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Identification of the specific microRNAs to fall dormancy in alfalfa

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Abstract
MicroRNA (miRNA) is a form of small, non-coding RNA, and they are generated from single-stranded precursors with unique hairpin structures consisting of 18–25 nucleotides (nt). MiRNAs regulate their target genes by targeting miRNA cleavage and translational repression. MiRNAs have been reported to play an important role in many biological systems in many organisms, but little is known about miRNA in fall dormancy regulation in alfalfa. In our study, miRNAs were identified in dormant and non-dormant alfalfa using deep sequencing and functional analysis to identify miRNAs that may impact fall dormancy. These miRNAs including some known miRNAs (e.g., miR172, miR166, and miRNA393) and novel miRNAs (*e.g*., novel-miR_1, novel-miR_3, novel-miR_18, novel-miR_42, novel-miR_77, and novel-miR_102,) were probably involved in fall dormancy.

Keywords: Deep sequencing, Expression pattern, Fall dormancy, miRNA

Introduction

MicroRNA (miRNA) is a form of small, non-coding RNA. MicroRNAs are generated from single-stranded precursors with unique hairpin structures consisting of 18–25 nucleotides (nt). MiRNAs regulate their target genes by targeting miRNA cleavage and translational repression (Jones-Rhoades *et al*., 2006). Recently, researchers have found that miRNAs play an important role in embryonic development. They also play a key role in many biological systems in many different kinds of organisms, making the quick and precise drafting of physiology and structures possible (Ying *et al*., 2008). These miRNAs were first reported in *Caenorhabditis elegans* as developmental timing regulators (Lee *et al*., 1993). In plants, functional analysis of conserved miRNAs revealed that they regulate many different processes, including plant development, signal transduction, response to environmental stress and pathogen invasion, and their own biogenesis (Jones-Rhoades *et al*., 2006; Chen, 2005; Zhang *et al*., 2006; Dugas and Bartel, 2004). Plant miRNAs were first identified in *Arabidopsis* (Llave *et al*., 2002; Park *et al*., 2002; Reinhart *et al*., 2002). Plants are sessile organisms that must bear stressful and changeable environments. Most plant genes are regulated in response to drought stress, soil salinity stress, and extreme temperatures (Shinozaki and Yamaguchi, 2007; Fowler and Thomashow, 2002; Zhu, 2002). Accumulating reports have suggested that plant miRNAs also play vital roles in biotic and abiotic stress responses (Fujii *et al*., 2005; Arazi *et al*., 2005; Bari *et al*., 2006; Aung *et al*., 2006; Navarro *et al*., 2006).

Alfalfa (*Medicago sativa* L.) a member of family Leguminosae is an important forage crop, however varieties grown over different geographical locations experience dormancy. Various studies have focused on studying genetic diversity and breeding more than the mechanisms regulating fall dormancy (Yadav *et al*., 2010a; Yadav *et al*., 2010b). Fall dormancy reflects the acclimation response of alfalfa to both shortening photoperiods and falling temperatures, and highly positive phenotypic correlations (r = 0.90) between autumn growth and winter hardiness have been found in previous studies (Stout and Hall, 1989; Schwab *et al*., 1996). Recently, an increasing number of reports have demonstrated that plant miRNAs are involved in the cold stress response (Mallory and Vaucheret, 2006; Sunkar *et al*., 2007). MiR393, miR397b, and miR402 activity was observed in response to cold stress and other kinds of stress treatment. Although significant progress has been made in the demonstration of plant miRNA function and in understanding the underlying mechanism, most of these studies were performed in plants whose whole-genome sequences were known, such as *Arabidopsis*, rice and *Populus*. Other evolutionarily and economically important species have yet to be examined.

In previous studies, plant miRNAs were identified through three major approaches: forward genetics, bioinformatic

prediction, and direct cloning and sequencing. In these previous studies, only a few miRNAs were identified using forward genetics (Aukerman and Sakai, 2003; Wang *et al*., 2005; Baker *et al*., 2005). It is difficult to use bioinformatics to predict species-specific miRNAs (Zhang *et al*., 2009). Thus, direct cloning and sequencing is the best way to identify plant miRNAs. Because of limited resources, only a few hundred miRNAs have been identified using this approach, which has led to the incomplete conclusion that there are only a few types of miRNAs in plants. However, the identification of numerous small RNAs is possible using high-throughput sequencing, with which many more species-specific miRNAs were revealed than had previously been believed to exist (Fahlgren *et al*., 2007; Yao *et al*., 2007; Lu *et al*., 2006; Zhu *et al*., 2008). Despite the numerous studies on the involvement of *M. truncatula* miRNAs in abiotic stress, there has been no report on alfalfa (*Medicago sativa* L.), especially for perennial miRNAs involved in fall dormancy. Alfalfa cultivars are classified by fall dormancy from classes (FDC) 1 to 11 (Brummer *et al*., 2000). Fall dormant alfalfa varieties generally have less fall growth, resulting in a reduced number of cuttings and less forage yield, and non-dormant alfalfa varieties produce higher biomass yield during autumn but usually die during the winter (Li and Wan, 2004). Although phenotypic correlations generally show winter hardiness and late summer and autumn growth (fall dormancy, FD) to be strongly associated in alfalfa (r = 0.90), the genetic relationship between the traits is not well understood (Cunningham *et al*., 2001; Cunningham *et al*., 1998). In order to identify miRNAs whose expression is specific to dormant alfalfa and to clarify their role in fall dormancy, small RNA populations in dormant variety alfalfa and non-dormant variety alfalfa were sequenced using high-throughput sequencing technology, and the function of the candidate miRNAs was analyzed.

Materials and Methods

Plant material: According to growth conditions (Zhang *et al*., 2015), the test sample-alfalfa leaves were collected from the standard variety Maverick (FDC1) and the standard variety CUF101 (FDC9) in late September. The collected alfalfa leaves were frozen immediately in the liquid nitrogen fourteen days after cutting and then stored at -80°C. The abbreviations DS and NS took the place of dormant alfalfa (Maverick) and non-dormant alfalfa (CUF101) which were collected in September, respectively.

Construction of small RNA library and high-throughput sequencing: Total RNA was isolated from the frozen alfalfa leaves (DS and NS) with Trizol (Invitrogen) in accordance with the manufacturer's protocol and used for construction of the DS and NS small RNA libraries. 3 mg total RNA (e" 100ng/ìL) each test sample was made as an input material for the small RNA sample preparations. Alfalfa small RNA sequencing libraries were produced using NEBNext® Multiplex for Illumina® (NEB, New England Biolab, and Ipswich, MA, U.S.). NEB 3' SR adaptor was ligated to 32 end of miRNA directly. The SR-RT primer was hybridized to the excess 3' SR adaptor (the adaptor remaining free after the 3' ligation reaction) and transformed from a single-stranded DNA adaptor to a double-stranded DNA (dsDNA) molecule. The dsDNAs were not suitable substrates for ligation mediated by T4 RNA ligase and therefore did not ligate to the 5' SR adaptor during the subsequent ligation step. Then a 5' end adapter was ligated to the 5' ends of miRNAs. First-strand cDNA was synthesized using M-MuLV Reverse Transcriptase (RNase H–). The cDNA was then PCR amplified using SR primer for Illumina and index primer containing one of 12 index sequences. The DNA fragments of 140-160 bp length (miRNA inserts plus the 3' and 5' adaptors) that were purified (6% TBE PAGE gel, 100 V, 60 min) were recovered in 10 ìL elution buffer and quantifed using Agilent high sensitivity DNA assay on the Agilent Bioanalyzer 2100 system. Index-coded test samples clustering were performed on a cBot Cluster Generation System using TruSeq SE Cluster Kit v3-cBot-HS (Illumina) following the instructions of manufacturer's. After cluster generation, the alfalfa small RNA library preparations were sequenced by the Illumina Hiseq 2000 platform and the test samples were run side by side. The raw data of alfalfa small RNA libraries have been uploaded to the web of the NCBI (https://trace.ncbi.nlm.nih.gov/Traces/sra/?study=SRP040470).

Analysis of small RNA sequencing data: Raw data (raw reads) of fastq format were first processed through custom perl and python scripts. Clean data (clean reads) were obtained by removing low-quality reads, reads containing poly-N, 5' primer contaminants, reads containing poly-A, T, G, and C sections, and reads without 3' primers and insert tags from raw data. Then a range of length from clean reads was chosen for downstream analysis. The alfalfa small RNA tags were mapped to a reference sequence using Bowtie with the alfalfa-transcriptome-sequence (Langmead *et al*., 2009) without

mismatch to analyze their expression and distribution on the reference material. Mapped small RNA tags were used to identify the known miRNA in alfalfa. MiRBase 20.0 was used as a reference to modify mirdeep2, to obtain the possible miRNA in alfalfa using sRNA-tools-cli, then the secondary structure of small RNA was drawn (Friedländer *et al*., 2012).The characteristics of hairpin structure of miRNA precursor can be used to predict novel miRNA. Novel miRNAs of alfalfa were predicted using the software packages miREvo and mirdeep2 through the way of evaluating its secondary structure (Friedländer *et al*., 2012; Wen *et al*., 2012). The minimal folding free energy of alfalfa small RNA tags and Dicer cleavage site were unannotated in the initial steps. In the current analysis pipeline, known miRNA was used with miFam.dat (http://www.mirbase.org/ftp.shtml) to look for families; novel miRNA precursors were submitted to Rfam (http://rfam.sanger.ac.uk/search/) to look for Rfam families. Predicting the target gene of miRNA was performed using psRNA Target (http://bioinfo3.noble.org/psRNATarget/) (Moxon *et al*., 2008). The analysis of gene-ontology (GO) enrichment in alfalfa was generated based on the target genes of candidate miRNAs which were expressed differentially (Mao *et al*., 2005). We assessed the statistical enrichment of the target genes of candidates miRNAs in alfalfa by KEGG pathways using the software of KOBAS.

Table 1. Primer sequences used for qRT-PCR

Primer	5' to 3'
GAPDH	CTGGAGAGGTGGAAGAGC
mtr-miR5255	GACUUGAUAGAGGACAUGGG
mtr-miR5205b	CUUAUAAUUAGGGACGGAGGG
mtr-miR5228	UCUGGUGUACAACUUGAUGG
mtr-miR172a	GAAUCCUGAUGAUGCUGCAG
mtr-miR2674	CACTCGCTTTGGAAGTCATGG
mtr-miR5287a	TTATAATAGTGATCGGAGGG
mtr-miR2593e	ACATCATTGATTGAATGAAC
mtr-miR5267l	AGGCATTTGCTAGGATACAC
mtr-miR5213-5p	TACGTGTGTCTTCACCTCTG
mtr-miR398b	TGTGTTCTCAGGTCGCCCCTG
mtr-miR2111l	ATCCTTGGAATGCAGATTATC
mtr-miR164a	TGGAGAAGCAGGGCACGTGC
mtr-miR1510a-5p	TTGTCTTACCCATTCCTCCCA
mtr-miR397-5p	TCATTGAGTGCAGCGTTGATG
mtr-miR5248	TTTTTAGTTGGCATGCATTCA
mtr-miR5261	TCATTGTAGATGGCTTTGGCT
mtr-miR5272a	GAATTGATTTATGTTTGGATACAC

Real-time quantitative PCR of miRNAs: To ensure the correctness of high - throughput sequencing result, 17 miRNAs were obtained from the results of deep sequencing and validated the accuracy using real time RT-PCR. RNA was collected using Trizol reagent (Invitrogen) following the manufacturer's instructions. Alfalfa RNA was reverse-transcribed using a One Step Prime Script miRNA cDNA Synthesis Kit (TaKaRa) in accordance with the manufacturer's instructions and diluted 10x before PCR. RT-qPCR was performed on LightCycler® 96 instrument (Roche) using SYBR Premix Ex TagII and all the primers used are listed in Table 1. *Medicago sativa* putative glyceraldehyde-3-phosphate dehydrogenase (*MsGAPDH*) was selected as a reference gene (Champagne *et al*., 2007). Small nuclear RNA U6 was used as an internal control.

Results and Discussion

Deep sequencing of small RNAs from alfalfa: Solexa sequencing of DS and NS generated a total of 7,035,161 and 7,145,001 raw reads, respectively. After removal of the low-quality sequences and the corrupted-adapter sequences (the length of reads <18nt), a total of 6,548,094 and 6,627,260 clean reads corresponding to 1,449,168 and 1,185,319 unique reads remained from the DS and NS libraries, respectively. The length distribution of the raw data's revealed that the reads majority excepting adapter sequences which were corrupted (the length of reads <18 nt) were 20–26 nt in size (Fig 1).The sequences specific to DS and specific to NS were analyzed. There were 17.15% total and 50.49% unique sequences in the dormant library and 11.83% total and 39.47% unique sequences in the non-dormant alfalfa library (Fig 2). Through preliminary processing, high-quality reads of the small RNA in alfalfa were mapped to alfalfa–transcriptome-sequence. The total sequences number which matched the transcriptome was 2,963,552 for the DS library and 2,012,514 for the NS library, and the numbers of unique sequences were 341,796 and 248,641, respectively (Fan *et al*., 2014). In plants, researchers have reported three types of small interfering RNA (siRNAs): transacting siRNAs (ta-siRNAs), natural antisense transcript-derived siRNAs (nat-siRNAs), and repeat-associated siRNAs (ra-siRNAs) (Vazquez *et al*., 2004). Usually, ra-siRNAs are 24 nt long and DCL3-dependent, ta-siRNAs are 21 nt in length and both DCL1- and DCL4-dependent, but nat-siRNAs are 21 or 24 nt in length and both DCL1- and DCL2-dependent (Vazquez, 2004). In the current study, a high percentage of the 21 nt small RNAs was observed in the dormant alfalfa (DS) library (Fig 1). It was inferred that some key miRNAs involved in fall dormancy might have been activated by the increased number of nat-si

RNAs. These miRNAs may cause dormancy in response to the shortening photoperiods and falling temperatures in autumn. In addition, the expression levels of miRNAs in the DS vs NS libraries indicated 80 differentially expressed miRNAs, including 43 up-regulated alfalfa miRNAs and 37 down-regulated alfalfa miRNAs. Among these miRNAs, some were found to play a role in developmental processes of which a majority of their targets were transcription factors and some were activated to different extents under different environmental conditions.

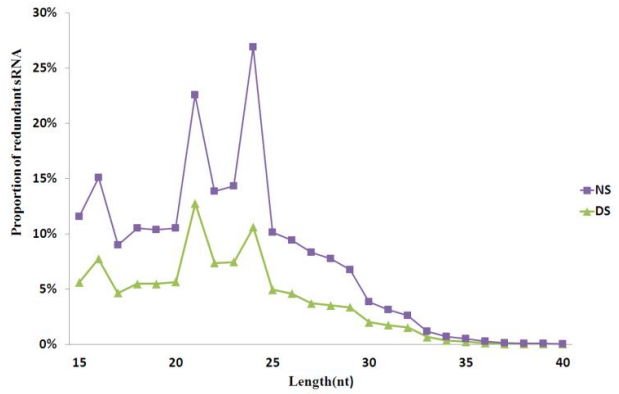

Fig 1. Size distribution of alfalfa small RNAs in (a) DS and (b) NS libraries

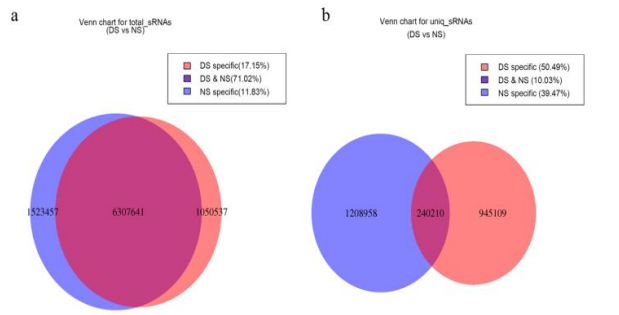

Fig 2. Venn diagram of DS versus NS for total small RNAs and unique small RNAs

The significance of miRNAs research in alfalfa: MiRNAs regulate their target genes by targeting miRNA cleavage and translational repression. MiRNAs have been reported to play an important role in many biological systems in many organisms, but little is known about miRNA in fall dormancy regulation in alfalfa. Reports show that between 26,656 and 844,110 small RNA reads have been identified in *M. truncatula* using Roche 454 sequencers (Jagadeeswaran *et al*., 2009; Lelandais-Brière *et al*., 2009). Szittya et al. obtained 3,948,871 reads and 1,563,959 unique sequences from two small RNA libraries of *M. truncatula* using Illumina-Solexa (2008). This was more than had previously been reported. Fall-dormancy (FD) is related to yield of herbages (biomass production) and winter survival in alfalfa, but the molecular mechanisms causing dormancy are still not clear (Rimi *et al*., 2014). However, little is known of identifying and analyzing dormancy responsive miRNAs in alfalfa. The auto-tetraploid plant *Medicago sativa* is different from the diploid plant *M. truncatula.* Alfalfa becomes dormant in autumn to adapt to the coming cold season. Fall dormancy reflects the acclimation response of alfalfa to both shortening photoperiods and falling temperatures (McKenzie *et al*., 1988). The acclimation of plant tissues prior to exposure to freezing conditions greatly improves survival (Guy, 1990). Non-dormant plants may have alleles at the major loci preventing the triggering of the various pathways leading to winter hardiness (Brummer *et al*., 2000). In contrast, dormant plants initiate all pathways involved in winter hardiness and begin their development well before autumn. The acclimation process in the late summer and autumn diverts fixed C from shoot and leaf growth and toward the various compounds involved in survival. Non-dormant genotypes may not survive well because their acclimation response is not activated. They continue autumn growth and do not accumulate sufficient winter-hardiness-related compounds in their roots and crowns. In the current study, genome-wide analysis of alfalfa miRNAs and their relationship to fall dormancy were conducted using high-throughput sequencing technology.

Identification of known miRNA: Mapped small RNA tags were used to identify the known miRNAs in alfalfa. MiRBase 20.0 served as a reference. With the software package mirdeep2 and the software RNA-tools-cli, 777 known miRNAs unique sequences were identified in the DS library and 797 known miRNAs unique sequences in the NS library, respectively. In these small RNA libraries, mtr-miR398b, mtr-miR1510a-5p, and mtr-miR167a were highly expressed in alfalfa and were sequenced more than 1,000 times each (Fig 3). Some miRNAs (mtr-miR5261, mtr-miR5213-5p, and mtr-miR2111l) were moderately expressed and were sequenced from 10 to 100 times each (Fig 3).

Identification of novel miRNA: Novel miRNA were predicted using the software MiREvo and mirdeep2 by the secondary structure of small RNA. Here, 50 novel hairpins not found in other plant species were identified in the two libraries. There were 460 and 455 unique sequences that mapped to miRNA DS and NS libraries, respectively. Exploration of the occurrence of miRNA

families identified from the samples in other species also showed that the known miRNA and novel miRNAs belonged to 66 miRNA families). Among the miRNA candidates, 23 miRNAs in the DS library and 21 in the NS were found to have complementary miRNAs, respectively. These candidate miRNAs were previously unknown miRNAs in alfalfa, even previously unknown among miRNA families in existence. Most novel miRNA sequences had lengths of 21 nt or 22 nt. The majority of the miRNAs started with a 52 uridine, a hallmark of miRNAs. MiRNA first nucleotide bias was also depicted (Fig 4).The minimum free energies of these miRNA precursors were -30 kcal/mol, which is similar to other reported values (Bonnet *et al*., 2004). The length of the predicted hairpin structures for the miRNA precursors ranged from 46 to 202 nt. These values were similar to those reported for other plants (Szittya *et al*., 2008; Jagadeeswaran *et al*., 2009; Lelandais-Brière *et al*., 2009). In this study, we found that some novel miRNAs were specific to dormant variety alfalfa and others to non-dormant variety alfalfa. These novel miRNAs included novel-miR_1, novel-miR_3, novel-miR_18, novel-miR_42, novel-miR_77, and novel-miR_102, whose expression levels were different between the dormant and non-dormant alfalfas. It has been suggested that these novel miRNAs may play a key role in response to autumn environment changes in alfalfa. However, these novel miRNAs functions are little known and need further study.

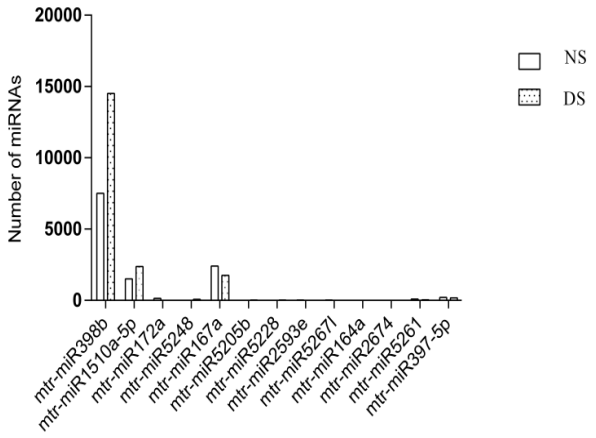

Fig 3. Abundance of conserved miRNAs sequences in DS and NS libraries

MiRNAs specified to different-dormant alfalfa: To investigate the special roles of miRNAs in dormant alfalfa grown in autumn, miRNAs expression in dormant alfalfa (DS) and miRNAs expression in non-dormant alfalfa (NS) were examined using the Solexa technology. In the DS and NS libraries, 21 nt and 24 nt length RNAs, respectively were the most abundant t (Fig 1). It was consistent with small RNAs distribution patterns in other species of plant (Lelandais-Brière *et al*., 2009). However, in the DS library, there were more small RNAs that were 21 nt in length than 24 nt. In the NS library, there were more small RNAs that were 24 nt in length than 21 nt. To identify miRNAs specific to dormant alfalfa, the expression of miRNA in the DS library was compared to that in the NS library. In the DS library, 43 up-regulated miRNAs and 37 down-regulated miRNAs were found. The normalized miRNAs expression level of dormant-variety Maverick and non-dormant-variety CUF101 were recorded (Fig 5). When assessed by RT-PCR and high-throughput sequencing (Fig 6), miRNA expression showed the same trends of up- and down-regulation, and the fold change was similar. Fig 6 showed mtr-miR5205b, mtr-miR5228, mtr-miR2674, mtr-miR5248, and mtr-miR164a to be up-regulated in dormant alfalfa. Conversely, mtr-miR52671, mtr-miR2593e, and mtr-miR172a were down-regulated in dormant alfalfa (Fig 6). Some miRNAs (such as mtr-miR5248, mtr-miR5205b, and mtr-miR5228) were expressed only in the DS library, and some miRNAs (such as mtr-miR172a, mtr-miR2593e, and mtr-miR5267l) were expressed only in the NS library (Fig 3). Some novel miRNAs were expressed very differently in different dormant varieties alfalfa. These novel miRNAs contained novel-miR_1, novel-miR_3, novel-miR_18, novel-miR_42, novel-miR_77, and novel-miR_102, which were highly expressed in the DS library but expressed only minimally or not at all in the NS library. From the differences of these miRNA expression profiles, we could indicate that every miRNA function was different in the dormancy regulation of alfalfa.

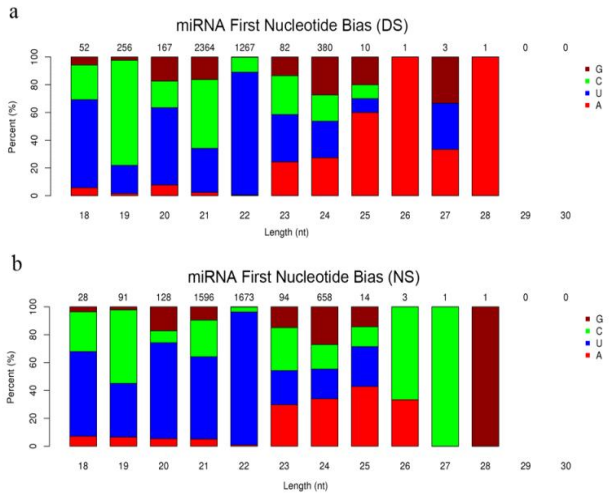

Fig 4. MiRNA first nucleotide bias (DS and NS)

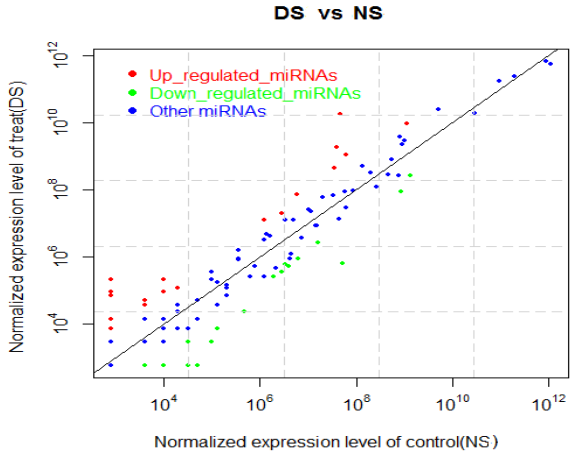

Fig 5. Normalized miRNA expression levels of two different standard alfalfa varieties (Maverick and CUF101)

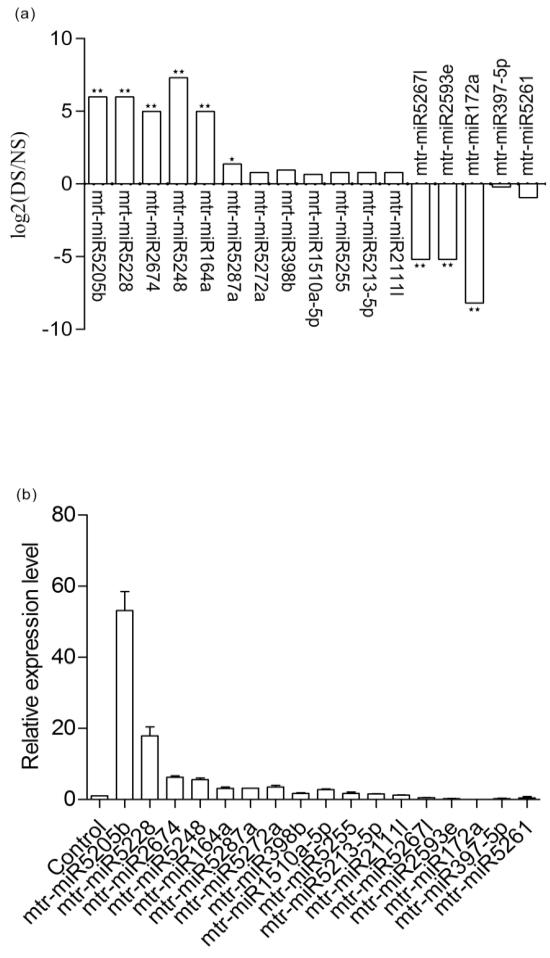

Fig 6. Differential expression analysis between DS and NS libraries

The analysis of conservative miRNA: According to the miRBase 20 (the newest miRNA database which was released in June, 2013), *M. truncatula* owns the richest amount of miRNAs of any plant (Sunkar *et al*., 2007; Lauter *et al*., 2005; Nair *et al*., 2010), even more than *Arabidopsis* and rice (Wang, 2014). However, there has been less functional research conducted on the miRNAs of *M. truncatula* than on those of *Arabidopsis*, maize and rice. Among these highly conserved miRNAs based on sequencing, miR398, miR399, miR393, miR166, miR164, and miR172, which are associated with fall dormancy, have been already investigated in other model plants. The predicted targets of miR172 are APETALA2 transcription factors, which include AP2 itself but also TARGET OF EAT (TOE1 and TOE2) or GLOSSY15 (GL15). Functional analyses of these genes indicated that they normally acted as floral repressors (Aukerman and Sakai, 2003; Kasschau *et al*., 2003; Chen, 2004). The previous study showed that the miR172 promoted the transition of maize from juvenile to adult through down-regulation of GL15, and the results suggested that this mechanism may be widespread for the regulation of changes in vegetative phase in higher plants (Lauter *et al*., 2005). In the current study, 4 miRNAs belonging to the 172 miRNA family were identified. Mtr-miR172a, mtr-miR172b, and mtr-miR172d-3p showed more expression in non-dormant than in dormant alfalfa, and mtr-miR172a was only detected in non-dormant alfalfa. It can be inferred that the short photoperiods and falling temperatures caused down-regulation of miR172 in dormant alfalfa. This might trigger AP2-like and GL15 genes and repress flowering and growth of dormant alfalfa in autumn. By contrast, increasing miR172 expression in non-dormant variety alfalfa may promote alfalfa growth and relieve floral repression. Mtr-miR172d-5p was found only in dormant alfalfa, and its expression was very low. MiR393 is predicted to target several F-box transcripts and it is already known to play an important role in the ubiquitination pathway (also known as ubiquitylation) (Bonnet *et al*., 2004; Jones-Rhoades and Bartel, 2004). Ubiquitination is a process of enzymatic and post-translational modification (PTM), and substrate protein attaches to ubiquitin protein. Ubiquitination affects all organismal activities mostly, such as gene expression, control of the cell cycle, regulation of transcription, and reproductive death. MiR393 has also been reported to be up-regulated in response to cold, dehydration, NaCl, and ABA, suggesting that these miRNAs may target genes involved in a more general response to stress (Martin *et al*., 2010). In this way, increasing levels of miR393 may reduce alfalfa growth velocity under stress conditions.

Table 2. Functions of conserved microRNAs (miRNAs) for which computational prediction and experimental evidence have been published

Function	miRNA families	Target gene	Changes in expression
Floral development and changes in vegetative phase	172	AP2-like transcription factors	Down (Jones-Rhoades *et al*., 2006; Arazi *et al*., 2005)
Developmental defects	393	TIR1	Up (Chen, 2004)
Organ separation and number	164	CUC transcription factors	Down (Mallory and Vaucheret, 2006; Martin *et al*., 2010)
Organ polarity, vascular and meristem development	166	HD-ZIPIII transcription factors	Up (Mallory and Vaucheret, 2006;
Oxidative stress	398	Copper superoxide dismutases	Guy, 1990; Mallory *et al*., 2004) Up (Combier *et al*., 2006)
Phosphate homeostasis	399	Phosphate transporter	Down (Fujii *et al*., 2005; Aufsatz *et al*., 2002)

The experimental result showed that miR393 expression in dormant variety alfalfa was higher than non-dormant variety alfalfa significantly. The function of miR164 is organ separation and determination of the number of organs (Mallory and Vaucheret, 2006; Laufs *et al*., 2004). MiR164 could control the transcription levels and patterns of expression. MiR164 was found to be down-regulated in the current study, suggesting that it might contribute to developmental robustness (Ambros *et al*., 2003; Sieber *et al*., 2007; Combier *et al*., 2006). The functions of miR398 focused on oxidative stress, and its target gene encoded copper superoxide dismutase (Aufsatz *et al*., 2002). MiR399 played a role in phosphate homeostasis, and it was found to target phosphate transporter (Fujii *et al*., 2005; Aufsatz *et al*., 2002). MiR166 targeted HD-ZIPIII transcription factors and its functions focus on organ polarity, vascular and meristem development (Mallory *et al*., 2004). The functional analysis of miR172, miR164, miR166, miR393, miR398, and miR399 showed that all of them were included in biological regulation. Based on the results of GO classification analysis and KEGG pathway analysis, the phenomenon of fall dormancy in alfalfa was strongly correlated to the metabolic pathway which response to the biological regulation.

GO enrichment and KEGG pathways analysis: As indicated by GO enrichment, the main GO enriched factors were biological-regulation, the regulation of biological-processes, response-to-stress, and cellular-responses to a stimulus. Results also showed that the main GO enriched factor was biological regulation, which indicated fall dormancy to be a main biological process in alfalfa (Fig 7). We assessed the statistical enrichment of the target gene candidates in alfalfa by KEGG pathways using the software of KOBAS. The KEGG pathways statistical enrichment showed that the majority clusters included glycerolipid-metabolism, cell-cycle, -meiosis, plant-hormone-signal-transduction, glycerophospholipid -metabolism, and ubiquitin-mediated-proteolysis.

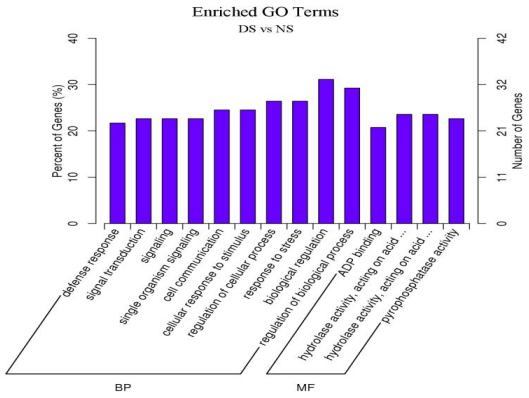

Fig 7. GO enrichment terms of genes differentially expressed in alfalfa

Functions of conserved plant microRNAs involved in alfalfa: Functions of conserved plant miRNAs for which computational prediction and experimental evidence have been published, were also gathered (Table 2). The DS library was compared to NS, and there were three up-regulated conserved miRNAs (miR393, miR166, and miR398) and three down-regulated conserved miRNAs (miR172, miR164, and miR399).

Conclusion

High-throughput sequencing analysis of miRNAs was performed on dormant variety alfalfa and non-dormant variety alfalfa grown in autumn environment. In the current study, the expression levels of miRNAs in the NS library were compared, and 80 miRNAs showed significant differences from their counterparts in the DS library.

Among these miRNAs, some were only found in dormant or in non-dormant alfalfa. Functional analysis of these miRNAs indicated that some known miRNAs (e.g., miR172, miR166, and miRNA393) and novel miRNAs (e.g., novel-miR_1, novel-miR_3, novel-miR_18, novel-miR_42, novel-miR_77, and novel-miR_102) might be very important in the response to dormancy in alfalfa. The current research provides useful information about further miRNAs functional analysis involved in dormancy of alfalfa.

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References

Ambros, V., B. Bartel, D.P. Bartel, C.B. Burge, J.C. Carrington, X. Chen, G. Dreyfuss, S.R. Eddy, S. Griffiths-Jones and M. Marshall. 2003. A uniform system for microRNA annotation. *RNA* 9: 277-279.

Arazi, T, M. Talmor-Neiman, R. Stav, M. Riese, P. Huijser and D.C. Baulcombe. 2005. Cloning and characterization of micro-RNAs from moss. *Plant Journal* 43: 837-848.

Aufsatz, W., M.F. Mette, J. van der Winden, M. Matzkeand A.J. Matzke. 2002. HDA6, a putative histone deacetylase needed to enhance DNA methylation induced by double stranded RNA. *The EMBO Journal* 21: 6832-6841.

Aukerman, M. and H.Sakai. 2003. Regulation of flowering time and floral organ identity by a microRNA and its APETALA2-like target genes. *The Plant Cell* 15: 2730-2741.

Aung, K., S.I. Lin, C.C. Wu, Y.T. Huang, C.l. Su and T.J. Chiou. 2006. PHo_2, a phosphate overaccumulator, is caused by a nonsense mutation in a microRNA399 target gene. *Plant Physiol*ogy 141: 1000-1011.

Baker, C.C., P. Sieber, F. Wellmer and E.M. Meyerowitz. 2005. The early extra petals1 mutant uncovers a role for microrna mir164c in regulating petal number in arabidopsis. *Current Biology* 15: 303-315.

Bari, R., B.D. Pant, M. Stitt and W.R. Scheible. 2006. PHO2, microRNA399, and PHR1 define a phosphate-signaling pathway in plants. *Plant Physiology* 141: 988-999.

Bonnet, E., J.Wuyts, P. Rouzé and P. Y. Van-de. 2004. Evidence that microRNA precursors, unlike other non-coding RNAs, have lower folding free energies than random sequences. *Bioinformatics* 20: 2911-2917.

Brummer, E.C., M.M. Shah and D. Luth. 2000. Reexamining the relationship between fall dormancy and winter hardiness in alfalfa. *Crop Science* 40: 971-977.

Champagne, C.E.M., T.E.Goliber, M.F.Wojciechowski, R.W. Mei, B.T. Townsley, K. Wang, M.M. Paz, R. Geeta and N.R. Sinha .2007. Compound leaf development and evolution in the legumes. *The Plant Cell* 19: 3369-3378.

Chen, X. 2005. MicroRNA biogenesis and function in plants. *FEBS Letters* 579: 5923-5931.

Chen, X. 2004. A microRNA as a translational repressor of APETALA2 in Arabidopsis flower development. *Science* 303: 2022-2025.

Combier, J.P., F. Frugier, F. de Billy, A. Boualem, F. El-Yahyaoui, S. Moreau, T. Vernié, T. Ott, P.Gamas and M. Crespi. 2006. MtHAP2-1 is a key transcriptional regulator of symbiotic nodule development regulated by microRNA169 in *Medicago truncatula*. *Genes and Development* 20: 3084-3088.

Cunningham, S., J. Gana, J. Volenec and L. Teuber. 2001. Winter hardiness, root physiology, and gene expression in successive fall dormancy selections from 'Mesilla'and 'CUF 101'alfalfa. *Crop Science* 41: 1091-1098.

Cunningham, S., J. Volenec and L. Teuber. 1998. Plant survival and root and bud composition of alfalfa populations selected for contrasting fall dormancy. *Crop Science* 38: 962-969.

Dugas, D.V. and B. Bartel. 2004. MicroRNA regulation of gene expression in plants. *Current Opinion in Plant Biology*7: 512-520.

Fahlgren, N., M.D. Howell, K.D. Kasschau, E.J. Chapman, C.M. Sullivan, J.S. Cumbie, S.A. Givan, T.F. Law, S.R. Grant and J.L. Dangl. 2007. High-throughput sequencing of Arabidopsis microRNAs: evidence for frequent birth and death of MIRNA genes. *PLoS ONE* 2: e219.

Fan, W., S. Zhang, H. Du, X. Sun and Y. Shi. 2014. Genome-wide identification of different dormant *Medicago sativa* L. microRNAs in response to fall dormancy. *PLoS ONE* 9: e114612.

Fowler, S. and M.F. Thomashow. 2002. Arabidopsis transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. *The Plant Cell* 14: 1675-1690.

Friedländer, M.R., S.D. Mackowiak, N. Li, W. Chen and N. Rajewsky. 2012. miRDeep2 accurately identifies known and hundreds of novel microRNA genes in seven animal clades. *Nucleic Acids Research* 40: 37-52.

Fujii, H., T.J. Chiou, S.I. Lin, K. Aung and J.K. Zhu. 2005. A mirna involved in phosphate-starvation response in *Arabidopsis*. *Current Biology* 15: 2038-2043.

Guy, C.L. .1990. Cold acclimation and freezing stress tolerance: role of protein metabolism. *Annual Review of Plant Physiology and Plant Molecular Biology* 41: 187-223.

Jagadeeswaran, G., Y. Zheng, Y.F. Li, L.I. Shukla, J. Matts, P. Hoyt, S.L. Macmil, G.B. Wiley, B.A. Roe and W. Zhang. 2009. Cloning and characterization of small RNAs from *Medicago truncatula* reveals four novel legume-specific microRNA families. *New Phytologist* 184: 85-98.

Jones-Rhoades, M.W. and D.P. Bartel. 2004. Computational identification of plant microRNAs and their targets, including a stress-induced miRNA. *Molecular Cell* 14: 787-799.

Jones-Rhoades, M.W., D.P. Bartel and B. Bartel. 2006. MicroRNAs and their regulatory roles in plants. *Annual Review of Plant Biology* 57: 19-53.

Kasschau, K.D., Z. Xie, E. Allen, C. Llave, E.J. Chapman, K.A. Krizan and J.C. Carrington. 2003. P1/HC-Pro, a viral suppressor of RNA silencing, interferes with arabidopsis development and miRNA function. *Developmental Cell* 4: 205-217.

Langmead, B., C. Rapnell, M. Pop and S.L. Salzberg. 2009. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biology* 10: 25.

Laufs, P., A. Peaucelle, H. Morin and J. Traas. 2004. MicroRNA regulation of the CUC genes is required for boundary size control in Arabidopsis meristems. *Development* 131: 4311-4322.

Lauter, N., A. Kampani, Carlson, M. Goebel and S.P. Moose. 2005. microRNA172 down-regulates glossy15 to promote vegetative phase change in maize. *Proceedings of the National Academy of Sciences of the United States of America* 102: 9412-9417.

Lee, R. C., R. L. Feinbaum and V. Ambros. 1993. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* 75; 843-854.

Lelandais-Brière, C., L. Naya, E. Sallet, F. Alenge, F. Frugier, C. Hartmann and M. Crespi. 2009. Genome-wide *Medicago truncatula* small RNA analysis revealed novel microRNAs and isoforms differentially regulated in roots and nodules. *The Plant Cell* 21: 2780-2796.

Li, X. and L. Wan. 2004. Alfalfa fall dormancy and its relationship to winter hardiness and yield. *Acta Prataculturae Sinica* 13: 57-61.

Llave, C., K.D. Kasschau, M.A. Rector and J.C. Carrington. 2002. Endogenous and silencing-associated small RNAs in plants. *The Plant Cell* 14: 1605-1619.

Lu, C., K. Kulkarni, F.F. Souret, R. MuthuValliappan, S.S. Tej, R.S. Poethig, I.R. Henderson, S.E. Jacobsen, W. Wang and P.J. Green. 2006. MicroRNAs and other small RNAs enriched in the Arabidopsis RNA-dependent RNA polymerase-2 mutant. *Genome Research* 16: 1276-1288.

Mallory, A.C., B.J. Reinhart, M.W. Jones-Rhoades, G. Tang, P.D. Zamore, M.K. Barton and D.P. Bartel. 2004. MicroRNA control of PHABULOSA in leaf development: importance of pairing to the microRNA 52 region. *The EMBO Journal* 23: 3356-3364.

Mallory, A.C. and H. Vaucheret. 2006. Functions of microRNAs and related small RNAs in plants. *Nature Genetics* 38: S31-S36.

Mao, X., T. Cai, J.G. Olyarchuk and L.Wei. 2005. Automated genome annotation and pathway identification using the KEGG Orthology.KO. as a controlled vocabulary. *Bioinformatics* 21: 3787-3793.

Martin, R.C., P.P. Liu, N.A.Goloviznina and H. Nonogaki. 2010. microRNA, seeds, and darwin?: diverse function of miRNA in seed biology and plant responses to stress. *Journal of Experimental Botany* 61: 2229-2234.

McKenzie, J.S., R. Paquin and S.H. Duke. 1988. Cold and heat tolerance. In: A.A. Hanson, D.K. Barnes and R.R. Hill (eds). *Alfalfa and alfalfa Improvement*. SSSA, Madison, WI. pp. 259-302.

Moxon, S., F. Schwach, T. Dalmay, D. MacLean, D.J. Studholme and V. Moulton. 2008. A toolkit for analysing large-scale plant small RNA datasets. *Bioinformatics* 24: 2252-2253.

Nair, S.K., N. Wang, Y. Turuspekov, M. Pourkheirandish, S. Sinsuwongwat, G. Chen, M. Sameri, A. Tagiri, I. Honda and Y. Watanabe. 2010. Cleistogamous flowering in barley arises from the suppression of microRNA-guided HvAP2 mRNA cleavage. *Proceedings of the National Academy of Sciences of the United States of America* 107: 490-495.

Navarro, L., P Dunoyer., F. Jay, B. Arnold, N. Dharmasiri, M. Estelle, O. Voinnet and J.D. Jones. 2006. A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. *Science* 312: 436-439.

Park, W., J. Li, R. Song, J. Messing and X. Chen. 2002. CARPEL FACTORY, a Dicer Homolog, and HEN1, a novel protein, act in microRNA metabolism in *Arabidopsis thaliana*. *Current Biology* 12: 1484-1495.

Reinhart, B.J., E.G. Weinstein, M.W. Rhoades, B. Bartel and D.P. Bartel. 2002. MicroRNAs in plants. *Genes and Develpoment* 16: 1616-1626.

Rimi, F., S. Macolino, B. Leinauer, L.M. Lauriault and U. Ziliotto. 2014. Fall dormancy and harvest stage impact on alfalfa persistence in a subtropical climate. *Agronomy Journal* 106: 1258-1266.

Schwab, P., D. Barnes and C. Sheaffer. 1996. The relationship between field winter injury and fall growth score for 251 alfalfa cultivars. *Crop Science* 36: 418-426.

Shinozaki, K. and S.K. Yamaguchi .2007. Gene networks involved in drought stress response and tolerance. *Journal of Experimental Botany* 58: 221-227.

Sieber, P., F. Wellmer, J. Gheyselinck, J.L. Riechmann and E.M. Meyerowitz. 2007. Redundancy and specialization among plant microRNAs: role of the MIR164 family in developmental robustness. *Development* 134: 1051-1060.

Stout, D.G. and J.W. Hall. 1989. Fall growth and winter survival of alfalfa in interior British Columbia. *Canadian Journal of Plant Science* 69: 491-499.

Sunkar, R., V. Chinnusamy, J. Zhu and J.K. Zhu. 2007. Small RNAs as big players in plant abiotic stress responses and nutrient deprivation. *Trends in Plant Science* 12: 301-309.

Szittya, G., S. Moxon, D.M. Santos, R. Jing, M.P. Fevereiro, V. Moulton and T. Dalmay. 2008. High-throughput sequencing of *Medicago truncatula* short RNAs identifies eight new miRNA families. *BMC Genomics* 9: 593.

Vazquez, F., H. Vaucheret, R. Rajagopalan, C. Lepers, V. Gasciolli, A.C. Mallory, J.L. Hilbert, D.P. Bartel and P. Crété. 2004. Endogenous trans-acting siRNAs regulate the accumulation of Arabidopsis mRNAs. *Molecular Cell* 16: 69-79.

Wang, J.W., L.J. Wang, Y.B. Mao, W.J. Cai, H.W. Xue and X.Y. Chen. 2005. Control of root cap formation by microRNA-targeted auxin response factors in Arabidopsis. *The Plant Cell* 17: 2204-2216.

Wang, T.Z. 2014. Recent research progress on micrornas in *Medicago truncatula*. *The Plant Cell* 18: 2051-2065.

Wen, M., Y. Shen, S. Shi and T. Tang. 2012. miREvo: an integrative microRNA evolutionary analysis platform for next-generation sequencing experiments. *BMC Bioinformatics* 13: 140.

Yao, Y., G. Guo, Z. Ni, R. Sunkar, J. Du, J.K. Zhu and Q. Sun. 2007. Cloning and characterization of microRNAs from wheat (*Triticum aestivum* L.). *Genome Biology* 8: R96.

Yadav, V. P., G. P. Shukla, R. V. Kumar, S. Kumar and H. C. Pandey. 2010a. Genetic divergence across the cutting management in alfalfa (*Medicago sativa* L.). *Range Management and Agroforestry* 31: 76-78.

Yadav, V. P., G. P. Shukla, R. V. Kumar, S. Kumar and H. C. Pandey. 2010b. Variability in different cutting management environments in alfalfa (*Medicago sativa* L.). *Range Management and Agroforestry* 31: 150-152.

Ying, S.Y., D.C. Chang and S.L. Lin. 2008. The microRNA (miRNA): overview of the RNA genes that modulate gene function. *Molecular Biotechnology* 38: 257-268.

Zhang, B., X. Pan, G.P. Cobb and T.A. Anderson. 2006. Plant microRNA: a small regulatory molecule with big impact. *Developmental Biology* 289: 3-16.

Zhang, J., Y. Xu, Q. Huan and K. Chong. 2009. Deep sequencing of Brachypodium small RNAs at the global genome level identifies microRNAs involved in cold stress response. *BMC Genomics* 10: 449.

Zhang, S., Y. Shi, N.Cheng, H. Du, W. Fan and C. Wang. 2015. *De novo* characterization of fall dormant and nondormant alfalfa (*Medicago sativa* L.) leaf transcriptome and identification of candidate genes related to fall dormancy. *PLoS ONE* 10: e0122170.

Zhu, J.K. 2002. Salt and drought stress signal transduction in plants. *Annual Review of Plant Biology* 53: 247-273.

Zhu, Q.H., A. Spriggs, L. Matthew, L. Fan, G. Kennedy, F. Gubler and C. Helliwell. 2008. A diverse set of microRNAs and microRNA-like small RNAs in developing rice grains. *Genome Research* 18: 1456-1465.