Drought tolerance dissection and molecular breeding in alfalfa (*Medicago sativa*)

Dong, Luo*; Qiang Zhou*; Xueming Dong*; Wenxian Liu*; Zhipeng Liu*,[1]
*State Key Laboratory of Herbage Improvement and Grassland Agro-ecosystems, Lanzhou University, Lanzhou 730000, China
[1]Correspondence author

Keywords: Alfalfa; drought tolerance; transcriptome analysis; functional analysis

Abstract

Drought stress is one of the leading impediments that limit the productivity of global alfalfa (*Medicago sativa*). The underlying molecular and genetic mechanisms for drought tolerance in alfalfa remain largely unclear. In order to fully reveal the transcriptional changes of alfalfa in response to abiotic stress, the alfalfa transcriptome database under mannitol (simulated drought stress), NaCl (simulated salt stress), or exogenous ABA application was built via various RNA-seq technologies. Through further screening of the transcriptome database, a number of genes significantly induced by drought stress, such as the *Nuclear Transport Factor 2-like* (*MsNTF2L*), *Drought-Induced Unknown Protein 1* (*MsDIUP1*), and *MsNST1*, were identified. These three genes were transferred into alfalfa by overexpression and RNAi techniques, and their physiological characteristics and transcriptional level response were synthetically studied. Alfalfa *MsNTF2L*-OE plants have been approved by the Ministry of Agriculture of China to carry out the field test in Gansu Province. Furthermore, we constructed a GWAS population and obtained 50 excellent plants with strong drought tolerance and high hay-yield. These studies provide a theoretical foundation for drought-tolerant molecular breeding of alfalfa.

Introduction

Cultivated alfalfa (*Medicago sativa*) is the most widely grown forage legume in pastoral agriculture, with a planting area of more than 40 million hectares worldwide (Luo *et al.* 2022). However, in many arid and semi-arid areas, drought is one of the main factors limiting the growth and yield of alfalfa. Molecular breeding based on genotyping has a high potential interest for improving alfalfa tolerance to drought stress conditions. Molecular breeding includes marker-assisted selection (MAS), genomic selection (GS), and genetic engineering. Approaches such as transcriptome analysis and genome-wide association study (GWAS) analysis help in identifying marker-trait associations that can guide MAS and/or GS, while transgenic or gene editing approaches were used to genetically confirm the numerous trait loci. In this study, we constructed a transcriptome database for alfalfa. Then, a number of drought-induced genes were systematically studied by analyzing physiological and transcriptional responses under drought stress. Finally, we constructed an alfalfa GWAS population and screened drought-tolerant alfalfa individuals. These results provide great potential to optimize drought tolerance trait in further alfalfa molecular breeding.

Methods and Study Site

In the past 12 years, our group has constructed a series of alfalfa transcriptome data,

including the development process of different tissues and the response to abiotic stress. Leaves, root tips, or whole seedlings from Zhongmu No.1 were usually collected to understand the regulatory network of alfalfa in response to abiotic stresses, such as drought, salt, and cold stress (Table 1). The drought-induced genes screened from the transcriptome database were used to assay their drought tolerance. Two-month-old different alfalfa plants were grown in pots, and then water was withheld until the WT plants showed wilting (about 14 days). Thereafter, the soil was rewatered to field water capacity for 7 days, and the survival rate was recorded. The GWAS analysis population including 258 individual alfalfa plants was constructed. All alfalfa plants were planted under normal irrigation and drought stress, respectively. The relative water content of leaves and aboveground biomass were measured before and after drought stress. In addition, drought-tolerant and high-yield individuals were screened from the 258 individuals to confirm their drought tolerance performance in 2019-2020.

Table 1. Transcriptome data of alfalfa collected by our team.

No.	Alfalfa varieties	Treatments	Tissues	References
1	Golden queen	No	15 different tissues (mixed sample)	Liu et al., 2013
2	Zhongmu No.1	Cow saliva deposition treatment	Leaves, four stages	Liu et al., 2015
3	Zhongmu No.1	Al stress (10 mM $AlCl_3$)	Root tips, four stages	Liu et al., 2017
4	Zhongmu No.1	Cold stress (4°C)	Whole seedling, five stages	Zhou et al., 2018
5	Zhongmu No.1	Drought stress (400 mM mannitol)	Root tips, seven stages	Luo et al., 2019a
6	Zhongmu No.1	Salt stress (250 mM NaCl)	Root tips, seven stages	Luo et al., 2019a
7	Zhongmu No.1	ABA stress (10 μM ABA)	Root tips, four stages	Luo et al., 2019b
8	Longzhong	No	Stems and leaves, five stages	Zhou et al., 2022
9	Golden queen	No	Seeds, six stages	Zhao et al., 2022
10	Golden queen	No	Anthers, seven stages	Unpublished

Results and Discussion

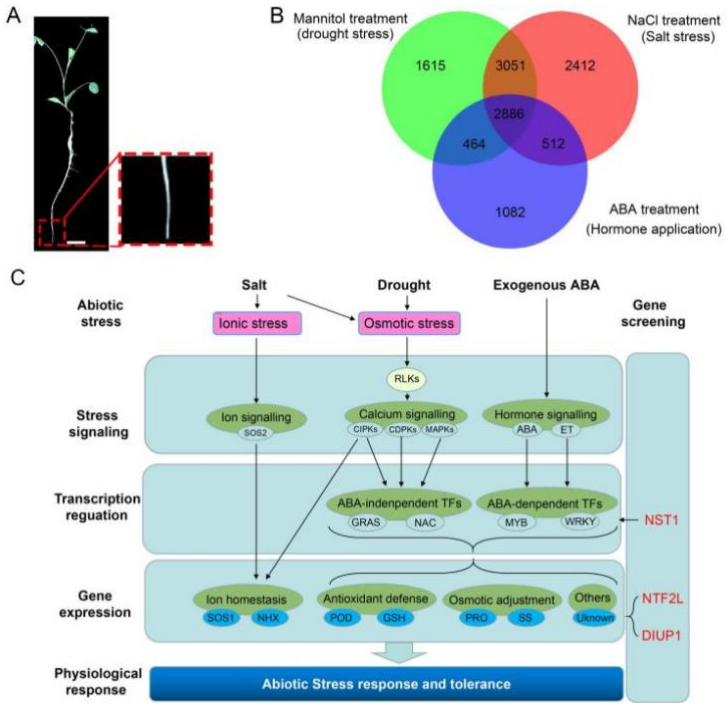

Figure 1. Transcriptome analysis of alfalfa seedlings under various abiotic stresses

including drought, salt, and ABA treatments. (A) Sampling strategy for alfalfa seedlings using root tips (1 mm). (B) Venn diagrams showing the DEGs common to or distinct in different treatments. (C) Models describing the regulated network in alfalfa response to abiotic stresses. The genes for functional analysis were marked with red color.

Based on the transcriptome data, we have obtained numerous differentially expressed genes (DEGs) of alfalfa in response to abiotic stresses. Among them, we found that 2886 DEGs co-regulated by mannitol, NaCl, and ABA, and these DEGs, whereas 1615, 2412, and 1082 DEGs were specifically regulated by mannitol, NaCl, and ABA in alfalfa root tips, respectively (Fig. 1A, B). These genes were involved in stress signaling, transcription regulation, antioxidant defense, and osmotic adjustment (Fig. 1C), which were consistent with previous studies on alfalfa and other plants (Postnikova *et al* 2013).

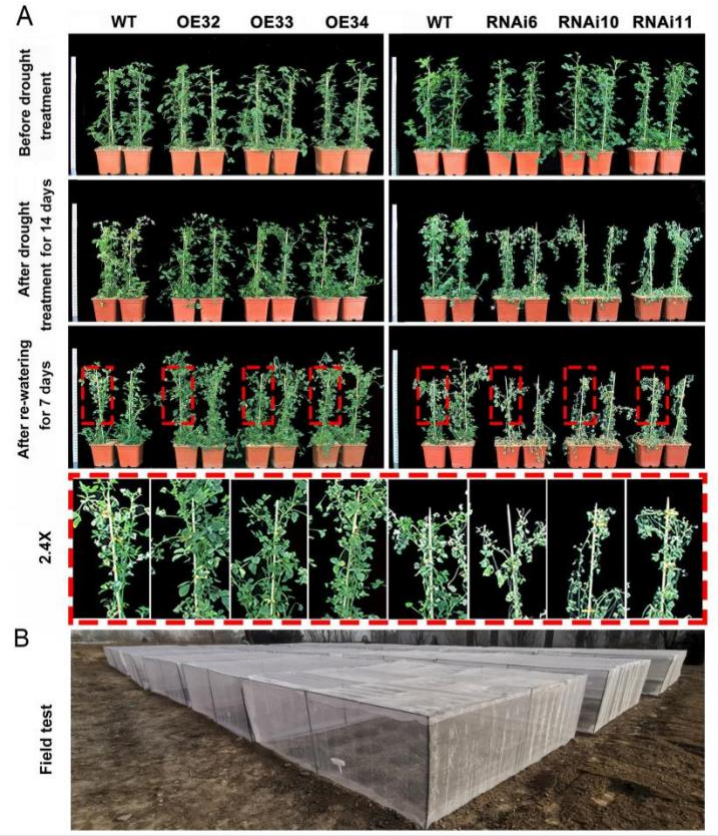

Figure 2. Growth of two-month-old alfalfa WT, *MsNTF2L*-OE, and *MsNTF2L*-RNAi plants during drought stress. (A) The phenotypes of all alfalfa plants were taken before drought stress, after withholding water for 14 days, or after rewatering for 7 days at the green house. (B) Evaluation of the drought tolerance of all alfalfa plants in field. When all alfalfa plants were grown the field, they were covered with white plastic nets.

Large-scale genes involved in drought response were screened from our transcriptome database, and three genes that were dramatically induced by drought stress were identified, *Nuclear Transport Factor 2-like* (*MsNTF2L*), *Drought-Induced Unknown Protein 1* (*MsDIUP1*), and *MsNST1*. Overexpression of *MsNTF2L*, *MsDIUP1*, and *MsNST1* in *Arabidopsis* resulted in increased tolerance to drought, with higher survival rate than in wild-type (WT) plants. Then, analysis of *MsNTF2L*, *MsDIUP1*, and *MsNST1* over-expression (OE)

and RNA interference (RNAi) alfalfa plants revealed that these three genes also increased tolerance to drought stress, accompanied by reduced malondialdehyde content, stronger antioxidant defense, and increased osmoprotectants accumulation, relative to the WT (Fig. 2A). These results suggested the positive roles for *MsNTF2L*, *MsDIUP1*, and *MsNST1* in regulating drought tolerance. Furthermore, the drought tolerance of *MsNTF2L* transgenic alfalfa plants was examined in the field (Fig. 2B). Once again, *MsNTF2L*-OE plants have a better performance of drought tolerance than WT in the field. However, these plants are still far from breeding commercial cultivars.

To more accurately locate drought tolerance related genes, we performed GWAS analysis with 258 alfalfa germplasm resources. A total of 50 excellent plants were screened with strong drought tolerance in the GWAS population (Fig. 3). However, whether *MsNTF2L*, *MsDIUP1*, and *MsNST1* have distinct single nucleotide polymorphisms between drought tolerant and drought sensitive populations is still unclear. In the future study, we will integrate our previous results of the transcriptome, genetic, and GWAS to deeply explore the mechanisms of drought tolerance genes, and therefore conduct alfalfa drought tolerance breeding at the whole genome level (Han et al., 2018).

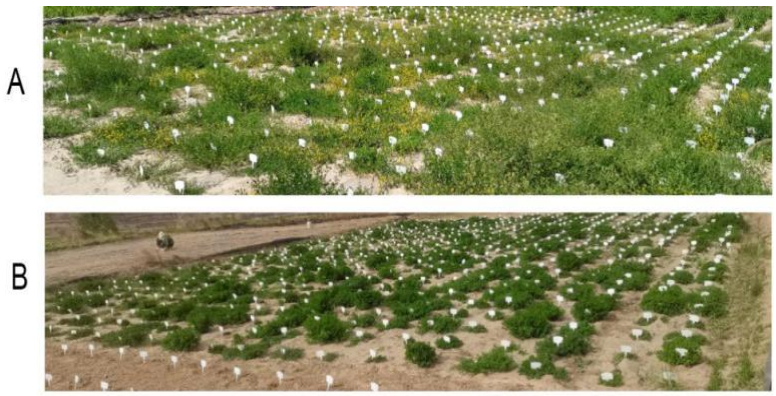

Figure 3. Evaluation of the drought tolerance of alfalfa GWAS population. (A, B) Alfalfa grown under normal irrigation (A) and drought treatment (B) experimental fields in Yuzhong county, Gansu province in 2022.

Conclusions

In this study, we constructed a comprehensive alfalfa transcriptome database. Based on the transcriptome database, three drought-induced *MsNTF2L*, *MsDIUP1*, and *MsNST1* genes were systematically genetically defined in alfalfa. In addition, 50 excellent plants were screened with strong drought tolerance in the GWAS population. These results provide valuable tools for future candidate gene identification, map-based gene cloning, and marker-assisted selection for alfalfa drought tolerance.

References

Han, K., Lee, H.Y., Ro, N.Y., Hur, O.S., Lee, J.H., Kwon, J.K., Kang, B.C., QTL mapping and GWAS reveal candidate genes controlling capsaicinoid content in *Capsicum*. 2018, *Plant Biotechnology Journal*., 16 (9): 1546-1558.

Luo, D., Liu, J., Wu, Y.G., Zhang, X., Zhou, Q., Fang, L.F., Liu, Z.P. 2022. *NUCLEAR TRANSPORT FACTOR 2-LIKE* improves drought tolerance by modulating leaf water loss in alfalfa (*Medicago*

sativa L.). *The Plant Journal*., 112(2): 429-450.
Postnikova, O.A., Shao, J., Nemchinov, L.G. 2013. Analysis of the alfalfa root transcriptome in response to salinity stress. *Plant and Cell Physiology*., 54(7): 1041-1055.