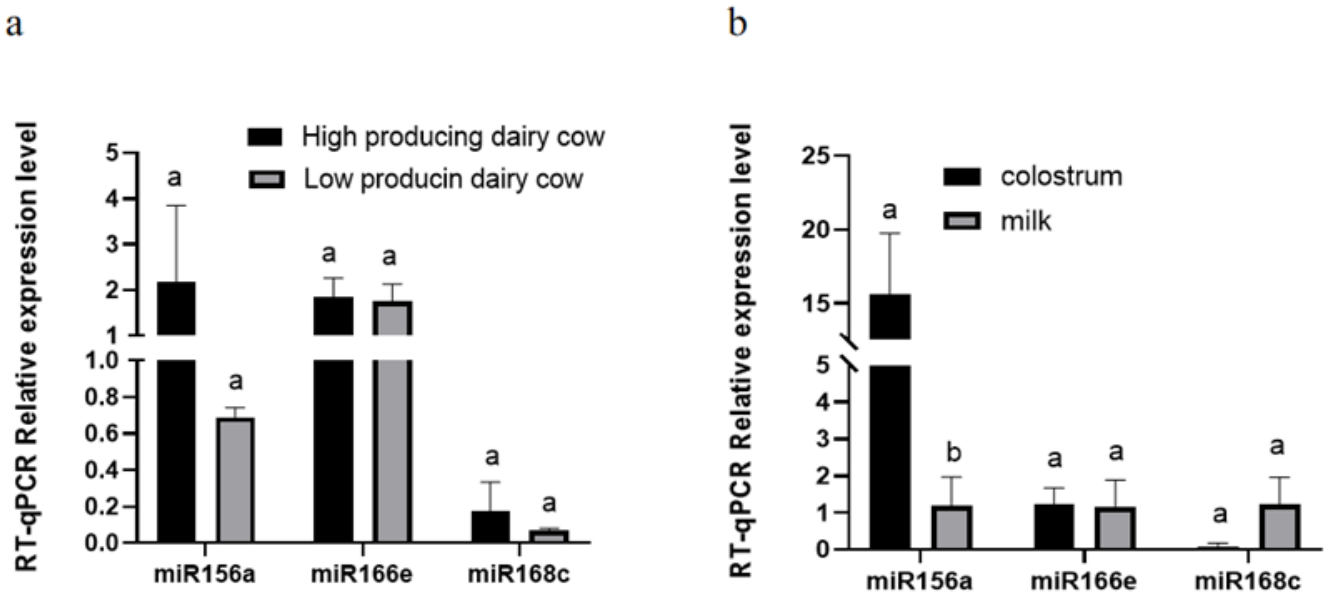

Figure 7

a.miR-156a、miR-166e、miR-168c expression in cow serum;

b. miR-156a、miR-166e and miR-168c expression in cows colostrum and milk.

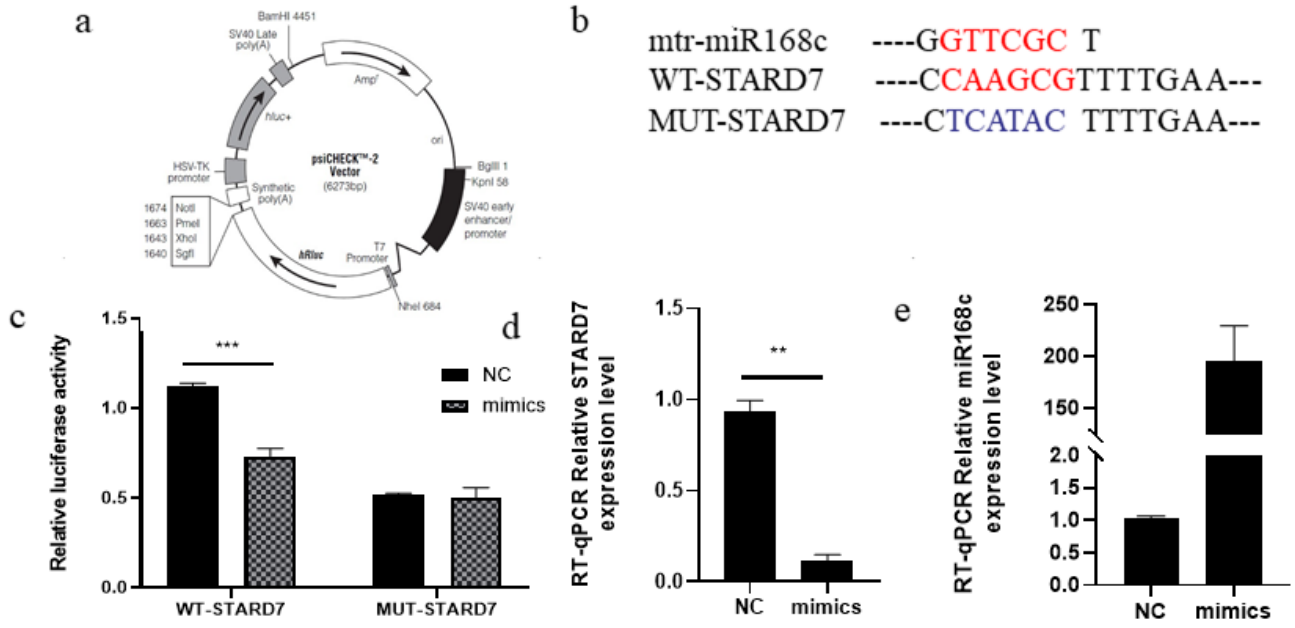

Figure 8

Dual-Luciferase Reporter Assay, a: Psi-check2 carrier, b:Target gene binding site, c:Luciferase activity, d and e: Overexpression efficiency

Supplementary Files

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