ABA-insensitivity of Alfalfa (Medicago sativa L.) during seed germination associate with plant drought tolerance

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Abstract

Abscisic acid (ABA) is a vital stress resistant hormone of plant in coping with adverse environmental conditions, such as drought stress. Sensitivity of seed germination to exogenous ABA treatment could link to different drought tolerance ability of different plant species. Here, we selected alfalfa seedlings (S0-50) from seeds germinated under 50 μM ABA treatment. The S0-50 plant showed more sensitivity in stomatal closure to exogenous ABA and PEG treatments, and also stronger drought tolerance than the plant of ABA-sensitive seed during germination (S0-0). Testing of ABA content in leaf indicated that the S0-50 had a higher ABA content in normal and under drought stress growth conditions than that of the S0-0 plants. The seed of S0-50 next generation (S1-50) showed significantly higher germination ratio under 50 μM ABA treatment, and also had longer root after 15% PEG6000 treatment than the segregated ABA-sensitive seed (S1-0). We found a cytosol-nucleus dual-localized PPR protein gene *MsSOAR1* was significantly highly expressed in S0-50 than in S0-0 plant. Overexpression of *AtSOAR1*, a negative regulator in ABA-mediated Arabidopsis seed germination inhibition, and also a homologous gene of *MsSOAR1*, significantly improved alfalfa drought tolerance, branch number and plant height, and reduced the expression level of ABA receptors *MsPYL5* and *MsPYL6*. The results suggest that ABA-insensitive during seed germination could associate with repression of ABA signaling transduction. Selection of alfalfa seedling during seed germination with exogenous ABA could be a way to obtain drought tolerance germplasm, at least in 'Zhongmu No.1' alfalfa cultivar the plant material we used in the experiment.

Introduction

Alfalfa (*Medicago sativa* L.), also known as "the queen of forage", is one of the most important forage crops and is widely planted woldwide due to its high yield, quality, and adaptability to a changing environment (Annicchiarico et al., 2015; Biazzi et al., 2017). Alfalfa with a high forage yield is one of the most important factors in livestock husbandry (Shi et al., 2017). However, the yield of alfalfa is seriously affected by various abiotic stresses such as drought (Mckersie et al., 1996; Singer et al., 2018). Although alfalfa has a deep-root system, the increasing prevalence of drought due to less rainfall and reduction in available irrigation water has limited alfalfa forage production (Ronald, 2011; Singer et al., 2018). Researchers have been trying to improve alfalfa drought tolerance by genetic transformation, but the achievement is limited (Gou et al., 2018; Tang et al., 2013; Wang et al., 2016; Zhang et al., 2005). Drought tolerance of alfalfa is a complex trait controlled by multiple pathways and genes, such as abscisic acid (ABA) biosynthesis and signal transduction pathways (Brookbank et al., 2021; Muhammad Aslam et al., 2022). Besides, highly heterozygous and outcrossing nature of alfalfa results in an extensive reservoir of genetic variation. Normally, it is hard to identify the key gene that plays a predominant role in alfalfa drought tolerance (Gou et al., 2018). In this study, we try to explore an effective method of alfalfa drought tolerance germplasm selection, and investigate the drought tolerant genes of alfalfa.

Drought stress restricts plant water-uptake and limits its growth and development. Plants have evolved a

wide range of strategies to resist drought stress. Among them, inducing stomata to close by rapidly increasing plant cellular ABA content to reduce water loss is a key strategy to enhance drought tolerance (Lim et al., 2015). Stress-induced ABA accumulation is regulated by the precise balance between its biosynthesis, catabolism, and reversible conjugation (Dong et al., 2015; Ma et al., 2018). Several reports have shown that overexpression of genes in ABA biosynthesis, such as zeaxanthin epoxidase (ZEP) (Zhang et al., 2016), 9-cis-Epoxycarotenoid dioxygenase (NCED) (Frey et al., 2012; Hao et al., 2009; Huang et al., 2019; Pedrosa et al., 2017), and molybdenum cofactor sulfurase (LOS5/ABA3) (Yue et al., 2011) up-regulated ABA content and significantly enhanced plant drought tolerance. Upregulating ABA β-glucosidase (BG, or BGLU) can rapidly increase ABA content in *Arabidopsis* by hydrolyzing ABA-glucosyl ester (ABA-GE) to free ABA and improve drought tolerance (Han et al., 2020). Stress induced ABA can redesign various physiological and biochemical signal transduction cascades in plants to coping with drought stress. The ABA signaling transduction contains three major components: ABA receptor pyrabactin resistance (PYR), PYR-like (PYL) and regulatory component of ABA receptor (RCAR), protein phosphatase 2C (PP2C) and sucrose non-fermenting (SNF) SNF1-related protein kinase 2 (SnRK2) (Dong et al., 2015; Ma et al., 2018). It has been reported that overexpression of PYLs or SnRK2s enhanced drought tolerance by improving ABA signaling transduction (Zhang et al., 2019; Zhao et al., 2016; Zhong et al., 2020). Overexpression of a single PP2C gene reduced plant ABA sensitivity, thus reducing plant drought tolerance (Miao et al., 2020). These results reveal improved ABA content and/or ABA signaling transduction could significantly improve plant drought tolerance.

ABA is a pivotal hormone in regulating seed dormancy, germination, and early post-germinative growth (Chen et al., 2020). Phenotyping of ABA inhibited seed germination is a reliable to identify mutants in endogenous ABA synthesis and/ or signaling transduction pathways, such as the mutant of *ABA INSENSITIVE 1 (ABI1)-ABI5* (Jin et al., 2018). However, ABA-sensitivity in seed germination stage is not always related to drought tolerance at the vegetative growth stage. For example, overexpression of a *VvNAC17* in *Arabidopsis*increased sensitivity to ABA during seed germination while improved plant drought tolerance (Ju et al., 2020). However, overexpression of a cytosol-nucleus dual-localized PPR protein SOAR1 repressed the expression of ABI5 to negatively regulate ABA signaling transduction in seed germination and significantly while enhanced salt, drought and cold tolerance of *Arabidopsis* plant (Jiang et al., 2014; Jiang et al., 2015; Mei et al., 2014;). It is largely unknown the relationship between ABA sensitivity during seed germination and drought tolerance of alfalfa.

In this study, we hypothesize that the seeds of alfalfa cultivar Zhongmu No.1 could exhibit different ABA sensitivity during the seed germination stage and also in ABA-mediated drought tolerance. We evaluated the drought tolerance of ABA- sensitive and insensitive alfalfa populations developed from two cycles of selection during seed germination. In addition, we investigated the potential molecular mechanisms and key responsive genes for drought tolerance in ABA-insensitive alfalfa. Our study proves that selection of ABA-insensitive seedlings during seed germination is a reliable method for selection of 'Zhongmu No.1' alfalfa drought tolerant germplasm.*MsBG1* and *MsSOAR1* genes may play key roles in the ABA rapidly increasing and signaling transduction after drought stress in alfalfa plants.

Materials and methods

Seeds sterilization and germination

Alfalfa cultivar 'Zhongmu No. 1' was used in this study. The mature seeds were soaked in 50% sulfuric acid for 10 minutes, rinsed 5 times with sterile water. Then sterilizing with 5% sodium hypochlorite for 40 minutes, and then washed with sterile water for 5 times. The sterilized alfalfa seeds (100 seeds/ plate) were germinated on filter paper soaked with sterile water containing 0 μM ABA or 50 μM ABA in an incubator with a photoperiod of 14h/ 10h (day/night), 25 for 7 days. The number of germinated seeds was counted. Three biological replicates were carried out.

Selection of ABA-insensitive and sensitive alfalfa populations

The germinated seeds were isolated and labeled as S0-50 under 50 μM ABA treatment, while the un-

germinated seeds were washed with sterile water and regerminated under ABA-free conditions to produce S0-0 plants. The isolated plants were grown further in flower pots (Φ=10 cm, h=15 cm) filled with 200 g soil in a greenhouse with a photoperiod of 14h/10h (day/night) for four months. Other S0-50 plants were planted in the field (Beijing, China, N40.09, E116.22). We harvested seeds from each individual S0-50 plant (S1) respectively, as biological replicates.

The mature seeds of S1 were germinated on wet filter paper containing 50 μM ABA after sterilization. Three days later, we picked out the germinating seeds (S1-50), while the ungerminated seeds (S1-0) were washed with sterile water. Then, S1-50 and S1-0 regenerated on wet filter paper for four days for PEG treatment.

Drought tolerance test

Four-month-old plants were used for drought tolerance test in a greenhouse. We weighed the total weight of nutrient soil and plant in the flower pot after watering to the maximum (100%) water holding capacity (WHC) of soil before drought treatment. Then, control water treatment was given till WHC was reduced to 50%. The date was recorded as 0 d of drought treatment. We tested the leaf physiological parameters at 0 d, 7 d, and 10 d after drought treatment. Nine S0-0 and nine S0-50 plants were randomly selected and divided into three groups as three biological replicates to evaluate plant drought tolerance. As to the transgenic plants overexpressing *AtSOAR1* , three plants propagated by cuttage of each line were treated as a biological repeat.

Physiological parameters tests

To evaluate the drought tolerance of plants, we detected the leaf electrolyte leakage (leaf EL), malondialdehyde (MDA) content, relative water content (RWC), and H_2O_2 content following our previous reports (Liu et al., 2019).

PEG treatment

Seven-day old alfalfa S1-50 and S0-50 seedlings were placed on filter paper soaked with 15% PEG6000 for four days. The root length of seedlings before and after treatment was measured using vernier calipers. Seeds harvested from one S0-50 plant were used as a biological repeat. One biological repeat contains fifteen seedlings, three biological repeats were given. Four-month-old plants were soaked in 15% PEG6000 to simulate a drought treatment. Leaves at 0h, 1h, 3h, 6h, and 12h were sampled after PEG6000 treatment for RNA extraction to detect the gene expression pattern after drought stress. Three biological repeats were given.

Plasmid and alfalfa transformation

The Zhongmu No. 1 cultivar was used to generate *AtSOAR1*(At5g11310) overexpressing transgenic plants. The full-length cDNA of*AtSOAR1* was constructed into a pCAMBIA1300 backbone and driven*Cauliflower mosaic virus* (CaMV) 35S promoter (Mei *et al* ., 2014). The construct was introduced into *Agrobacterium tumefaciens* strain EHA105 and transformed into alfalfa following our previous reports (Zhang and Wang, 2015). Transgenic plants were verified by PCR, RT-PCR, and qRT-PCR using *AtSOAR1* gene specific primers (Liu et al., 2019).

ABA content determination

ABA content in the leaves from the third internode (from the top down) of four-month old plants or seven-day old seedlings were measured by enzyme-linked immunosorbent assay (ELISA) following the manufacture's instruction (mlbio ml077235 kit, Shanghai, China, https://www.mlbio.cn/). Briefly, 0.1g plant material was ground with liquid nitrogen, then 2 ml extract solution (80% methanol add 1 mM Di-tert-butyl p-phenol) was added and placed at 4 for 4 hours (Mei et al., 2014). Then, the samples were centrifuged at 4 (3500r/min, 8min) to take the supernatant and store at 4. Then 1ml of ABA extract solution was added to the precipitate and placed at 4 for 1 hour. The supernatant was collected by centrifuged at 4 (3500 r/min, 8min). Two supernatants were combined and lyophilized using an integrated vacuum concentrator (MC-2, JM CV2000), then dissolution with 200 μl dissolved liquid of the kit as ABA extraction liquid (mlbio

ml077235 kit, Shanghai, China). Fifty µl of ABA extraction liquid was used to determine the ABA content by an ELISA kit (Mei et al., 2014).

Quantitative real-time PCR

Total RNA was extracted by Trizol reagent, and one µg RNA was used for inverse transcription to synthesize the first strand of cDNA following the manufacture's instruction (Takara RR047 kit, Dalian, China). The cDNA was used to test gene expression level using Starlighter SYBR Green qpcr Mix (Beijing Qihengxing Biotechnology Co., LTD, FS-Q1002 kit) with gene specific primers (Table S2) with an qTOWER^{3}G (analytik jena). Amplification of alfalfa β-actin gene (JQ028730.1) was used as an internal control to normalize the gene expression. The $2^{-[?][?]CT}$ measurement method was used to calculate the relative expression level (Liu et al., 2019). The data of the relative expression levels were means derived from three biological replicates.

Stomatal conductance and stomatal movement assay

Leaves of the second internode from the top of four-month-old plants were used for analyzing the stomatal movement. Firstly, the adaxial surface of leaf was immersed in a buffer (50 mM KCl, 10 mM MES, and 0.1 mM $CaCl_2$, pH 6.15) under a cold light source (200 µmol m^{-2} s^{-1}) for 4h. Then, the leaf was transferred to a new buffer with additional 0 µM ABA (CK), 25µM ABA, or 15% PEG6000. The leaf was fixed in the FAA solution after treatment for 40 min. The photograph of the stoma on the abaxial surface of leaves was captured under an optical microscope. The size of the stomatal aperture was measured using Image J software. At least 80 stomata were measured from three biological repeats (each one contained three leaves), that were used for statistical analysis.

Stomatal conductance was measured with a LI-COR/LI-6400 portable photosynthesis measurement system with an LED light source at 200 µmol m^{-2} s^{-1}, airflow was set to 500 µm and the CO_2 concentration at 800 ppm (Pan et al., 2020). Eight leaves were treated as one biological replicate, and three biological replicates were given in the tests.

Statistical analysis

In the experiment, data from biological repeats were analyzed by one-way ANOVA through the proc GLM for ANOVA of SAS 8.2 (SAS Institute, Cary, NC, USA). The comparison of treatments was separated by Duncan's multiple range test (P ¡0.05).

Results

Isolation of ABA-insensitive alfalfa seedlings

Exogenous 50µM ABA significantly inhibited alfalfa seed germination (Figure 1A). Only about 5% of the seeds were germinated in the presence of 50 µM ABA (S0-50) (Figure 1B). The seedlings obtained during re-germination after washing to get rid of ABA were labeled as S0-0. There were no significant differences were found, except that the S0-50 plants were slightly but not significantly higher than the S0-0 plants when we compared their phenotypes after four-month growth in pot (Table S1). The stomata of detached leaves from S0-0 and S0-50 were noticed to close obviously after treatment with 25µM ABA or 15% PEG6000 (Figure 1C). However, the stomatal apertures of S0-50 were significantly smaller than those of S0-0 after ABA or PEG treatment (Figure 1C, D). The relative stomatal conductance of S0-50 was lower but there was no significant difference with that of S0-0 in the presence of 25 µM ABA for 40 min (Figure 1E), and was significantly lower than S0-0 after 15% PEG6000 treatment (Figure 1F). Taken together, the alfalfa plants that were insensitive to ABA-mediated seed germination inhibition were more sensitive to ABA or PEG treatments in leave stomatal movement.

S0-50 plant showed enhanced resistance to drought stress

To compare the drought tolerance of S0-0 and S0-50 plants, nine S0-0 plants and nine S0-50 plants were randomly selected to perform a drought tolerant test (Figure 2A). When drought treatment for seven days, the leaves of S0-0 plants showed obvious wilting and chlorosis, while S0-50 plants had healthier leaves (Figure

2A). After drought treatment for 10 days, both the S0-0 and S0-50 plants showed obvious wilting, but S0-50 looked better than S0-0. Then, we cutted off the aboveground parts of the plants and rewatered them. The S0-50 plants were recovered quickly and had healthy shoots developed after rewatering for three days, in comparison the S0-0 plants recovered much slower (Figure 2A).

We also tested the physiological parameters, including leaf EL, RWC, MDA, and reactive oxygen content of S0-0 and S0-50 plants during drought treatment. The results showed that the tested leaf physiological parameters had no significant difference at 0d between S0-0 and S0-50 (Figure 2B-E). The RWC content of leaves decreased obviously with the prolonging of stress time. However, the RWC content of S0-50 plants at 7d and 10d was significantly higher than that of S0-0 plants (Figure 2B). Leaf electrolyte leakage of S0-0 plants was also sharply increased after drought treatment. The EL of S0-0 plants was dramatically higher than that of S0-50 plants after drought treatment for 7 days (Figure 2C). Leaf MDA content reflects the degree of leaf lipid peroxidation of the cell membrane after drought stress. As shown in figure 2D, the MDA content of plants significantly increased after drought treatment compared to 0d. However, the MDA content of S0-50 was significantly lower than that of S0-0 plants (Figure 2D). The H_2O_2 content of the leaf was significantly increased after drought treatment, and the S0-0 always had a significantly higher H_2O_2 content than the S0-50 (Figure 2E). The results indicated S0-50 plants had higher water retention and ROS scavenging capacity.

A higher proportion of seeds from S0-50 plants were insensitive to ABA and PEG treatment during germiation

To further verify the drought tolerance of ABA-tolerant alfalfa progeny, we planted S0-50 in the field and collected the seeds from each individual plant (the first generation after ABA selection, S1). The germination rate of S1 seeds was found to be significantly lower (approximately 25%) than that of control seeds (about 80%) under normal condition (Figure S1A, B). About 50% S1 seeds were ABA-insensitive to 50 μM ABA treatment during germination (S1-50), which was significantly higher than that of 'Zhongmu No.1' alfalfa seeds (Figure 3C). The seedlings obtained with a re-germination after washing to get rid of ABA were isolated and recorded as S1-0. Seven-day old seedlings of S1-0 and S1-50 were treated with sterile water or 15% PEG6000 for four days. There were no significant differences observed between S1-0 and S1-50 seedlings when treated with sterile water (Figure S1C, D). However, after PEG treatment for 4 days the root length of S1-0 seedlings was significantly shorter than that of S1-50 and showed a curling phenotype (Figure 3A, D). The seedlings of S1-0 showed remarkable brown color than thaose of S1-50 after DAB staining, which indicated that the roots of S1-0 seedlings accumulated more reactive oxygen species (ROS) than S1-50 (Figure 3B).

S0-50 plants contain higher content of ABA

We detected the ABA content in leaves from the second internode of four-month-old S0 plants and seven-day-old S1 seedlings. As shown in Figure 4, the ABA content of S0-50 and S1-50 was significantly higher than S0-0 and S1-0, respectively. ABA content was rapidly increased after PEG treatment for 3 h, especially for S0-50 and S1-50. As shown in Figure 5A, five tested ABA biosynthesis genes (*MsAAO3* ,*MsABA3* , *MsNNCED5* , *MsBG1* and *MsBG2*) showed significantly higher expression in S0-50 compared to that of S0-0 under normal conditions. And, those genes also showed induced expression after PEG treatment. Intriguingly, the expression level of the*Ms BG1* gene was significantly higher in S0-50 than that in S0-0 after PEG treatment. ABA catabolism genes, ABA 8'-hydroxylase genes *MsCYP70A1/4* and*MsUGT71B6/8* showed significantly lower expression levels in S0-50 compared to that of S0-0 plant after PEG treatment (Figure 5B). There were no differences in the expression of ABA long-distance transportation related genes between S0-0 and S0-50, such as*MsABCG5* and *MsNRT1.2* (Figure 5B). The results suggested that the S0-50 has higher internal ABA content by activating ABA biosynthesis and inhibiting ABA catabolism.

Expression of ABA signaling transduction genes in S0-0 and S0-50

ABA signaling transduction related genes in S0-0 and S0-50 had similar expression patterns after PEG treatment (Figure 5C). The *MsPYL5* and *MsPYL6*ABA receptors were significantly induced after one hour,

then sharply decreased after PEG treatment for 3h. Expression of the other tested genes in ABA signaling transduction was gradually increased during PEG treatment. Most of them, such as *MsPYL5* , *MsPYL6* ,*MsPYL7* , *MsPYL9* , *MsSnRK2.2* , *MsABI1* and*MsABF4* , were more highly expressed in S0-50 than in S0-0 under normal conditions, but showed no significant difference in expression level between S0-0 and S0-50 after PEG treatment. The expression level of the *MsSOAR1* , a homology gene of *Arabidopsis* ABA signaling inhibitor, was significantly higher in S0-50 than in S0-0. The results indicated *MsSOAR1* could play a key role in drought resistance of the S0-50 plant.

Overexpression of *AtSOAR1* in alfalfa plant improved biomass yield and sensitive to ABA-induced stomatal closure

To explore the effects of *MsSOAR1* overexpression in alfalfa, we introduced a homology gene of *MsSOAR1* , *AtSOAR1* , into alfalfa (Figure S2A, B). Transgenic (TG) plants with different*AtSOAR1* expression levels were generated (Figure S2C, 6A, 6B). Four-month-old TG plants showed a significant increase in plant height and primary branch number (Figure 6C, 6D), and above-ground biomass (Figure 6E) than that of WT plants. We found that the TG plants had similar ABA content as WT plants under normal growth conditions (Figure S2D), whereas the TG plants had smaller stomatal aperture than WT after 25μM ABA treatment for 40min (Figure 6F, 6G). The relative stomatal conduction of TGs was significantly lower than that of WT in the presence of 25μM ABA (Figure 6H). The results indicated overexpression of *AtSOAR1* positively regulated ABA-mediated alfalfa plant stomatal closure.

Overexpression of *AtSOAR1* improved drought tolerance of transgenic alfalfa

We also tested the effect of overexpression of *AtSOAR1* on alfalfa drought tolerance (Figure 7A). At the beginning of drought treatment, WT and TG plants had similar content of leaf RWC, MDA, H_2O_2, and EL (Figure 7B-E). However, after drought treatment for 7 days WT plants showed obvious wilting compared to TGs. After drought treatment for 10 days, all the plants showed wilting on the top leaves. In comparison, the TG plants looked much better. The TG plants had new shoots and leaf growth after rehydration for 3 days, but the WT did not (Figure 7A). During the drought treatment, the leaf RWC content of WT was significantly lower than that of TG plants (Figure 7B), and the EL (Figure 7C), MDA (Figure 7D), and leaf H_2O_2 content (Figure 7E) of WT were significantly higher than those of TG plants. The results suggested that overexpression of *AtSOAR1* enhanced drought tolerance in alfalfa.

Overexpression of *AtSOAR1* affected drought tolerance related genes expression of alfalfa

To explore the potential mechanisms of overexpression of *AtSOAR1*improving drought tolerance in alfalfa, we detected expression of drought tolerance and also ABA signaling transduction related genes in WT and TG plants. The results showed that expression of ABA receptors*MsPYL5* and *MsPYL6* was significantly down regulated in the TG lines compared to that of the WT plant. Expression of *MsSnRK2.3* and *MsPYL7*was significantly up-regulated in TG2, and *MsSnRK2.3* and*MsMYC2* were highly expressed in TG3. The ABA-dependent drought response gene *MsABF1* and the ABA-independent drought response genes *MsCBF1* and *MsCBF4* showed up-regulated expression by*AtSOAR1.* Expression of *MsMYB2* was down-regulated in*AtSOAR1* transgenic plants (Figure 8). The results suggested*AtSOAR1* inhibited ABA signaling transduction by repressing ABA receptor *MsPYL5* and *MsPYL6* , and regulated the ABA-dependent and ABA-independent pathways to improve alfalfa drought tolerance.

Discussion

ABA is a premier hormone for plants to respond to drought and plays a critical role in seed germination, plant growth and development (Brookbank et al., 2021; Ma et al., 2018; Muhammad Aslam et al., 2022). Alfalfa, a cross-pollinated autotetraploid plant species, it is largely unknown whether there is a distinct separation of ABA content and signaling among different alfalfa plant, and the relationship between ABA-mediated inhibition of seed germination and drought tolerance in the adult vegetative stage. In this study, we isolated ABA-insensitive and sensitive alfalfa seedlings from alfalfa cultivar 'Zhongmu No. 1', and tested their drought tolerance. Intriguingly, the plants that were ABA-insensitive during the seed germination

showed obviously stronger drought tolerance than the ABA-sensitive plants in the vegetative stage, which may be due to, at least in part, the rapid increase of ABA content and the higher expression level of ABA signaling transduction related genes.

Exogenous ABA significantly inhibited alfalfa seed germination and post-germination growth (Mei et al., 2014). Adding exogenous ABA during seed germination is an effective method to distinguish seeds with different ABA biosynthesis and/ or signaling transduction pathways (Huang et al., 2019; Jin et al., 2018). In the study, we found about 5% of alfalfa seeds showed a strong insensitive phenotype in ABA-mediated seed germination inhibition and post-germination growth arrest. It was reported that ABA sensitivity in seed germination is not always consistent with drought tolerance in adult plants. For example, overexpression of *VvNAC17* or *ARABIDOPSIS F-BOX PROTEIN HYPERSENSITIVE TO ABA 1* mutant delayed seed germination in the presence of ABA but showed stronger drought tolerance (Ju et al., 2020; Kim et al., 2021). Overexpression of *FOF2* or *AtSOAR1* in*Arabidopsis* , on the other hand, demonstrated strong ABA-insensitivity during seed germination and enhanced drought tolerance in adult plants (Jiang et al., 2015; Qu et al., 2020). Similarly, in this study, we found that the early germinated seeds in the presence of ABA showed sensitivity with regard to ABA-mediated stomatal closure and were tolerant to drought stress in the adult vegetative stage in the 'Zhongmu No.1' alfalfa. Similar results were also found in the second cycle of slection of ABA- sensitive and insensitive seedlings for seeds harvested from S0-50 plant. Our results clearly showed it is an effective way to improve drought tolerance of 'Zhongmu No.1' alfalfa by isolating ABA-insensitivity seedlings during seed germination, but the wide applicability of this method still needs further verification in other cultivars.

ABA accumulation plays an important positive role in plant resistance to drought stress by inducing stomatal closure, regulating ROS homeostasis and inducing ABA-dependent drought tolerance gene expression (Muhammad Aslam et al., 2022). Endogenous ABA level in plant cells is mainly determined by ABA biosynthesis and catabolism. In comparison with the S0-0 plant, the ABA biosynthesis genes *MsAAO3* , *MsABA3* , and*MsNCED5* were significantly more highly expressed in the S0-50 plant. On the other hand, ABA catabolism genes such as *MsCYP70A1* ,*MsUGT71B6* and *MsUGT71B8* showed significantly lower expression than that of the S0-0 plant after drought treatment. That could be the reason the of S0-50 plant had a higher ABA level and stronger drought tolerance (Huang et al., 2019; Ma et al., 2018; Pedrosa et al., 2017). It has been reported that *CYP70A* genes encod ABA 8'-hydroxylase and are considered the key catabolic genes reducing ABA levels (Umezawa et al., 2006). ABA-uridine diphosphate (UDP) glucosyltransferases (UGTs), AtUGT71B6 and AtUGT71B8, catalyse free ABA to an inactivated conjugate form ABA-glucose ester (ABA-GE) (Priest et al., 2006). Plants ABA-GE can be reversed and catalyzed to free ABA by β-glucosidase homologue BG1 in the endoplasmic reticulum (ER), which is the main pathway for rapid increase of ABA in plants in response to environmental stresses (Lee et al., 2006; Ondzighi-Assoume et al., 2016). Overexpression of *BG1* reportedly improved drought tolerance in *Arabidopsis* (Han et al., 2020). Interestingly, during PEG treatment for 12 h, the expression of *MsBG1* in S0-50 was always higher than that in S0-0, which may be the key reason for its higher ABA content and stronger drought resistance. Overall, the increased ABA content in S0-50 may be due to enhanced ABA de novo biosynthesis and BG-mediated hydrolysis of ABA-GE pathways and a reduced ABA catabolism pathway (Dong et al., 2015).

PYLs-ABA-PP2C-SnRKs is a core pathway of ABA signaling transduction, which can be induced by stress-induced ABA, then activate the expression of abiotic stress response genes, playing a positive role in regulating plant drought tolerance (Gonzalez-Guzman et al., 2012). Here, we found that not only the positive regulators of ABA signaling, such as*MsPYLs* , *MsSnRK2.2/2.8* , *MsABI5* and *MsABF4* , but also the inhibitors of ABA signaling, such as *MsSOAR1* and*MsABI1* , were significantly highly expressed in S0-50 plants under normal conditions. It is well known that higher ABA content and fast signaling transduction enhanced plant drought tolerance but impaired plant development, which promoted plants to develop an optimal internal resilient system for survival and growth (Tan et al., 2017). We speculate that the ABA signal transduction of strong ABA-insensitive alfalfa may have such a trade-off strategy to keep normal development and also have strong drought-tolerant capacity.

After drought treatment, we noticed a cytosol-nucleus dual-localized PPR protein gene *MsSOAR1* was significantly highly expressed in S0-50 than in S0-0. Its homologous gene *AtSOAR1* was reported to play a negative role in ABA signaling in seed germination and a positive role in abiotic tolerance in *Arabidopsis* (Jiang et al., 2015; Mei et al., 2014). We verified that heterologous expression of *AtSOAR1*significantly improved alfalfa drought tolerance. In *Arabidopsis* ,*AtSOAR1* reportedly functions at the downstream of the ABA receptor and probably upstream of ABI5 (Mei et al., 2014). However, we found that the ABA receptors*MsPYL5* and *MsPYL6* were significantly downregulated in *AtSOAR1* transgenic plants. And,*MsPYL5 /6* showed a different expression pattern than the other tested *MsPYLs* , which negatively correlated with*MsSOAR1* expression after drought tolerance. Several reports showed *PYL5* and *PYL6* had specific functions and characteristics. For example, *AtPYL5* is expressed in guard cells and has a strongly negative response to ABA treatment (Dittrich et al., 2019). And, *OsPYL5/6* negatively regulated rice growth and panicle branching, but *OsPYL7/8/9* didn't (Miao et al., 2018).We observed that the *AtSOAR1* transgenic alfalfa plant was higher and had more branches than the WT plant. Those results indicated *MsPYL5* and*MsPYL6* might be the main downstream genes of *SOAR1* in regulating alfalfa drought resistance and promoting alfalfa growth.

Drought tolerance related genes such as *AtRD29B* , *AtMYC2*and *ZmRD22* usually contain single or multiple abscisic acid responsive element (ABRE). Their expression would be induced by elevated ABA content under drought stresses (Abe et al., 2003; Hua et al., 2006; Phillips and Ludidi, 2017). ABRE can be recognized by the basic leucine zipper-transcription factors (TFs), namely, ABA responsive element-binding protein (AREB)/ABA-binding factor (ABF) to activate downstream genes expression, such as *ZmbZIP72* and *AtABF1*(Ying et al., 2012). We found overexpression of *AtSOAR1*significantly increased the expression of ABA-dependent genes,*MsMYC2* , and *MsABF1* , and down-regulated *MsMYB2* . In addition, ABA-independent drought responsive genes, such as*MsCBF1* and *MsCBF4* , were upregulated in *AtSOAR1*transgenic plants (Yang et al., 2011; Yang et al., 2020). The results suggest *AtSOAR1* regulates expression of both the ABA-dependent and independent drought responsive genes.

Endogenous ABA content and signaling transduction play an important role in seed physiological dormancy (Chen et al., 2020). Several mutations with ABA deficiency or reducing ABA signaling transduction, such as *aba1* , *aba2/3* ,*nced6nced9* , and *pyl123456789101112* , all showed attenuated seed dormancy (Lefebvre et al., 2006; Nakashima et al., 2009; Zhao et al., 2018). Mutants of ABA metabolism genes *CYP707A1* ,*CYP707A2* , and *CYP707A3* , which encode abscisic acid 8'-hydroxylases, showed increased seed dormancy (Okamoto et al., 2006). In this study, we noticed that S1-50 seeds harvested from S0-50 plants showed significantly physical dormancy (40% hardseededness) with a water-impermeable seed coat and can't absorb water. We haven't tested the seed coat structure of S1-50 seeds, but there may be a potential relationship between the morphological structure and composition of the water-impermeable layer of seed and endogenous ABA content, which is also worth studying in the future.

Overall, our results indicate that the phenotyping of ABA-insensitive during seed germination is correlated with drought tolerance in the adult vegetative stage of the 'Zhongmu No.1' alfalfa. Isolating the ABA-insensitive seedlings in seed germination is an effective way to select drought-stress resistant alfalfa germplasm for breeding. Overexpression of *AtSOAR1* , a negative regulator in ABA-mediated inhibition of seed germination, could improve alfalfa's drought tolerance.

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Conflict of Interest Statement

The authors have no conflict of interest to declare.

Author contributions

Y.L. and D.J. analyzed data and drafted the manuscript; D.J., Y.L., J.Y., K.W., S.L. performed the experiments and data collection. W.Z. and Y.L. guided the experiments and revised the manuscript.

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Figure legends:

Fig. 1. Insensitve (S0-50) and sensitve (S0-0) alfalfa plants to ABA during seed germination and detached leaf stomatal movement assay.

Fig. 2. S0-50 plant showed improved drought tolerance.

Fig. 3. Phenotype comparison of S1-0 and S1-50 alfalfa seedlings after PEG treatment.

Fig. 4. ABA content of four-month-old S0 plant leaves and seven-day-old S1 seedlings before and after 15% PEG6000 treatment.

Fig. 5. Gene expression tests of second internode leaves from four-month-old S0-0 and S0-50 alfalfa after PEG treatment.

Fig. 6. Overexpressing *AtSOAR1* reduced alfalfa ABA sensitivity.

Fig. 7. Overexpression of *AtSOAR1* enhanced drought tolerance of alfalfa transgenic plants (TGs).

Fig. 8. Expression of ABA-dependent and ABA-independent drought response genes in WT and *AtSOAR1* alfalfa transgenic plants.

Supplymentary data

Table S1 Phenotype comparison of four-month old S0-50 and S0-0 plants.

Table S2 The primers used in the study.

Fig. S1. S1 seeds germination and phenotype comparison of S1 seedlings.

Fig. S2. Generation and verification of overexpression *AtSOAR1*transgenic alfalfa plants (TGs).

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