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# Integrative Analysis of miRNA and mRNA in Dormant Alfalfa under Different Growth States

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## ABSTRACT

**Background:** Alfalfa is one of the most important legume forages in the world; it includes three fall dormancy (FD) types. There is previous difference between FD alfalfa and non-FD alfalfa. However, the molecular basis of these differences remains largely dim. MicroRNAs (miRNAs) have critical roles in the regulation of gene expression; the reverse differential expression of genes and miRNAs can help us to reveal the regulation mechanism of fall dormancy in alfalfa.

**Methods:** We carried out integrative analysis of miRNA and mRNA in dormant alfalfa under different growth conditions based on miRNA and transcriptome sequencing analysis. The differential genes were obtained by transcriptome analysis and the differential miRNAs were obtained by small RNA analysis. Then the integration analysis of miRNAs and mRNAs was performed to screen reverse different expressions between genes and miRNAs to construct the key miRNA-mRNA regulatory network.

**Result:** Our results indicated the biological process was the key factor in the fall dormancy of alfalfa, 24 miRNAs downregulated with transcript genes upregulated and 24 miRNAs upregulated with transcript genes downregulated, respectively. Key factors of the regulatory network showed that MiR5287b and miR2643a had a more complex network. MiR5287b had 22 corresponding regulatory transcript factors and miR2643a had 17 corresponding regulatory transcript factors and some conserved miRNAs (*e.g.*, miR172a, miR156e and miRNA169h). Most of them play the vital role in plant growth and development and also participate in regulating fall dormancy in alfalfa.


**Key words:** Alfalfa, Fall-dormancy, Integrative analysis, miRNA, mRNA.

## INTRODUCTION

Fall dormancy (FD) (Barnes *et al*., 1979) is an adaptive trait in alfalfa (*Medicago sativa* L.), a forage legume cultivated worldwide. It is indexed by fall dormancy classes (FDC) one to 11 and generally categorized into three types, dormant (FDC 1-4), semi-dormant (FDC 5-7) and non-dormant (FDC 8-11) (Teuber *et al*., 1998). Fall dormant types usually have a slow regrowth pace compared to non-dormant types after harvest in autumn (Ariss and Vandemark, 2007; Wang *et al*., 2009). Fall dormancy induced by shortening day length and falling temperature in late summer or early autumn (McKenzie *et al*., 1988), FD plays an essential role in winter survival and biomass accumulation of alfalfa in mid or high-latitude regions (Weishaar *et al*., 2005; Ariss and Vandemark, 2007). A lot of effort has been made to investigate FD in alfalfa. Our previous study showed that the shortening day length rather than the falling temperature was the main factor causing FD in alfalfa (Wang *et al*., 2008). Other studies are mainly concerned with the sugar content in alfalfa, which related to the cold resistance and overwintering rate (Volenec *et al*., 1991; Cunningham and Volenec, 1998; Cunningham *et al*., 2001). However, cold resistance and fall dormancy are different growth characteristics (Li and Wan, 2004), the details molecular mechanisms of FD have not been studied in detail. Gene expression regulation involving microRNAs (miRNAs) has been a research hot spot in recent years (Li *et al*., 2014). MiRNAs are small non-coding RNAs (about 22 nucleotides) that were found in plants and animals (Chen and Rajewsky, 2007). As post-transcriptional regulators, miRNA could bind to the 3′ end of the untranslated region (UTR) of a target mRNA through base pairing and lead to the degradation or translation inhibition (Sales *et al*., 2010; Kenneth *et al*., 2014). miRNAs may have multiple target mRNAs (Sales *et al*., 2010) and miRNAs act to regulate target mRNAs negatively. Previous studies have shown that miRNAs regulate genes with different biological processes, including but not limited to organ separation, polarity, identity miRNAs biogenesis and function (Dugas and Bartel, 2004).

Recently, transcriptome sequencing and small RNA sequencing of two standard alfalfa varieties (Maverick, FDC 1; CUF101, FDC 9) at two-time points (May and September) were done in our lab. Transcriptome and small RNA sequencing results provided us with a valuable resource for

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fall dormancy research in alfalfa. In this work, we present the integration analyses of miRNA and mRNA in dormant alfalfa.

## MATERIALS AND METHODS

### Plant materials

The experimental variety of Maverick was the standard variety (FDC1). DM and DS were used as abbreviations for dormant type (Maverick) in May under normal growing condition and in September under dormant growing condition, respectively.

Details of growth condition, sequencing process can be found in this article (Zhang *et al*., 2015). Integrative analysis of miRNA and mRNA have been carried out by the Gideo Biology Co., Ltd in 2021.

### Sequencing data availability

Raw transcriptome sequencing reads have been deposited at NCBI Sequence Read Archive (SRA) under accession number SRA057663. The *de novo* assembled transcriptome data have been deposited at DDBJ/EMBL/GenBank under the accession GAFF00000000. Small RNA sequencing reads have been deposited at Gene Expression Omnibus (GEO) under NCBI accession number SRP040470.

### Genes and miRNAs expression analysis

All sequence reads from mRNA sequencing were used to get a *de novo* assembled transcriptome using Trinity (Grabherr *et al*., 2011). This *de novo* assembled transcriptome was treated as the reference transcriptome in the downstream analysis. mRNA sequencing reads were mapped to the reference transcriptome using Bowtie (Langmead *et al*., 2009) individually and quantified using RSEM (Li and Dewey, 2011). Differential gene expression analysis was performed using the Simbiot® platform (Umylny and Weisburd, 2012). Benjamini *et al*. (1995) false discovery control procedure was used in the gene expression analysis. sRNA reads were aligned to the reference transcriptome using Bowtie and the expression levels were quantified by TPM (transcript per million) (Zhou *et al*., 2010). Expression analysis was conducted using DEG-seq (Wang *et al*., 2010).

Both *P*-values of gene and miRNA expression analysis were adjusted using q-value (Storey, 2003). Significant differential gene and miRNA expression was accepted if the q-value $\leq 0.01$ and the absolute value of $Log_2$ (Fold change) > 1.

### MiRNA target prediction

Targets of miRNAs were performed using both psRobot (version 1.2) (Wu *et al*., 2012) and psRNATarget (Dai *et al*., 2018). The intersection of miRNA targets predicted by both software were accepted as miRNA targets.

### Gene ontology and pathway analysis

Gene ontology (GO) functional analysis was performed using go-seq (Young *et al*., 2010). KOBAS was used to perform the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis (Chen *et al*., 2011).

### Construction of miRNA mRNA regulatory network

According to the target action relationship data file between differentially expressed miRNAs (upregulation/ downregulation, q-value) and mRNAs provided, this file could be directly imported into Cytoscape software for visual editing.

## RESULTS AND DISCUSSION

### Differential expressed of genes and miRNAs

We performed genes and miRNAs expression analysis; the up and down regulation analysis results of different genes and miRNAs were sorted out between DM and DS. Statistics of expression analysis were depicted in this study. We identified thousands of differentially expressed genes across the groups. After filtering, 2787 genes were upregulated and 3422 genes were downregulated (Fig 1A). Only small portion of genes were annotated (known).

Both psRobot and psRNATarget were used to predict miRNA targets in our sequencing project. No more than 100 differential expressed miRNAs identified in each group. After filtering, 45 miRNAs were upregulated and 45 miRNAs were downregulated (Fig 1B).

To adapt to cold in autumn end and winter, dormant alfalfa becomes dormant state in autumn. Fall dormancy reflects the acclimatization response of alfalfa to both shortening photo periods and falling temperatures in some way (McKenzie *et al*., 1988). FD is the overall growth performance in alfalfa; it is different from the bud dormancy of the plants.

### The reverse different expressions between genes and miRNAs

In plants, most miRNAs cut off the mRNA molecules of their target genes-miRNAs and target genes complement and combine totally. The mode of action and function are very similar to siRNAs, finally, cut the target mRNA.

To find the target genes predicted by miRNAs, we first needed to find the miRNAs and target genes with contrasting traits in the two samples. In this experiment, we found 24 miRNAs downregulated with transcript genes upregulated and 24 miRNAs upregulated with transcript genes downregulated, respectively (Table 1).

### GO enrichment and KEGG pathways analysis

According to the corresponding relationship between miRNAs and their target genes, we performed GO and KEGG enrichment analysis on the collection of target genes of differentially expressing miRNAs in each group.

GO has three ontologies, which describe the molecular function, cellular component and biological process of genes respectively. As indicated by GO enrichment, the main GO-enriched factors were metabolic process, cellular process, binding and catalytic activity (Fig 2). Results also showed that the main GO enriched factor was biological regulation (Fig 2), which in some way indicated that biological process was the critical factor in the fall dormancy of alfalfa, so we zoomed Directed Acyclic Graph (DAG) of biological process and each box represented a GO term in the figure (Fig 3).

KEGG pathways were used to assess the statistical enrichment of the target gene candidates *via* KOBAS software. The results showed that the most significantly enriched key factors were Metabolic pathways and the greater the degree of enrichment factor was Cyanoamino acid metabolites (Fig 4).

After analyzing GO enrichment and KEGG pathways-rich factors, we inferred that fall dormancy in alfalfa was the biological regulation process in the metabolic pathway.

**Construction of miRNA mRNA regulatory network**

The target interaction relationship between miRNA and mRNA could directly import into Cytoscape software for visual editing. After filtering, in the different reverse expression between genes and miRNAs, we chose seven key miRNAs to make the regulatory network. In Fig 5, green represents significant upregulation, red represents significant downregulation, the triangle represents miRNA and the circle represents the target gene. From Fig 5 and Table 2, we could see miR5287b and miR2643a had a more complex network. MiR5287b had 22 corresponding regulatory transcript factors and miR2643a had 17 corresponding regulatory transcript factors.

Little was known about miRNA-mRNA mediated regulation of fall dormancy in alfalfa and most function annotations were hypothetical proteins. MiR5287 was considered that targets a cytochrome P450 family protein and fructose-bisphosphate aldolase (Li *et al*., 2018).

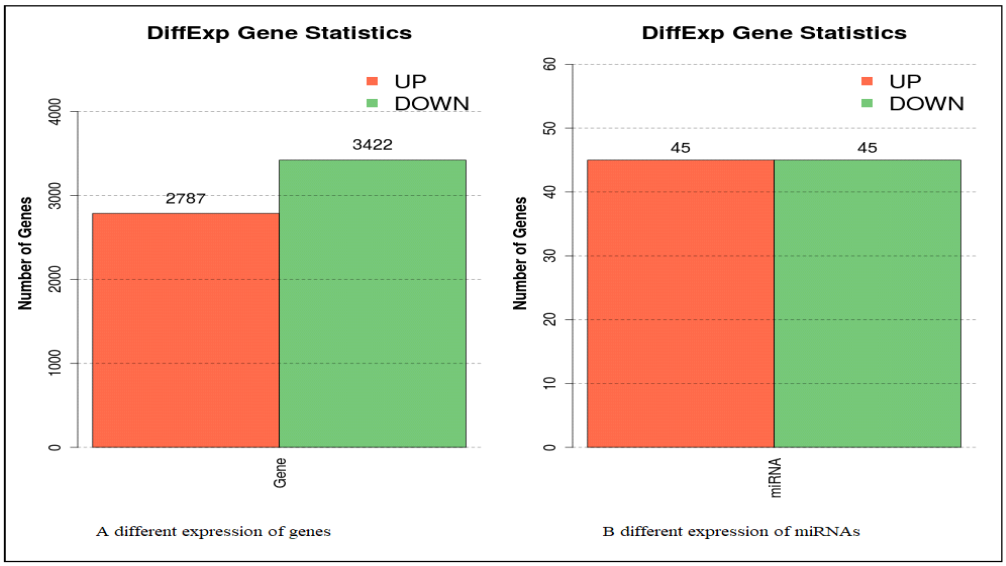


**Fig 1:** Differential expressed of genes and miRNAs.
Different expressed genes and miRNAs were sorted out between DM and DS.

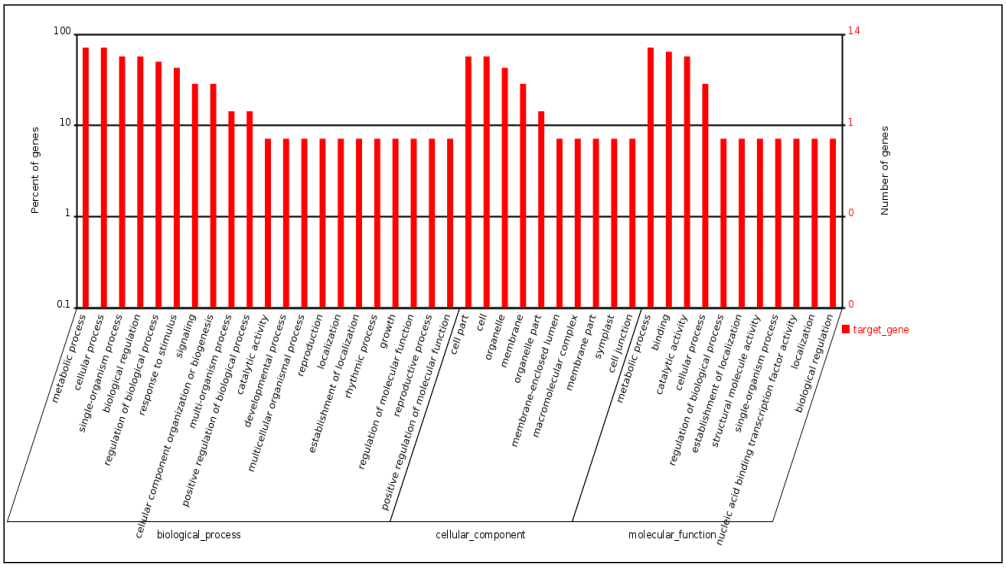


**Fig 2:** Gene Ontology (GO) function classification diagram.
GO Slim terms were into three groups: (a) biological process, (b) cellular component, (c) molecular function.

**Table 1:** The reverse different expressions between genes and miRNAs.

| miRNA | Transcript | miRNA expression | transcript expression |
|---|---|---|---|
| mtr-miR172a | comp60642_c0 | Down | Up |
| mtr-miR5287b | comp491576_c0 | Down | Up |
| mtr-miR169h | comp62644_c0 | Down | Up |
| mtr-miR2591 | comp22340_c0 | Down | Up |
| mtr-miR5287a | comp66494_c1 | Down | Up |
| mtr-miR5287b | comp66494_c1 | Down | Up |
| mtr-miR5287b | comp53655_c0 | Down | Up |
| mtr-miR2586a | comp37431_c0 | Down | Up |
| mtr-miR2586a | comp32246_c0 | Down | Up |
| mtr-miR1507-3p | comp72157_c0 | Down | Up |
| mtr-miR172a | comp9219_c0 | Down | Up |
| mtr-miR2586a | comp33123_c0 | Down | Up |
| mtr-miR2634 | comp64481_c0 | Down | Up |
| mtr-miR1510a-3p | comp10523_c0 | Down | Up |
| mtr-miR2591 | comp1191508_c0 | Down | Up |
| mtr-miR1507-3p | comp65742_c0 | Down | Up |
| mtr-miR2586a | comp1765981_c0 | Down | Up |
| mtr-miR2586a | comp6805_c0 | Down | Up |
| mtr-miR2634 | comp689986_c0 | Down | Up |
| mtr-miR2629a | comp737703_c0 | Down | Up |
| mtr-miR2593e | comp57061_c0 | Down | Up |
| mtr-miR1510a-3p | comp71858_c0 | Down | Up |
| mtr-miR5281a | comp2089_c0 | Down | Up |
| mtr-miR5281a | comp65945_c2 | Down | Up |
| mtr-miR156e | comp522911_c0 | Up | Down |
| mtr-miR5299 | comp686501_c0 | Up | Down |
| mtr-miR2643a | comp71995_c0 | Up | Down |
| mtr-miR5272a | comp61687_c0 | Up | Down |
| mtr-miR398b | comp14275_c0 | Up | Down |
| mtr-miR1509b | comp762722_c0 | Up | Down |
| mtr-miR2655b | comp4773_c0 | Up | Down |
| mtr-miR408-3p | comp1339099_c0 | Up | Down |
| mtr-miR408-5p | comp892738_c0 | Up | Down |
| mtr-miR5205b | comp67236_c0 | Up | Down |
| mtr-miR156g-3p | comp961951_c0 | Up | Down |
| mtr-miR2643a | comp62724_c0 | Up | Down |
| mtr-miR2603 | comp911634_c0 | Up | Down |
| mtr-miR2655b | comp911634_c0 | Up | Down |
| mtr-miR164a | comp904743_c0 | Up | Down |
| mtr-miR2655b | comp1263283_c0 | Up | Down |
| mtr-miR2603 | comp47189_c0 | Up | Down |
| mtr-miR5248 | comp4999_c0 | Up | Down |
| mtr-miR5299 | comp670317_c0 | Up | Down |
| mtr-miR156g-3p | comp67179_c0 | Up | Down |
| mtr-miR5752a | comp70904_c0 | Up | Down |
| mtr-miR2655b | comp25040_c0 | Up | Down |
| mtr-miR5237 | comp60898_c0 | Up | Down |
| mtr-miR5752a | comp51137_c0 | Up | Down |

The target genes or protein of the mir2643a is the F-box protein interaction domain protein and the partner efflux family protein of Abscisic acid receptor PYL9-like protein. The functions described were as follows: shoot development and leaf senescence, drought resistance and citric acid secretion (Pokoo *et al*., 2018). MiR172a gene functions described wereAP2-like transcription factors and in the improvement of salt tolerance by functioning as a signal through degradation of the transcription suppressor Gene SSAC1 (Pan *et al*., 2016). Flowering is a pivotal event in the life cycle of angiosperm plants; miR172 has been widely confirmed to play critical roles in flowering time control by regulating its target gene expression (Wang *et al*., 2016; Luan *et al*., 2018). MiR169 regulates stomatal development by targeting MADS-box protein, while miR169 is upregulated by phytochrome, which participates in light signal transduction and affects photosynthetic efficiency. Light signals mainly regulate plant growth and development by

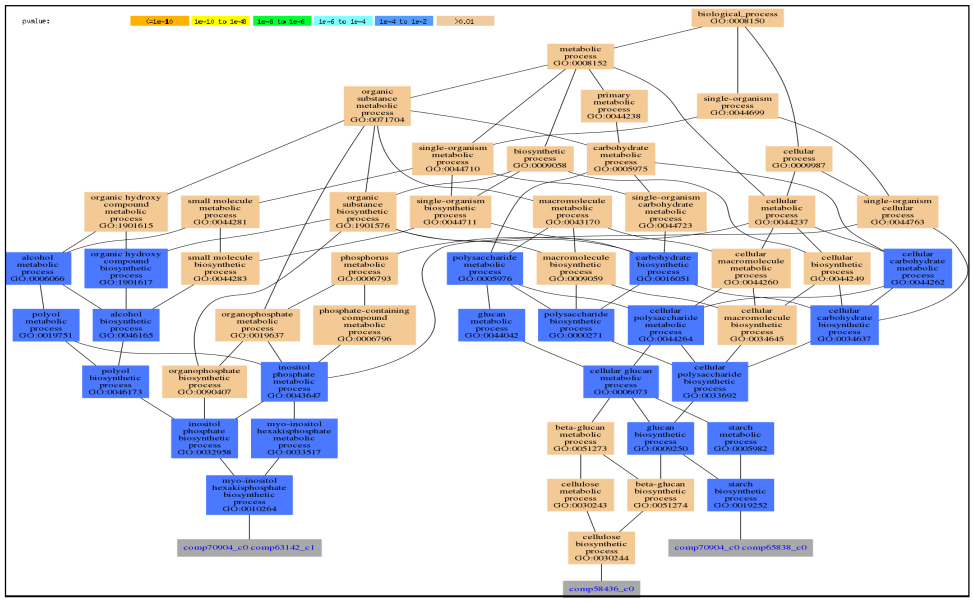


**Fig 3:** Directed acyclic graph in biological process of candidate target genes.

Each box represents a GO term. After zooming, the contents and the meanings are from top to bottom: the ID of the GO term, the description of the GO, the p-value, the number of candidate target genes and background genes.

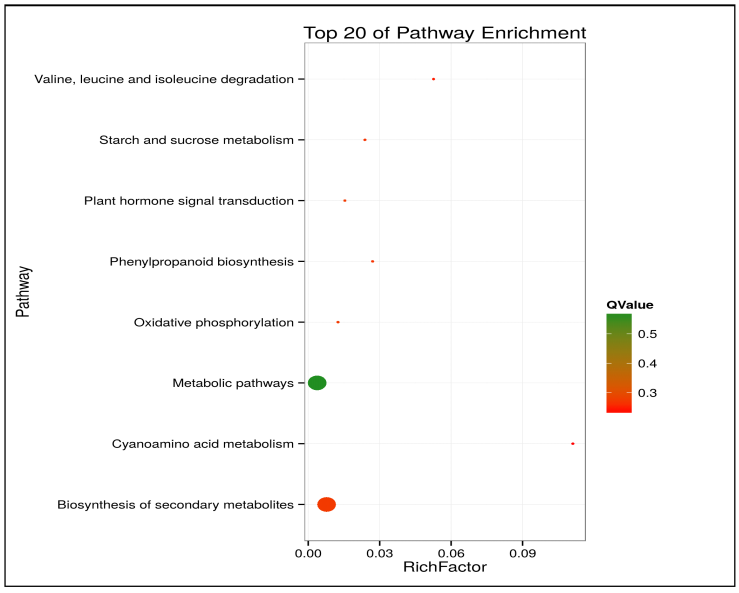


**Fig 4:** Scatter plot of candidate target genes pathway enrichment.

The vertical axis represents the path name, the horizontal axis represents the rich-factor, the size of the point represents the number of candidate target genes in the path and the color of the point corresponds to different Q-value ranges.

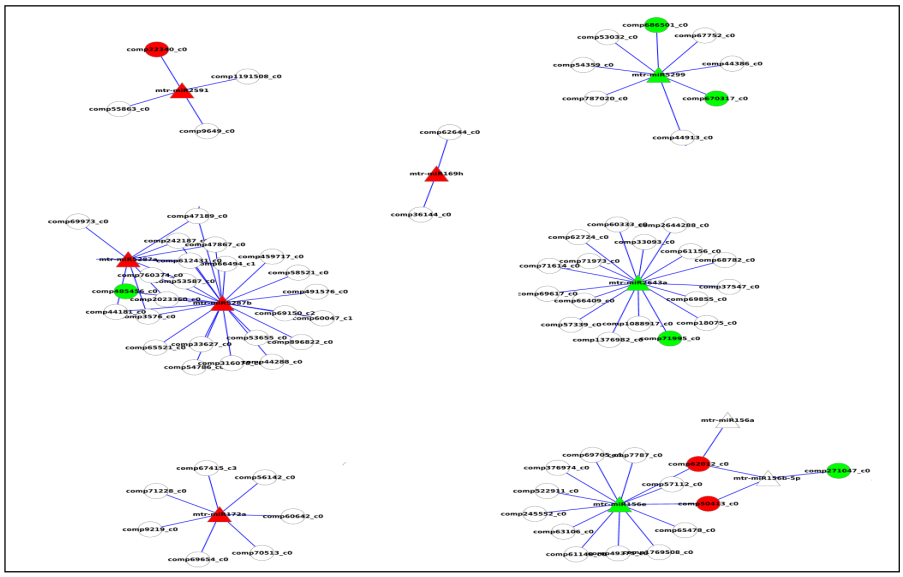

**Fig 5:** Construction of miRNA-mRNA regulatory network in DM and DS.
Seven key miRNAs from the whole network to make regulatory network in the reverse different expression between genes and miRNAs.

**Table 2:** Construction of key miRNA-mRNA regulatory network.

| miRNA | miRNA expression | Targets transcripts of regulatory network | Function |
|---|---|---|---|
| mtr-miR172a | Down | comp60642_c0, comp67415c_3, comp56412_c0, comp70513_c0, comp69654_c0, comp9219_c0, comp71228_c0 | AP2-like transcription factors; salt tolerance; flowering time control. |
| mtr-miR5287b | Down | comp491576_c0, comp65521_c0, comp33627_c0, comp54786_c0, comp3160_c0, comp53655_c0, comp44288_c0, comp896822_c0, comp60047_c0, comp69150_c0, comp58521_c0, comp459717_c0, comp47867_c0, comp47189_c0, comp242187_c0, comp612431_c0, comp53587_c0, comp760374_c0, comp485456_c0, comp2023360_c0, comp44181_c0, comp3576_c0. | Targets a cytochrome P450 family protein and fructose-bisphosphate aldolase. |
| mtr-miR169h | Down | comp62644_c0, comp36144_c0. | Root development, flower organ formation, lateral organ formation, stomatal formation and stress response. |
| mtr-miR2591 | Down | comp22340_c0, comp1191508_c0, comp9649_c0, comp55863_c0. | No hit. |
| mtr-miR156e | Up | comp522911_c0, comp245552_c0, comp63106_c0, comp6114_c0, comp49371_c0, comp769508_c0, comp22340_c0,50413, comp57112_c0, comp7787_c0, comp6970_c0, comp376974_c0. | Regulates the expression of miR172 *via* SPL1; Squamosa promoter-binding-like protein contig_52418. |
| mtr-miR5299 | Up | comp686501_c0, comp67752_c0, comp44386_c0, comp670317_c0, comp44913_c0, comp787020_c0, comp54359_c0, comp53032_c0. | Hypothetical protein chr4. |
| mtr-miR2643a | Up | comp71995_c0, comp18705_c0, comp69855_c0, comp37547_c0, comp68782_c0, comp61156_c0, comp2644288_c0, comp33093_c0, comp60333_c0, comp62724_c0, comp71973_c0, comp71614_c0, comp69617_c0, comp66409_c0, comp57339_c0, comp1376982_c0, comp1088917_c0. | Target gene or protein are F-box protein, interaction domain protein, Abscisic acid receptor PYL9 -like protein and MATE efflux family protein. |

transcriptome driving dramatic shifts, such as protein far-red impaired response 1(FAR1, comp1262167_c0), Cryptochrome-2 (CRY-2, comp46970_c0). MiR169h is widely and relatively conserved, which regulates a class of conserved transcription factors NF-YA (nuclear transcription factor Y subunit A) in plants. MiR169h participates in plant growth and development, such as root development, flower organ formation, lateral organ formation, stomatal formation and stress response. It has been shown that miR156 regulates the expression of miR172 *via* SPL1 (comp50413_c1), which directly promotes the transcription of miR172b by squamosa promoter-binding-like protein contig_52418 (Wu *et al*., 2009). Most of the miRNAs may play an important role in plant growth and development through interacting with their target genes and also participate the regulation of fall dormancy in alfalfa.

Exploring some dormancy-responsive miRNAs and mRNAs may be crucial for understanding the mechanism of fall dormancy in alfalfa. These integrative analysis of miRNA and mRNA could play crucial roles during the dormancy of alfalfa and our analysis provides valuable information regarding further functional genes involved in fall dormancy in alfalfa.

## CONCLUSION

Integrative analysis of miRNA and mRNA in dormant alfalfa under different growth conditions showed that biological process was the key factor in the fall dormancy of alfalfa; After filtering, 2787 genes were upregulated and 3422 genes were downregulated, 45 miRNAs were upregulated and 45 miRNAs were downregulated, among them, 24 miRNAs downregulated with transcript genes upregulated and 24 miRNAs upregulated with transcript genes downregulated, respectively. Key factors of the regulatory network showed that miR5287b and miR2643a had a more complex network, miR5287b had 22 corresponding regulatory transcript factors and miR2643a had 17 corresponding regulatory transcript factors and some conserved miRNAs (*e.g*., miR172a, miR156e and miRNA169h). Most of them play an important role in plant growth, development and participate in the regulation of fall dormancy in alfalfa.

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**Conflict of interest:** None.

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