Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Overexpression of *Medicago sativa cv. Pianguan* miR397-5p gene enhances drought tolerance in K326 tobacco

Huisen Zhu (zhuhuisen@126.com)
Shanxi Agricultural University
Rong Jia
Shanxi Agricultural University
Jiali Tao
Shanxi Institute of Technology
Junbing Jiang
Shanxi Institute of Technology
Kuanhu Dong
Shanxi Agricultural University

Research article

Keywords: Medicago sativa cv. Pianguan, miR397-5p, recombinant plasmid vector pBWA(V)KS-miR397, tobacco

Posted Date: November 11th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-76070/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License

Abstract

Background

Alfalfa (*Medicago sativa cv. Pangua*n) is one of the most widely cultivated perennial leguminous forage. Drought is one of the major abiotic factors that affect alfalfa productivity. MicroRNAs (miRNAs) have been proved playing important roles in plant growth, development and stress response.

Results

In this study, High-throughput sequencing (HTSeq) was used to identify the miRNAs and their target related to drought stress from polyethylene glycol treated and control alfalfa samples, differential expression analysis showed that a large number of miRNAs were down-regulated or up-regulated as a result of PEG-6000 stress. qRT-PCR and sequencing analysis results showed that the relative expression of 9 miRNAs and their target genes related to drought stress, 3 miRNAs were considered potential drought-responsive miRNAs, including miR159b, miR397-5p and unconservative_6_4438. The recombinant plasmid vector pBWA(V)KS-miR397 was constructed and introduced into tobacco successfully. 31 positive seedlings were obtained through the process of infection, co-culture, differentiation and rooting. The physiologieal variation of transgenic tobacco under drought stress was researched. Finally, a new tobacco variety with high drought resistance was obtained.

Conclusion

These finding although need further studies to confirm, these data provide a useful evidence for the possible involvement of miRNAs in the process of drought response in the alfalfa plant which could help explain the drought stress responses and to alleviate the adverse effects of drought stress on plant growth and development.

Background

Alfalfa (*Medicago sativa*) is one of the most valuable fodders of all common hay crops. It is used for grazing, hay, silage, cover crop, as well as green manure, and cultivated in many countries around the world [1]. Since the plant growth largely depends on adequate water supply, drought stress has been considered as the most important abiotic stress limiting alfalfa production and quality.

In plant-environment co-evolution, plants have evolved a series of regulatory mechanisms in the cellular, physiological, biochemical, and molecular processes to protect themselves from different kinds of stress conditions [2]. The major environmental factor that constrains the productivity and stability of plants is water [3]. More and more research results support that plant responses to drought stress are regulated both at transcriptional and post-transcriptional level. As a post-transcriptional gene regulator, MicroRNAs (miRNAs) are small noncoding regulatory RNA widely found in plants, animals and some viruses, that functions in RNA silencing and post-transcriptional regulation of gene expression [4–5]. miRNAs can bind to target messenger RNA (mRNA) transcripts of protein-coding genes and negatively control their translation or cause mRNA degradation [6]. Recent studies indicate that plant miRNAs show pivotal roles in plant response to many abiotic stresses, like high salinity [7], low temperature [8], oxidative stress [9] and drought [10]. It also has been indicated that a certain number of miRNAs involve in response to drought stress by altering the gene expression [11]. Deciphering the molecular genetic mechanisms involved in drought tolerance has certainly made a significant progress in revealing complex biological response of plants at the molecular levels against the drought. For the first time in Medicago truncatula, members of conserved miRNA families miR165, miR181 and miR397 were found [12]. A research from Sunkar showed a significant up-regulation of miR397 expression in Arabidopsis after drought, indicating that miR397 was involved in the stress response [13]. Zhou et al. found that miR397 had significantly lower expression in drought-treated rice than in control group [14]. Compared with that in the control group, miR397-5p had significantly higher expression in drought-treated sugarcane [15] and sorghum [16]. Expression patterns of the same miRNA are different in plant from different species. An over-expressing of tomato Sly-miR397 plasmid vector was constructed and introduced into Arabidopsis thaliana successfully. Under drought stress, the transgenic lines grew better than the wild type. Over-expressing Sly-miR397 significantly improved the drought tolerance of Arabidopsis thaliana, indicanting its important role in plant drought resistance [17].

Medicago sativa cv. Pianguan, known as “the King of grazing grasses”, is a famous, fine local breed of Shanxi. It has the characteristics of cold resistance, barren resistance and high nutritional value. It is significance to improve drought resistance and obtain new varieties with high drought resistance. Until now, there are few reports on the drought-related miRNAs of *Medicago sativa cv. Pianguan*, which also means that there are many drought-related miRNAs waiting for us to discover.

Previous studies on the stress tolerance of miRNA in plants mainly focused on model plants such as Arabidopsis and tobacco. In this study, a native variety of alfalfa, *Medicago sativa cv□Pianguan*, was selected to explore its molecular mechanism in against drought stress based on HTSeq and qRT-PCR. An over-expressing of alfalfa miR397 plasmid vector was constructed and introduced into tobacco successfully. To study the drought tolerance and function analysis of miR397-5p under abiotic stress. These data provide a useful evidence for the possible involvement of miR397-5p in the process of drought response in the alfalfa plant.

Results

HTSeq and qRT-PCR analysis of drought-related miRNAs and their predicted targets

There are 275 miRNAs have changed in 0 h and 72 h, which included 168 miRNAs with down-regulation expression pattern and 107 miRNAs with up-regulation expression pattern. According to the target gene annotation information, qRT-PCR validation of 9 differentially expressed miRNAs in drought-treated and control alfalfa. The results showed that 8 miRNAs had significantly lower expression in drought-treated alfalfa than in control group (**p<0.01; ***p<0.001), compared with that in the control group, miR397-5p had significantly higher expression (**p<0.01) (Fig.1a). We also validated the expression of 6 target genes in drought-treated and control alfalfa with qRT-PCR. In which the expression levels of MTR_1g021230 and MTR_2g020900 were significantly higher in drought-treated than in control alfalfa (**P<0.01), and MTR_3g052450 had higher expression in drought-treated alfalfa than in control one (*P<0.05). There was marked decrease the expression of MTR_4g015120 when treated by drought stress (***p<0.001). The expression of MTR_1g055255 was significantly decreased when drought-treated (**P<0.01). In contrast to control group, the expression level of the target gene MTR_3g052430 had no significantly difference under drought-treated (Fig.1b).

Verification of the selected 9 miRNAs and their potential target genes related to drought-treated from HTSeq and qRT-PCR (Fig.1c). Comparison of the miRNA from HTSeq and qRT-PCR analyses revealed that the relative expression trends of 9 miRNAs in response to the drought treatment were the same (conservative_8_17329, conservative_8_17330, conservative_8_17332, miR159b, miR2590h, miR2590i, miR2590j, and unconservative_6_4438 showed down-regulated trends; miR397-5p showed up-regulated trends). The target genes MTR_3g052430, MTR_3g052450, MTR_1g021230, MTR_1g055255, MTR_2g020900 for conservative_8_17329, conservative_8_17330, conservative_8_17330, miR159b, miR2590h, miR2590i, miR2590j, unconservative_6_4438 were down-regulated under drought-treated, while target genes MTR_4g015120 for miR397-5p was up-regulated. qRT-PCR and HTSeq analysis showed that the relative expression trends of 9 miRNAs and their target genes, of which the expression patterns of 6 miRNAs were the same for both methods, and their target genes have a negatively correlated expression pattern. In contrast to control group, the expression levels of the target genes MTR_3g052430 and MTR_3g052450 were not significantly different under drought-treated. Those left 3 miRNAs were considered to be potential drought-responsive miRNAs, including miR159b, miR397-5p, and unconservative_6_4438. By means of analyzing the similarity of miR397-5p and its target genes between in alfalfa and tobacco, we finally selected miR397-5p to construct the expression vector.

The result of recombinant plasmid pBWA(V)KS-miR397 into tobacco and identification

As shown in Fig. 2 , the results of PCR and qRT-PCR showed that among the 65 tobacco seedlings, there were 31 positive seedlings, the positive rate was 47.7%. After drought treatment, the relative expression level of miR397-5p in transgenic tobacco leaves was significantly higher than that in K326 tobacco (***p<0.001). The above results indicate that we have established a relatively complete tobacco conversion system.

Physiological responses under drought stress

In this study, Fig. 3a shows the conductivity of transgenic tobacco leaves was lower than that of K326 tobacco leaves after drought stress treatment (*p<0.05). As shown in Fig. 3b, after drought treatment, MDA content in K326 tobacco leaves was significantly higher than that in transgenic tobacco leaves (**p<0.01). Fig. 3c shows compared with K326 tobacco, the SOD activity and POD activity of transgenic tobacco leaves were significantly increased (**p<0.01). As shown in Fig. 3d, the content of proline in the leaves of transgenic tobacco was higher than that in K326 tobacco after drought treatment (*p<0.05). As shown in Fig. 3e, After drought treatment, the relative expression level of *P5CS* gene in transgenic tobacco leaves was higher than that in K326 tobacco (*p<0.05). The relative expression level of *δ-OAT* gene in transgenic tobacco leaves was significantly higher than that in K326 tobacco (***p<0.001); the relative expression level of *ProDH* gene in transgenic tobacco leaves was significantly lower than that of K326 tobacco (**p<0.01).

Discussion

Many studies have shown that miRNAs were involved in various biological processes by HTSeq technology [18], such as plant growth and development, cell differentiation, stress resistance and other biological processes, especially the mechanism of miRNA involvement in plant stress response was paid much attention [19]. Currently, miRNA research on Arabidopsis, rice and other model plants remained dominant, but there are still a large number of miRNAs have not been explored in *Medicago sativa cv. Pianguan.*

Wang et al. identified miRNA of drought treated four *Medicago truncatula* (*cv Jemalong A17*) and control group through HTSeq and qRT-PCR methods [20]. The results showed that miR399, miR2118, miR2089, miR2111 being up-regulated and miR164, miR169, miR171, miR396, miR398, miR1510 being down-regulated for drought stress, respectively. Partially differentially expressed miRNAs were also included in the results of our tests. In this study, qRT-PCR and sequencing analysis results showed that the relative expression of 9 miRNAs and their target genes, miR159b, miR397-5p and unconservative_6_4438 were considered potential drought-responsive miRNAs. It has been reported that the expression of ssp-miR397 (target of ssp-miR397 is a gene encoding a laccase) was increased in sugarcane under drought treatment for 48 h, and laccase expression levels had a reduction [15]. This research also obtained similar results, the expression level of miR397-5p was significantly increased under drought treatment for 72 h, while the expression of target gene MTR_4g015120 (laccase family protein) was significantly decreased. Indicating that miR397-5p might regulate drought stress response and tolerance by regulating the expression of the target gene MTR_4g015120.

In this study, an over-expressing of alfalfa miR397 plasmid vector was constructed and introduced into tobacco successfully. The present study investigated the responses of tobacco plants to drought stress induced by the addition of PEG 6000 to the nutrient solution. To study the drought tolerance and function analysis of miR397-5p under abiotic stress. Conductivity through cell membrances is commonly considered an index of membrance damage or deterioration. So, the conductivity is an important indicator of the extent of membrance integrity impairment in plant cells during abiotic stress [21]. Malondialdehyde (MDA) content can represent the extent of lipid peroxidation and membrane injury that has occurred. In transgenic tobacco, the relative conductivity and the MDA content were lower than in K326 tobacco, indicating that introduction of the miR397-5p gene protected the cell membranes and decreased membrane damage under the drought stress. This result is consistent with the previous findings by Wu et al. [22]. Wu et al. found that overexpression of the Arabidopsis 2-aldol reductase gene was significantly reduced the MDA content of tobacco under drought stress [22]. Antioxidant enzymes including SOD and POD maintains the

balance of ROS in plant cells, massive ROS accumulation which damages to cell membranes and accelerates leaf senescence [23]. The results of this test showd that compared with K326 tobacco, the SOD activity and POD activity of transgenic tobacco leaves were significantly increased. Drought, high temperature and high salt will lead to the accumulation of proline content in plants, and its level varies with the severity of stress and the level of stress tolerance of plant species [24]. These result showed that the content of proline in the leaves of transgenic tobacco was higher than that in K326 tobacco after drought treatment. Proline accumulation can improve the tolerance to abiotic stress such as drought, salt, heat, cold, in which the closely related key enzymes Delta-1-pyrroline-5-carboxylate synthase (P5CS) and δ-ornithine amino transferase synthase (δ-OAT), respectively. The proline dehydrogenase (ProDH) is the major rate-limiting enzyme in proline degradation [25]. Our results are consistent with those reported that P5CS and δ-OAT contribute to the drought stress-induced proline accumulation in tobacco. a negative correlation was found between proline and ProDH accumulation.

In summary, physiological analyses of plants under under drought stress condition demonstrated that miR397-5p could improve drought tolerance through regulating multiple physiological processes. These results highlight the beneficial roles of miR397-5p in drought responses. Future research will focus on the role of miR397-5p as stress signaling molecules on differential expression of genes and proteins, understanding molecular mechanism of miR397-5p in combating drought stress.

Conclusion

In summary, HTSeq and qRT-PCR analysis showed that miR397-5p gene was involved in the stress response by regulating the expression of the target gene MTR_4g015120. Further, the recombinant plasmid vector pBWA(V)KS-miR397 was constructed and introduced into tobacco successfully. Overexpression of *Medicago sativa cv. Pianguan* miR397-5p gene enhances drought tolerance in K326 tobacco. These data provide a useful evidence for the possible involvement of miRNAs in the process of drought response in the alfalfa plant which could help explain the drought stress responses and to alleviate the adverse effects of drought stress on plant growth and development.

Methods

Strains, vectors and plants

Escherichia coli strain DH5α and *Agrobacterium tumefaciensL*. strain EHA105 were used in this study. The plasmid vector pBWA(V)KS-ccDB was used to construct the expression vector. *Medicago sativa cv. Pianguan* was provided from the grass laboratory of the Agricultural University of Shanxi. The miR397-5p gene was amplified from the *Medicago sativa cv. Pianguan*. The tobacco (*Nicotiana tabacum L*.) cv. K326 was used for genetic transformation, which was provided by BioRun company of wuhan.

HTSeq and qRT-PCR analysis of drought-related miRNAs and their predicted targets

The native variety of alfalfa (*Medicago sativa cv*□*Pianguan*) were grown in a greenhouse under a photoperiod of 14 h at 20˚C to 25˚C for 4 weeks old, then the alfalfa were divided into two groups, and treated with the nutrient solution Hoagland (include 20%PEG-6000) for 0 h (control) and 72 h, respectively. The whole alfalfa samples were collected and frozen in liquid nitrogen, and then stored at -80˚C for RNA extraction. Three biological repeats were taken in each group to enhance the accuracy of the experimental result. The total RNA were extracted by Trizol (Invitrogen, USA) from those three repeats and then mixed with equal amount. Small RNA libraries were constructed as described by Eldem et al. [26].

The sequencing data were analyzed as described by Zhang et al. [27]. TargetFinder software was used to predict target genes, which based on gene sequence information of corresponding species from known miRNAs and novel miRNAs, the predicted target gene sequences and the databases from GO were compared by Blast software to obtain target gene annotation information (Additional file). Validation of drought-related miRNAs and target gene expression with qRT-PCR. RNA samples were reverse transcribed into cDNA as initial templates. The cDNA was synthesized by reverse transcription from 1 μg total RNA using the One Step PrimeScript miRNA cDNA Synthesis Kit (Takara, Japan) and Thermo First cDNA Synthesis Kit (Thermo, USA). The mixture was incubated at 37˚C for 60 min, and the reaction was stopped by heating at 85˚C for 10 min. Primer 5 software was used to design primers of 9 miRNAs and 6 target genes which related to drought resistance. Detailed information is shown in Table 1(Reverse primers of 9 miRNAs were provided with the kit). The cDNA was used as template, U6 and actin were used as internal genes for qRT-PCR. The qRT-PCR was performed using SYBR® Premix Ex Taq II Kit (Takara, Japan) on an ABI-7500 (Life Technologies, USA) under the following conditions, at 95˚C for 30 s, 40 cycles of 95˚C for 5 s and 60˚C for 34 s, one cycle of 95˚C for 15 s, 60˚C for 1 min, 95˚C for 30 s and 60˚C for 15 s. Reaction system was 20 μL, consisting 10 μL SYBR® Premix Ex Taq II (2×), 100 ng cDNA, 1 μL each primer (10 μmol/L), and ddH_2O to total volume 20 μL.

Table 1 Primers for 9 miRNAs and their 6 target genes

miRNA	Primer Seq(5'-3')	Target	Primer Seq(5'-3')
conservative_8_17329	ATTGGATTGAAGGGAGCTCC	MTR_3g052430	TGCCTTCTTCCACAATGCAA
	ATTGGATTGAAGGGAGCTCC		GCCATCACCACACCACTAGA
conservative_8_17330	ATTGGATTGAAGGGAGCTCC	MTR_3g052450	TAGTGGTGGTTGGTGATGAGG
conservative_8_17332			GCATTCCAATTCCCTTCACCA
miR159b	ATTGGAGTGAAGGGAGCTCCA	MTR_1g021230	CTGTGGGAAAAGCTGTCGTC
			ATTCGGCGTTCTTCTTCAGC
miR2590h	GCGAGAATGACATGGCAGAATAATCAC	MTR_1g055255	AGTGCCGGTGTCATCTTGTA
	GCAGAATGACATGGCAGAATAATCAC		
miR2590i	AGAATGACATGGCAGAATAATCAC		ACTAGGCCATGGATCCGATG
miR2590j			
miR397-5p	TCATTGAGTGCAGCGTTGATG	MTR_4g015120	AACTACCTGTGGAGAGTGGC
			TGTGAGTTTGTGGCCAGCTA
unconservative_6_4438	GCGTTAGCTCAGTTAGTAAGGACAATG	MTR_2g020900	TCCCTGGAGCTATCAATCGC
			ATACATGACCCAAGTGCCCA
U6	CCTGCGCAAGGATGACACGCAT	Actin JQ028731.1	TCGAGACCTTCAATGTGCCT
			ACTCACACCGTCACCAGAAT

Construction of recombinant plasmid pBWA(V)KS-miR397 vector

Alfalfa were grown in a greenhouse under a photoperiod of 14 h at 20˚C to 25˚C for 4 weeks old, then the alfalfa were treated with the nutrient solution Hoagland (include 20%PEG-6000) for 72 h. The leaves of drought-treated alfalfa were collected from the greenhouse. The total RNA were extracted and reverse transcribed into cDNA as initial templates for miR397-5p sequence amplification.

The miR397-5p precursor sequence was miR397 TTGGAGAAACATCATTGAGTGCAGCGTTGATGAAATCTTATGTAGCATCTAGCTATCTTATAGATATCAAATATTGCATGCTTAATTTCATCTACGCTACACTCAATTATGTT the miR397-5p mature sequence was TCATTGAGTGCAGCGTTGATG. The primers of miR397 are as follows,

Forward Primer: cagtGGTCTCacaacttggagaaacatcattgagt;

Reverse Primer: cagtGGTCTCatacattaaaaaaacataattgagt.

The PCR procedure was as follows, pre-denaturation at 94˚C for 5 min, denaturation at 94˚C for 10 s, annealing at 58˚C for 30 s, extension at 72˚C for 30 s and 30 cycles, and extension at 72˚C for 10 min, 16˚C for 30 min. Reaction system was 50 μL, consisting 1 μL Template, 1 μL each primer (10 μmol/L), 1 μL KOD, 10 μL dNTP mix, 5 μL 10 x buffer, and ddH_2O to total volume 50 μL. The 1% agarose gel electrophoresis was performed to detect the amplified product fragment and to determine its length. The appropriate fragment was recovered through DNA gel extraction kit (OMEGA, USA), then extraction product was cloned into the digested vector pBWA(V)KS-ccDB. Reaction system was 10 μL, consisting 2 μL miR397-5p, 1 μL T_4 DNA Ligase, 1 μL 10×T_4 DNA Ligase buffer, 1 μL pBWA(V)KS-ccDB, and ddH_2O 5 μL for 12 h at 4˚C. The target fragment was connected to the vector to form a new vector that included the target gene. The connection product was introduced into *Escherichia coli* strain DH5α through thermal activation, and *E. coli* DH5α was grown on kanamycin Luria-Bertani (Kan LB) solid medium and was cultured for 12 h at 37˚C when a single colony was created.

Detection of recombinant plasmid pBWA(V)KS-miR397 vector

Recombinant plasmid pBWA(V)KS-miR397 vector was identified by polymerase chain reaction (PCR), restriction enzyme digestion and sequencing. A single colony was picked and further cultured at 37˚C in LB liquid medium supplemented with Kan (100 μg/mL). The plasmid of included the target gene was extracted as a template for PCR amplification, Primer 5 software was used to design primers (F: 5′-ttggagaaacatcattgagt-3′, R: 5′-caagaccggcaacaggattcaatc-3′). The 1% agarose gel electrophoresis was performed to detect the amplified product fragment and to determine its length. Positive colony was further confifirmed by restriction enzyme digestion and sequencing. The plasmid of included the target gene was extracted, and the concentration was tested through NanoDrop 1000 (NanoDrop Technologies, USA). The restriction enzyme digestion reaction system was 10 μL, consisting 3 μL plasmid , 0.5 μL Eco32I, 1 μL 10×buffer, 5.5 μL ddH_2O for 2 h at 37˚C, the 1% agarose gel electrophoresis was performed to detect the product fragment and to determine its length. The PCR production which shows the brightest band was sent to Huada Company for DNA sequencing.

Tobacco transformation by Agrobacterium-mediated leaf disc method

The recombinant plasmid pBWA(V)KS-miR397 was introduced into *Agrobacterium tumefaciens* L. strain EHA105, and EHA105 was grown on Kan LB solid medium and was cultured for 2 days at 28˚C. A single colony was picked and further cultured in MS liquid medium supplemented with Kan (100 μg/mL) until the OD_{600} reached at 0.8. Sterile, fresh, and green tobacco leaves were cut into 0.5 cm × 0.5 cm leaf discs along their veins. These leaves were infected with the bacterial liquid for 10 min. The leaf discs were dried using a sterile blotting paper and then were inoculated in the co-culture medium (Murashige and Skoog (MS) + 2.0 mg/dm^3 6-benzylaminopurine (BA) + 0.1 mg/dm^3 indole-3-butytric acid (IBA)). After dark culture for 2 days at 25˚C, the leaves were transferred to the screening culture medium (MS + 2.0 mg/dm^3 6-BA + 0.1 mg/dm^3 IBA + 50 mg/dm^3 kanamycin (Kan) + 400 mg/dm^3 sodium cefotaxime (Cef)). After growing to 2 cm, the resistant buds were transferred to the rooting medium (MS + 75 mg/dm^3 Kan+ 400 mg/dm^3 Cef for screening). The rooting plants were propagated and acclimatized before they were transplanted into the greenhouse. The leaves of transgenic tobacco and non-transgenic tobacco (control) were collected from the greenhouse. Transgenic tobacco were identified by PCR and qRT-PCR. Five transgenic tobaccos were chosen from that was transformed by vector pBWA(V)KS-miR397. Following the principle of relative quantification, the initial number of copies of the miR397-5p in transgenic tobacco was measured using the real-time fluorescence quantitative PCR technique. Aactin gene was used as internal genes for qRT-PCR.

Physiological analyses

4 weeks old K326 tobacco and positive transgenic tobacco seedlings were treated with the nutrient solution Hoagland (include 20%PEG-6000) for 72 h, and the leaves of the tobacco were collected for physiological analyses and total RNA extraction. The relative conductivity of K326 tobacco and transgenic tobacco leaves was detected as described by Wang et al. [28]. The concentration of malondialdehyde (MDA) was calculated according to the formula described by Dhindsa et al. [29]. The activities of SOD and POD were determined by using the methods of Giannopolitis et al. [30] and Chance et al. [31]; Free proline content in the leaves was determined according to the method of Bates et al. [32]; Relative expression level of *P5CS*, *δ-OAT* and *ProDH* gene were detected by qRT-PCR, primers of *P5CS*, *δ-OAT* and *ProDH* gene information is shown in Table 2.

Table 2 Sequence of primers

Name of primer	Sequence of primers（5′-3′）	Length (bp)
P5CS	CTTGCAAGGGGAACTGGACA	204
	CATAGTCAGTTCCAGGACGGG	
δ-OAT	TTCTGGTCGATTGCTGGCTT	156
	AAGGTGCTTCCATGCTCTCC	
ProDH	AGCTATGTGCGTAGCCTCTT	158
	ATTTCGGCTCCACTTTGGGT	
β-actin	AACATCGTATTGAGTGGTGGTA	231
	CATGGTCCGGGTATTGTTTC	

Data analysis

T-test was used to determine if there was a significant difference between the means of two groups. The *t*-test was completed by GraphPad Prism (GraphPad Software, Inc. California, USA) software. Average threshold (Ct) values per triplicate were used to calculate the relative amounts of mRNA using the $2^{-\Delta\Delta Ct}$ method. Data were expressed as mean±SEM, $P<0.05$, $P<0.01$ and $P<0.001$ were considered to be significantly different at three levels.

Abbreviations

BA, benzylaminopurine; bp, base pair(s); Cef, sodium cefotaxime; HTSeq, High throughput sequencing; IBA, indole-3-butytric acid; Kan, kanamycin; LB, Luria-Bertani (medium); miRNA, miroRNA; MS, Murashige and Skoog.

Declarations

Acknowlegements

Not applicable.

Author's Contributions

HSZ and JBJ conceived and supervised the study; HSZ and KHD designed experiments; RJ performed experiments; RJ and JLT analyzed data; HSZ and RJ wrote the manuscript; JBJ made manuscript revisions.

Funding

This work was sponsored by the National Natural Science Foundation of China (31402131), and the fifty-sixth batch of China Postdoctoral Science Foundation (2014M561204, 124434).

Availability of data and materials

All data generated or analysed during this study are included in this published article (and its supplementary information files). The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

[1]College of Animal science and Veterinary Medicine, Shanxi Agricultural University, Taigu, 030801, Shanxi, China. [2]Department of Geological and Environmental Engineering, Shanxi Institute of Technology, Yangquan, 045000, Shanxi, China.

References

1. Osborn TC, Brouwer D, Mccoy TJ, Mckersie BD, Brown DCW. Molecular marker analysis of alfalfa. Biotechnology and Improvement of Forage Legume 1997;91-109.
2. Covarrubias AA, JOSé L, Reyes. Post-transcriptional gene regulation of salinity and drought responses by plant micrornas. Plant Cell and Environment. 2010;33(4):481-489.
3. Araus JL, Slafer GA, Reynolds MP, et al. Plant breeding and drought in C3 cereals: what should we breed for? Annals of Botany. 2002;89(7):925-940.
4. Ambros V. The functions of animal micrornas. Nature. 2004;431(7006):350-355.
5. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004;116(2):281-297.
6. Zheng H, Fu R, Wang JT, Liu Q, Chen H, Jiang SW. Advances in the techniques for the prediction of microrna targets. International Journal of Molecular Sciences. 2013;14:8179-8187.
7. Lu SF, Sun YH, Shi R, Clark C, Li LG, Chiang VL. Novel and mechanical stress–responsive micrornas in populus trichocarpa that are absent from arabidopsis. Plant Cell. 2005;17(8):2186-2203.
8. Zhou X, Wang G, Sutoh K, Zhu JK, Zhang W. Identification of cold-inducible micrornas in plants by transcriptome analysis. BBA-Gene Regulatory Mechanisms. 2008;1779(11):780-788.
9. Sunkar R. Posttranscriptional induction of two cu/zn superoxide dismutase genes in arabidopsis is mediated by downregulation of mir398 and important for oxidative stress tolerance. Plant Cell. 2006;18:2051-2065.
10. Kantar M, Unver T, Budak H. Regulation of barley mirnas upon dehydration stress correlated with target gene expression. Functional & Integrative Genomics. 2010;10(4):493-507.
11. Arenashuertero C,Pérez B, Rabanal F, Blancomelo D, Rosa CDL, Estradanavarrete G. Conserved and novel mirnas in the legumephaseolus vulgarisin response to stress. Plant Molecular Biology. 2009;70(4):385-401.
12. Eyles RP, Williams PH, Ohms SJ, Weiller GF, Ogilvie HA, Djordjevic MA, Imin N. Microrna profiling of root tissues and root forming explant cultures in medicago truncatula. Planta. 2013;238(1):91-105.
13. Sunkar R. Novel and stress-regulated micrornas and other small rnas from arabidopsis. The plant cell online. 2004;16(8):2001-2019.
14. Zhou LG, Liu YH, Liu ZC, Kong DY, Duan M, Luo LJ. Genome-wide identification and analysis of drought-responsive microRNAs in Oryza sativa. Journal of Experimental Botany. 2010;61(15):4157-4168.
15. Ferreira TH, Gentile A, Vilela RD, Costa GGL, Dias LI, Endres L, Menossi M. microRNAs associated with drought response in the bioenergy crop sugarcane (Saccharum spp.). Plos One. 2012;7(10):e46703.
16. Hamza NB, Sharma N, Tripathi A, Sanan N. Microrna expression profiles in response to drought stress in sorghum bicolor. Gene Expression Patterns. 2016;20:88-98.
17. Xiang J, Lin P, Li XS, Li ST, Liu MY, Zhang L, Guo YD. Overexpression of tomato Sly-miR397 gene enhances drought tolerance in Arabidopsis thaliana. Journal of China Agricultural University. 2016;21:51-58. (in Chinese)
18. Carrington JC, Ambros V. Role of micrornas in plant and animal development. Science. 2003;301(5631):336-338.
19. Hake S. Micrornas: a role in plant development. Current Biolog. 2003;13:851-852.
20. Wang T, Chen L, Zhao M, Tian Q, Zhang WH. Identification of drought-responsive microRNAs in Medicago truncatula by genome-wide high-throughput sequencing. BMC Genomics. 2011;12(1):367-367.
21. Lu Y, Xu ZS, Zhang RY. Over-expression of W6 gene increases salt tolerance in transgenic tobacco plants. Acta Agronomica Sinica. 2008;34:984 -990.(in Chinese)

22. Wu X, Wang SW, Cao D, et al. Influence ofoverexpres-sion of 2-alkenal reductase gene on drought resistance in tobacco. Acta Bot Boreal-Occidental Sin. 2015;35:166-172.
23. Xu J, Duan X, Yang J, Beeching JR, Zhang P. Enhanced reactive oxygen species scavenging by overproduction of superoxide dismutase and catalase delays postharvest physiological deterioration of cassava storage roots. Plant Physiol. 2013;161(3):1517-1528.
24. Yamada M, Morishita H, Urano K, Shiozaki N, Yamaguchi-Shinozaki K, Shinozaki K, Yoshiba Y. Effects of free proline accumulation in petunias under drought stress. Journal of Experimental Botany. 2005;56(417):1975-1981.
25. Zhu HS, Yu XJ, Zhao X, Yang WD, Dong KH. The activity and gene expression levels of P5CS and δ-OAT in Medicago Sativa cv. Pianguan under drought stress. Pakistan Journal of Botany. 2016;48:137-142.
26. Eldem V, Çelikkol Akçay U, Ozhuner E, et al. Genome-wide identification of miRNAs responsive to drought in peach (Prunus persica) by high-throughput deep sequencing. PloS One. 2012;7(12):e50298.
27. Zhang N, Yang JW , Wang ZM, et al. Identification of Novel and Conserved MicroRNAs Related to Drought Stress in Potato by Deep Sequencing. Plos One. 2014, 9(4):e95489.
28. Wang Y, Jiang J, Zhao X, Liu J, Yang C. A novel lea gene from Tamarix androssowii confers drought tolerance in transgenic tobacco. Plant Science. 2006;171:655–662.
29. Dhindsa RS, Plumb-Dhindsa P, Thorpe TA. Leaf Senescence: Correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. Journal of Experimental Botany. 1981;32:93-101.
30. Giannopolitis CN, Ries SK. Superoxide Dismutases: I. Occurrence in Higher Plants. Plant Physiology. 1977;59(2):309-314.
31. Chance B, Maehly AC. Assay of catalases and peroxidases. Methods in Enzymology, 1955, 2(55):764-775.
32. Bates LS, Waldren RP, Teare ID. Rapid determination of free proline for water-stress studies. Plant Soil. 1973;39(1):205–207.

Supplementary Information

Additional file: How to screen and obtain miRNA by gene annotation?

Additional file: Table S1. Gene ontology (GO) enrichment was conducted to analyze the functions of target genes for the miRNAs. **Table S2** Screen and obtain target genes related to drought stress by gene annotation. **Table S3.** Screen and obtain miRNA related to drought stress by gene annotation. **Table S4.** The expression of miRNAs responsive to drought stress. **Table S5.** 9 miRNAs and their potential target genes related to drought stress.

Figures

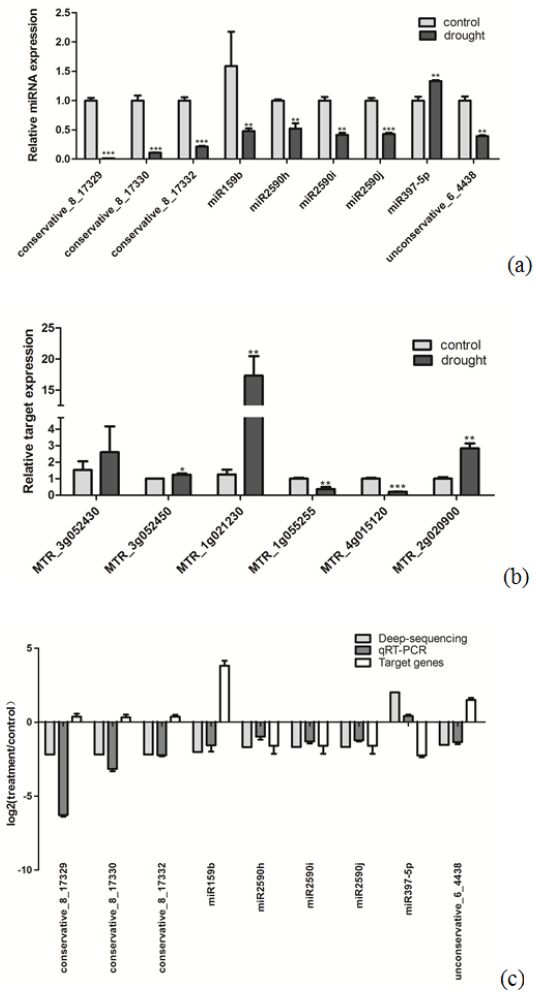

Figure 1

Fig.1a qRT-PCR validation of 9 differentially expressed miRNAs in drought versus control Alfalfa. Note: Bars in each panel represent the mean ± standard error (n=3).* means significant differentiale at the level of significance set at 0.05; ** means significant differentiale at the level of significance set at 0.01; *** means significant differentiale at the level of significance set at 0.001. Fig. 1b qRT-PCR validation of 6 target genes in drought versus control Alfalfa. Note: Bars in each panel represent the mean ± standard error (n = 3). * means significant differentiale at the level of significance set at 0.05; ** means significant differentiale at the level of significance set at 0.01; *** means significant differentiale at the level of significance set at 0.001. Fig. 1c Verification of the selected 9 miRNAs and their potential target genes related to drought stress. Note: Relative expression levels of the selected 9 miRNAs (conservative_8_17329, conservative_8_17330, conservative_8_17332, miR159b, miR2590h, miR2590i, miR2590j, miR397-5p and unconservative_6_4438) and their potential target genes (MTR_3g052430, MTR_3g052450, MTR_1g021230, MTR_1g055255, MTR_4g015120, MTR_2g020900) were measured by qRT-PCR. The expression level is expressed as the mean of relative fold changes of triplicate biological replicates and the vertical bars represent standard derivation of the mean (n=3).

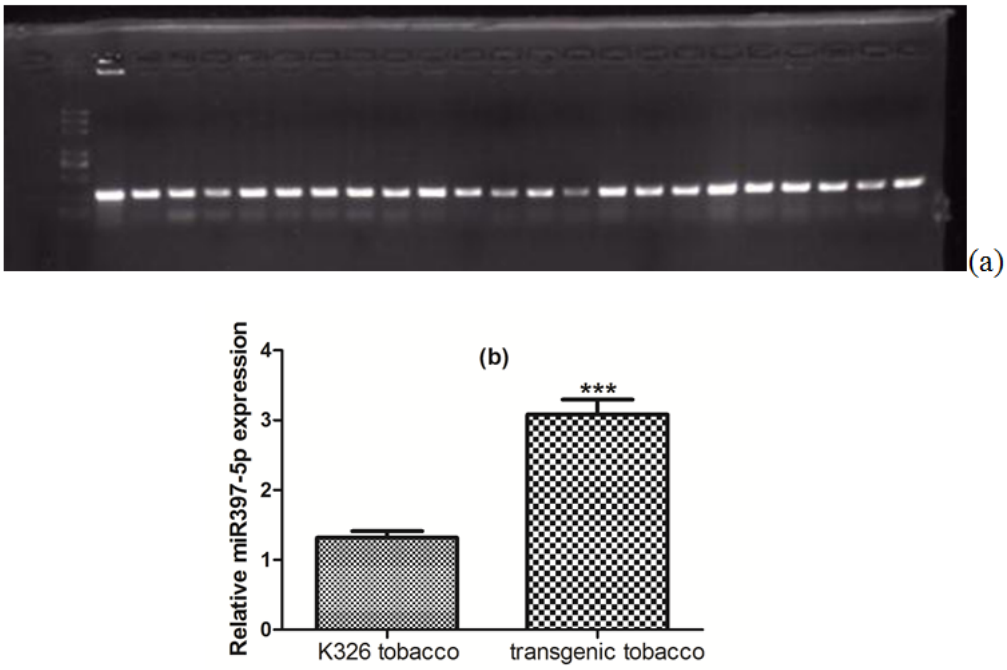

Figure 2

Result of PCR (a) and qRT-PCR (b) of transgenic tobacco. Data were expressed as mean±SEM, P<0.05, P<0.01 and P<0.001 were considered to be significantly different at three levels.

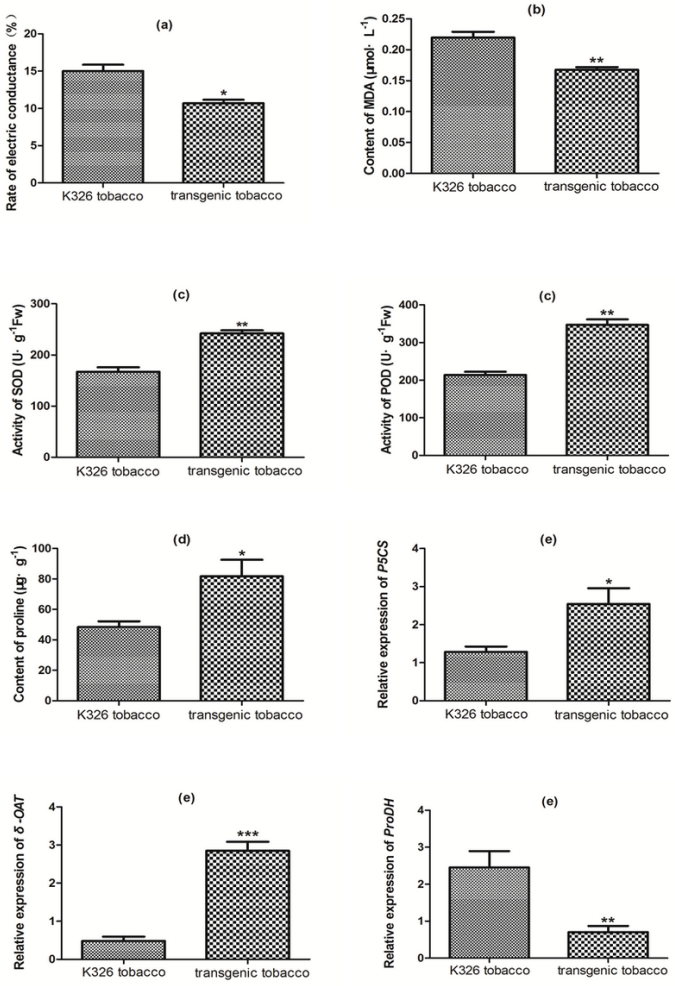

Figure 3

Effects of drought stress on leaves of K326 tobacco and transgenic tobacco. Note: Effects of drought stress on leaves of K326 tobacco and transgenic tobacco, including the rate of electric conductance (a), content of MDA (b), activity of SOD / POD (c), content of proline (d), relative expression level of P5CS / δ-OAT and ProDH gene (e). Data were expressed as mean±SEM, $P<0.05$, $P<0.01$ and $P<0.001$ were considered to be significantly different at three leves.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile.xls