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Transcriptome Analysis Reveals the Vital Role of ABA Plays in Drought Tolerance of the ABA-Insensitive Alfalfa (*Medicago sativa* L.)

Mingzhi Xu [1,†], Zhenpeng Xu [2,†], Yanrong Liu [1], Yaling Liu [3], Jinghui Liu [4,*] and Wanjun Zhang [1,*]

[1] College of Grassland Science, China Agricultural University, Beijing 100193, China; 15689780174@163.com (M.X.); liuyr_cgst@cau.edu.cn (Y.L.)
[2] Ulanqab Agricultural and Forestry Science Research Institute, Ulanqab 012000, China; zhenpengxu7607@163.com
[3] National Center of Pratacultural Technology Innovation (Under Preparation), Hohhot 010010, China; liuyaling0719@163.com
[4] College of Grassland, Resources and Environment, Inner Mongolia Agricultural University, Hohhot 010011, China
* Correspondence: cauljh@163.com (J.L.); wjzhang@cau.edu.cn (W.Z.)
† These authors contributed equally to this work.

Abstract: Drought stress severely affects alfalfa (*Medicago sativa* L.) growth and production. It is particularly important to analyze the key networks of drought in alfalfa through physiological and molecular levels. However, how to quickly screen drought-tolerant alfalfa germplasm and how to elucidate the molecular pathways of alfalfa responding to drought are less studied. In this study, based on our previous research, we further verified the association between the heritability of ABA sensitivity during seed germination and drought tolerance of plants and identified the key pathways of drought tolerance differences between ABA-sensitivity (S1-0) and -insensitivity (S1-50) plants via RNA-seq and analysis. The results showed that the sensitivity to ABA in alfalfa seeds can be inherited and that plants that are insensitive to ABA during germination show stronger drought tolerance. An analysis of the differentially expressed genes (DEGs) revealed that ABA biosynthesis and signaling, amino acid metabolism, LEA, and wax synthesis-related pathways may be the key pathways that can be used for drought tolerance improvement in alfalfa. DEGs such as *NCED*, *PYR/PYL*, and *PP2C* may contribute to drought tolerance in the S1-50 plant. The study further confirms that screening with ABA at the seed germination stage can select alfalfa lines with good drought tolerance, which provides a new theoretical basis for alfalfa drought tolerance breeding. The expression of the key genes of alfalfa in response to drought stress was also tested.

Keywords: alfalfa; ABA sensitivity; drought stress; transcriptome; genetic expression

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Citation: Xu, M.; Xu, Z.; Liu, Y.; Liu, Y.; Liu, J.; Zhang, W. Transcriptome Analysis Reveals the Vital Role of ABA Plays in Drought Tolerance of the ABA-Insensitive Alfalfa (*Medicago sativa* L.). *Agronomy* **2024**, *14*, 406. https://doi.org/10.3390/agronomy14030406

Academic Editor: Yong-Bao Pan

Received: 15 January 2024
Revised: 15 February 2024
Accepted: 18 February 2024
Published: 20 February 2024

1. Introduction

Alfalfa (*Medicago sativa* L.) is a leguminous forage crop planted widely in the world. It has the characteristics of high content of protein and good palatability, and it is known as the "Queen of Forage" [1]. However, harsh climate conditions, such as drought stress, severely affected alfalfa biomass yield losses, hence impacting the sustainable development of animal husbandry [2,3]. Although alfalfa has a relatively lush root system, frequent drought stress still causes yield reduction [4]. Therefore, it is of great importance to study the physiological responses and molecular mechanisms of alfalfa subjected to drought stress in order to breed a highly drought tolerant alfalfa cultivar [5].

Drought stress reduces plant water uptake, impairs plant growth and development, and also could lead to plant death [6,7]. To adapt to drought stress, plants have evolved drought-tolerant morphological structures and molecular strategies. Therein, the function of the stress hormone abscisic acid (ABA) in plants to respond to drought stress has

been widely reported. For instance, it has been reported that *NCED*, a gene encoding a 9-cis-epoxycarotenoid dioxygenase, enhances the synthesis of ABA and thus induces the expression of drought-responsive genes [8]. Regarding the pathways of ABA inactivation in plants, it is broadly divided into two categories. ABA can be irreversibly degraded to PA by ABA 8′-hydroxylase, and the inhibition of the activity of ABA 8′-hydroxylase improves the drought tolerance of rice, maize, and Arabidopsis [9]. The other pathway of inactivation is through the glycosylation of ABA's C1′ position. Up-regulating the expression of BG or BGLU, two enzymes in β-glucosidase of ABA, can quickly mobilize stored ABA and enhance plant drought tolerance [10]. The main regulatory pathways of ABA signaling mainly include *PYR/PYL*, protein phosphatase 2C (*PP2C*), and SNF1-related protein kinase 2 (*SnRK2*). PYR/PYL can relieve the inhibition of SnRK2s by PP2C, improve the stability of the protein, and thereby improve drought tolerance [11,12]. And the phosphorylation of these proteins will activate downstream genes such as *SLAC1*, thereby reducing stomatal aperture and water evaporation [13,14]. In addition, ABA improves physiological changes such as plant ROS scavenging capacity and osmotic species accumulation in response to drought stress [15–17].

Plants have also evolved a certain memory for drought stress. For single-generation drought-stressed plants, ABA, LEA, and osmotic regulators can be increased to improve plant drought tolerance [18]. The inheritance of drought tolerance in future generations is caused by DNA methylation or non-coding RNAs being passed on to the next generation [19]. As a reproductive offspring, there is a certain correlation between the sensitivity of seeds to ABA and the drought tolerance in the vegetative growth stage. For example, the seeds of the rice mutant SAPK3 have reduced sensitivity to ABA, but they have stronger drought tolerance at the seedling stage [20]. However, the expression of *VvNAC17* in Arabidopsis increased not only ABA sensitivity during seed germination but also improved plant drought tolerance [21]. It remains unknown whether this association between sensitivity to ABA during seed germination and drought tolerance can be inherited by the next generation. Our previous study found that the screened "Zhongmu No.1" alfalfa seedlings that were insensitive to ABA treatment during seeds' germination stage showed better drought tolerance [22]. However, it is largely unknown what the molecular mechanisms are that lie behind why the ABA-insensitive alfalfa showed stronger drought tolerance.

In this study, we hypothesized that the insensitivity to ABA during alfalfa seeds germination is heritable and that the corresponding drought tolerance can be maintained. To this end, based on our previous studies, we generated half-sib family lines by screening the S1 generation seeds with ABA during seeds germination and evaluated their drought tolerances by testing the physiological parameters after drought treatment, as well as performing transcriptome analyses. Then, the key molecular pathways which may play a vital role in drought tolerance between the two populations were analyzed based on the transcriptome results and qRT-PCR. In this study we verified the heritability of alfalfa seed sensitivity to ABA, further confirmed the reliability of our previous studies through the evaluation of drought tolerance in S1-generation plants, and provided a theoretical basis and candidate genes for the drought-tolerance modification of alfalfa and possibly other leguminous plants.

2. Materials and Methods

2.1. Seeds Sterilization and Germination

Seeds of the "Zhongmu No. 1" (ZM) cultivar and the seeds harvested, respectively, from two phenotype elite plants, ZMAD2 and ZMAD3, that were insensitive to 50 μM ABA treatment during seed germination were used in this study. The mature seeds were soaked in 50% sulfuric acid for 10 min and rinsed 5 times with sterile water. After sterilization with 5% sodium hypochlorite for 40 min, seeds were washed five times with sterile water and germinated on filter paper (100 seeds/plate) soaked in an incubator containing 0 μmol/L or 50 μmol/L ABA (Coolaber, Cat# CA1010, Beijing, China), with a photo period of 14 h/10 h

(day/night) and stored at 25 °C for 7 days. Three biological replicates were given in the experiments.

2.2. Isolated Seeds of ABA Insensitive (S1-50) and Sensitive (S1-0)

About 500 seeds that were harvested from ZMAD2 and ZMAD3 plants were picked out for sterilization and seed germination in a germination box with two layers of filter paper soaked with 0 or 50 μmol/L ABA solution. The seeds germinated in a lighted incubator with 14 h/10 h light/dark, at 25 °C. The seeds that were swelling but not germinated were picked out and placed in a germination box with sterile water for regermination to generate the S1-0 population. The seedlings that sprouted under 50 μmol/L ABA treatment were marked as the S1-50 population.

2.3. Determination of Water Loss Rate in Alfalfa Leaves

The leaves of the third internode from the top of four-week-old plants were harvested and placed on filter paper in a Petri dish and then placed in a constant-temperature incubator, at 28 °C. Three replicates of each plant were set up and weighed in order. The first weight was recorded as the initial fresh weight and weighed at 1 h intervals until nine hours passed. The initial fresh weight is subtracted from the fresh weight at each point in time to give the current water loss. The water loss rate was defined as the water loss at a certain point divided by the initial fresh weight.

2.4. Drought Tolerance Test

Four-month-old alfalfa plants were used for the drought tolerance test in a greenhouse with natural light. We weighed the total weight of nutrient soil and plant in the flowerpot after watering to the maximum (100%) water holding capacity (WHC) of soil before drought treatment. Then, control water treatment was given till the WHC was reduced to 50%. The date was recorded as 0 d of drought treatment. At 0 d and 18 d after drought treatment, We tested physiological parameters, including the leaf electrolyte leakage (leaf EL) and relative water content (RWC), as previously reported [23]. Nine ABA-insensitive and nine ABA-sensitive plants were randomly selected and divided into three groups as three biological replicates to evaluate plant drought tolerance.

2.5. RNA Isolation

The mature leaves of S1-0 and S1-50 plants isolated from ZMAD-3 were sampled before (recorded as S1-0/50) and 18 days after drought treatment (recorded as DS1-0/50). Then, the samples were used for total RNA extraction with TRIzol reagent. Qubit 4.0 was used for the quality control of the concentration and total amount. The integrity of the RNA samples was assessed using Agilent 2100. The assays included RIN values, 28S/18S, the presence or absence of uplift in the baseline of the graph, and 5S peaks. A Nano Drop spectrophotometer was used to QC the samples for purity.

2.6. Library Preparation and Sequencing

Eukaryotic mRNA was enriched from Total RNA, using magnetic beads with Oligo (dT), using the Hieff NGS® mRNA Isolation Master Kit (Yeasen Cat#12603). RNA samples were then used for cDNA library preparation. After library construction, Qubit was used for quantitative quality control, and the libraries were sequenced on the machine after passing the quality. The raw sequence data are publicly available in the NCBI database, under the accession number PRJNA1052612.

2.7. Reference-Based Mapping and Differential Gene Expression Analysis and Annotation

The raw data obtained from sequencing were filtered, and the filtered, clean reads were compared to the alfalfa reference genome sequence (*M. sativa* cultivar XinJiangDaYe) [24]. Based on the alignment results, analyses such as de novo transcript prediction, differentially spliced gene detection, and fusion gene detection were performed. A quantitative analysis

of known and novel genes was also performed. A differential expression analysis was performed based on the expression of genes in different sample sets, and more in-depth mining analyses were performed on the screened differentially expressed genes, such as the Gene Ontology (GO) functional analysis, Pathway functional analysis, clustering analysis, protein interaction network, and transcription factor coding ability prediction. KEGG and GO annotation analyses were performed using the website https://modms.lzu.edu.cn/ (accessed on 14 January 2024) for commonly differentially expressed genes [25]. An analysis of gene expression values and pairwise comparisons were performed using the DESeq R packages. We used a p-value < 0.05 and |log2FC| > 1 as thresholds for the DESeq analysis of differentially expressed genes (Supplementary Data S2). Volcano and Venn diagrams were drawn using the ggplot2 package (3.4.4), the ggrepel package (0.9.5), and the VennDiagram package (1.7.3) in the R language.

2.8. Quantitative Real-Time PCR

First, 1 μg of total RNA was used for reverse transcription to synthesize the first strand of cDNA, following the manufacturer's instructions (Takara RR047 kit, Dalian, China), and genomic DNA was removed using DNase, which was supplied within the kit. The cDNA was used to test gene expression level, using Starlighter SYBR Green qPCR Mix (Beijing Qihengxing Biotechnology Co., LTD, FS-Q1002 kit, Beijing, China) with gene-specific primers with a qTOWER3G (analytik jena). The alfalfa β-actin gene (JQ028730.1) was used as an internal control to normalize the gene expression. The $2^{-\Delta\Delta CT}$ measurement method was used to calculate the relative expression level [23]. All the primers are shown in Supplementary Table S2. The data of the relative expression levels were the means derived from three biological replicates.

2.9. Statistical Analysis

In the experiment, at least three biological repeats were given. The data were analyzed with Student's t-test, and SPSS 27.0 was used for statistical analyses. Statistically significant differences were called at $p < 0.05$.

3. Results

3.1. Seeds Germination of ZM and ZMAD under 0 μM or 50 μM ABA Treatment

The germination rate of seeds (S1 generation) harvested from two ABA-insensitive plants (ZMAD-2 and ZMAD-3) was about 40% if there was no dormancy-broken treatment, which was significantly lower than that of the Zhongmu No. 1 (ZM) seeds (Figure 1A,B). Under the 50 μM ABA treatment, however, the generation rate of S1 seeds was found to be between 20% and 60%, which was significantly higher than that of ZM cultivar 10% (Figure 1C). These results indicate that the ABA-insensitivity is heritable. The seedlings germinated under 50 μM ABA treatment were marked as S1-50 plants, and the seedlings germinated after the removal of ABA inhibition were marked as S1-0. These two half-sibling populations were used for drought tolerance tests and the transcriptome sequencing analysis.

3.2. The S1-50 Alfalfa Plants Showed Better Drought Tolerance Than S1-0 Plants

To compare the drought tolerance of S1-0 and S1-50 plants, we first evaluated the water loss rates of the detached leaves. As shown in Figure 2A, wilting of leaves was noticed after the 6 h dehydration treatment, and the fresh weight of leaves was stabilized after the 9th hour. We noticed that the leaves from S1-0 ZMAD3 wilted more rapidly than those of S1-50 alfalfa (Figure 2A). We also found that the detached leaves of S1-50 ZMAD3 had a significantly lower water loss rate than S1-0 ZMAD3 (Figure 2B). To confirm the result, we also compared the drought tolerance of four-month-old S1-0 and S1-50 alfalfa plants screened from ZMAD3. After drought treatment for 18 d, leaves and branches showed severe wilting in S1-0 plants compared to that of S1-50 plants. After being rewatered for 3 days, the S1-50 plants recovered rapidly, and the branches resumed their normal development; meanwhile most of the leaves and branches died in

S1-0 plants (Figures 2C and S1A). We also tested leaf RWC and EL during the drought treatment. The results showed that there was no significant difference between S1-0 and S1-50 before the drought treatment. The water content of the leaves decreased significantly with the prolonged time of drought treatment (Figures 2D and S1B). The leaves' relative water content for S1-50 was significantly higher than that of S1-0, while the electrolyte permeability of S1-0 increased significantly, from 24% to 68%, compared to that of S1-50 (Figures 2E and S1C). All of these results indicated that S1-50 exhibited stronger drought tolerance compared to S1-0.

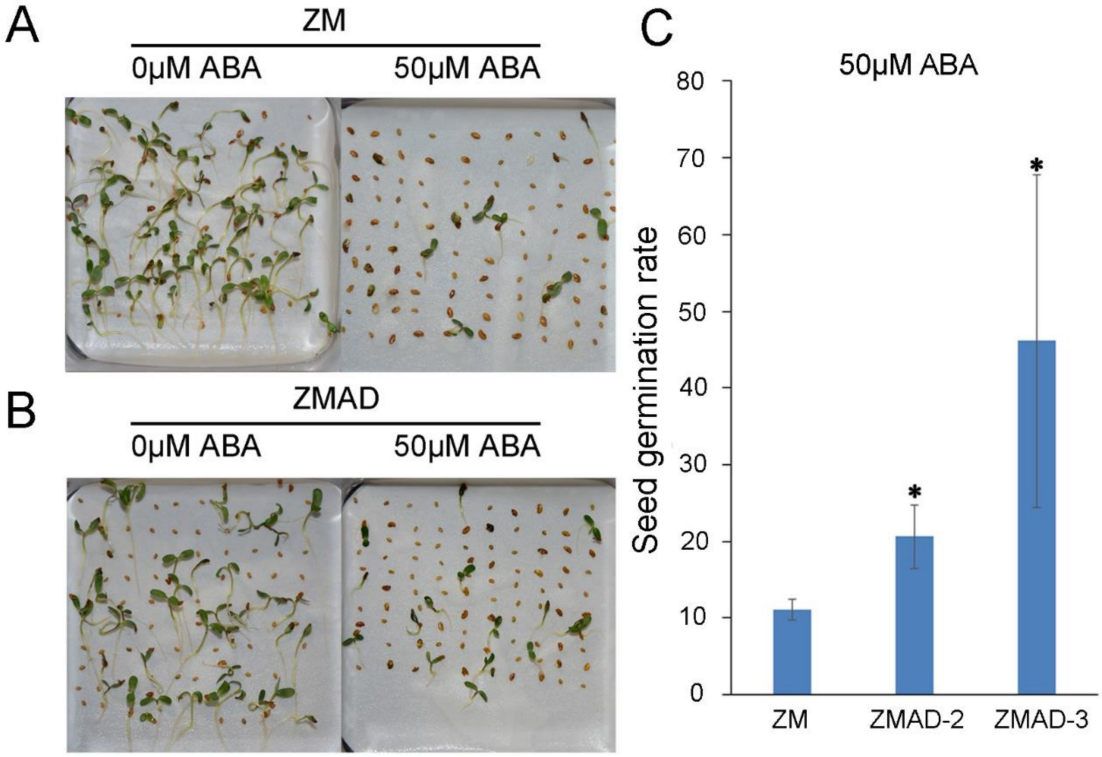

Figure 1. Germination rate of alfalfa seeds harvested from cultivar "Zhongmu No. 1" (ZM) and ABA-insensitivity plant during seed germination stage (ZMAD) under 0 μM and 50 μM ABA treatment. (**A**,**B**) Typical photograph of germination of ZM (**A**) and ZMAD. (**B**) under 0 μM and 50 μM ABA treatment. (**C**) Germination rate comparison of ZM, ZMAD-2, and ZMAD-3 under 50 μM ABA treatment. The asterisk represents the statistically significant difference ($p < 0.05$).

3.3. Data Quality and Summary of Reads

To further investigate the molecular mechanisms of drought tolerance in the ABA-insensitive plant, we performed a transcriptome analysis of the leaves from three S1-0 and three S1-50 plants screened from the ZMAD3 line before and after 18 d of drought treatment, respectively. A total of 12 samples were measured using the MGI T7 platform, yielding an average of 7.61 Gb of data per sample. On average, 96.6% of the reads were aligned to the alfalfa reference genome (*M. sativa* cultivar XinJiangDaYe) [24]. After sequencing reads to the reference genome and reconstructing the transcripts, a total of 101,706 new transcripts were detected. Subsequently, reads were aligned to genes, using bowtie2, and an average of 71,459 genes were detected per sample (Table 1). We used the criteria of a p-value < 0.05 and $|\log2FC| > 1$ to determine the differential genes obtained by different comparisons.

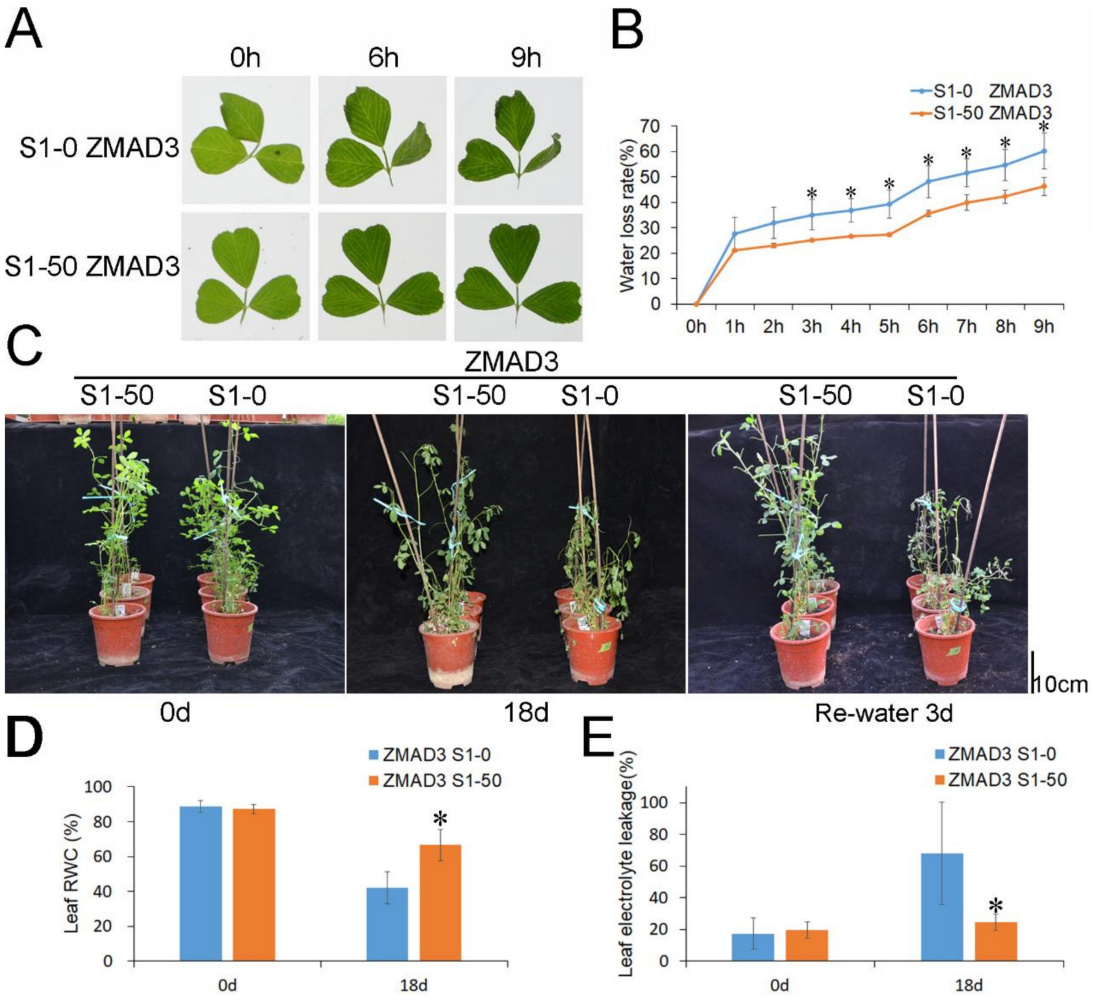

Figure 2. ABA-insensitivity plants isolated from ZMAD-3 showed stronger drought tolerance. (**A**) Photograph of detached leaves in a water loss treatment; photos were taken at 0 h, 6 h, and 9 h of ABA-sensitivity (S1-0) and ABA-insensitivity (S1-50) plants isolated from ZMAD3. (**B**) The water loss rate at observed time points for detached leaves. (**C**) Phenotype of S1-0 and S1-50 before and after 18 d drought treatment and 3 d after a rehydration treatment. Scale bars equal 10 cm. (**D**,**E**) Leaf RWC (**D**) and leaf electrolyte leakage (**E**) of ZMAD3 leaves before and after 18 d drought treatment. The asterisk indicates that the difference between S1-0 and S1-50 is statistically significant ($p < 0.05$).

Table 1. Reference genome-matching results.

Sample	Treatment	Biological Replicate	Total Reads	Total Mapped	Mapping Rate (%)
S1-0	Control	1	44,542,818	43,091,372	96.74
		2	44,707,562	43,175,678	96.57
		3	110,570,548	106,908,754	96.69
DS1-0	Drought (18 d)	1	44,868,990	43,253,798	96.40
		2	46,082,556	43,690,094	94.81
		3	44,924,794	42,946,096	95.60
S1-50	Control	1	44,114,614	42,721,328	96.84
		2	45,108,312	43,688,472	96.85
		3	46,831,492	45,535,672	97.23
DS1-50	Drought (18 d)	1	46,928,416	45,547,974	97.06
		2	44,681,506	43,377,766	97.08
		3	45,269,416	43,987,334	97.17
Total			608,631,024	587,924,338	96.60

3.4. Deferentially Expressed Genes between S1-0 and S1-50 Plants under Drought Stress Treatment

Compared to before drought treatment, 613 deferentially expressed genes (DEGs), including 267 up-expressed genes and 346 down-expressed genes, were detected in S1-0 leaves after drought treatment. Meanwhile, there were 309 DEGs under drought stress in S1-50, including 154 up-expressed genes and 155 down-regulated expression genes (Figures 3A and S2A,B). When analyzing the GO annotation of the differential genes, we found that the entries of S1-0 and S1-50 in signal transduction, mRNA precursor processing, and hormone anabolism were significantly increased before and after drought treatment, which indicated that drought affected the normal life activities of the plant and triggered the stimulus response to make the plant responsive to drought stress (Supplementary Figure S2B,E). The same analysis of DEGs annotated to KEGG revealed that DEGs in S1-0 focused on plant signal transduction, photosynthesis, and MAPK signaling pathways (Supplementary Figure S2C). However, DEGs in S1-50 focused on amino acid synthesis and degradation, as well as fatty acid metabolism (Supplementary Figure S2F). We then analyzed the genes that were commonly differentiated in both S1-0 and S1-50 before and after drought and found that 95 genes were differentiated in both materials (Figure 3B). The GO enrichment analysis of these genes showed that they were enriched in abiotic stimulus response, hormone-mediated signaling pathways, and hormone metabolism processes, suggesting that hormone response was most rapid during the onset of drought stress in alfalfa (Figure 3C). Meanwhile, the KEGG analysis revealed that those genes were enriched in plant hormone signal transduction and the MAPK signaling pathway—plant and zeatin biosynthesis (Figure 3D). These results indicate that plant hormones and their signaling play a key role in response to drought stress in alfalfa.

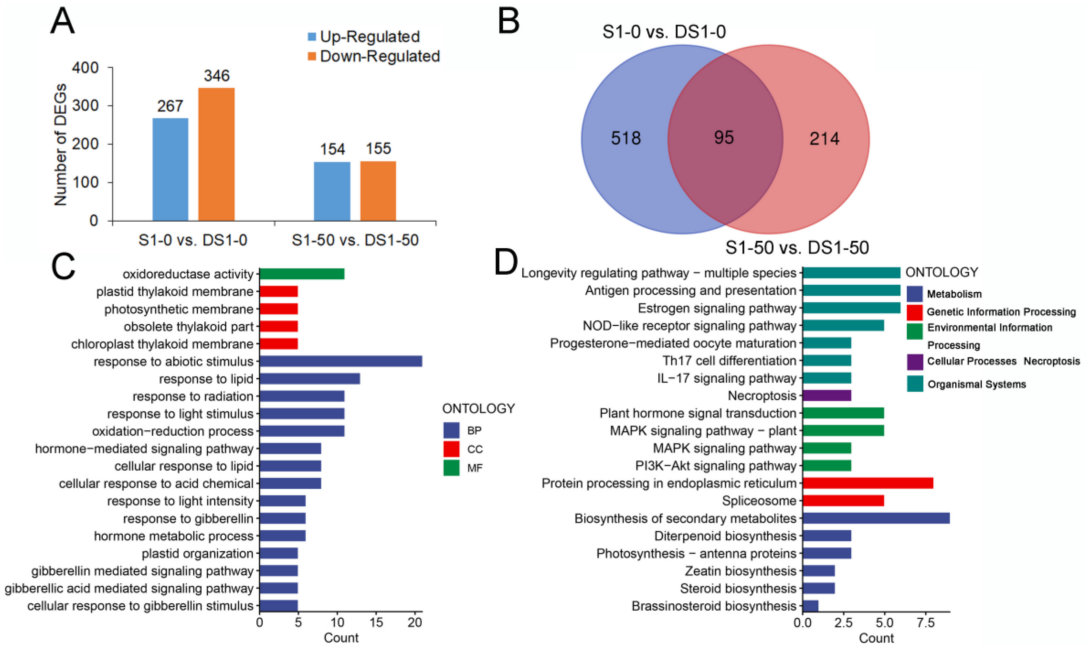

Figure 3. Transcriptome analysis of S1-0 and S1-50 leaves at 0 d and 7 d after drought treatment. (**A**) Number of differential genes between 0 d and 7 d after drought treatment at S1-0 and S1-50, respectively. (**B**) Venn diagram of the number of co-differentially expressed genes. (**C**) GO enrichment histogram of co-differentially expressed genes. In the figure, BP represents biological processes, CC represents cellular components, and MF represents molecular functions. (**D**) KEGG enrichment histogram of co-differentially expressed genes.

3.5. DEGs between S1-0 and S1-50 before and after Drought Stress Treatment

We further analyzed the genes that were jointly up- and down-regulated by S1-0 and S1-50 before and after drought treatment to try to understand why S1-50 alfalfa had better drought tolerance.

Transcriptional comparisons between S1-0 and S1-50 revealed that more genes were enriched in areas such as cellular metabolism and mRNA precursor processing, whereas S1-50 was more responsive in photosynthesis, carbon and nitrogen metabolism, and hormone responses after drought treatment (Supplementary Figure S3). An analysis of the genes that co-occurred in the differences between the two comparisons revealed that 48 genes continued to be up-regulated, while 31 genes continued to be down-regulated (Figure 4A–C). The genes were further analyzed based on their functions, and we found that the up-regulated expressed genes tended to favor the regulation of seed germination, osmotic stress, photosynthesis, and early development, while the down-regulated expressed genes were tended to the process of responding to external stimuli (Figure 4D,E). Those pathways were possibly contributed to improving the drought tolerance of S1-50.

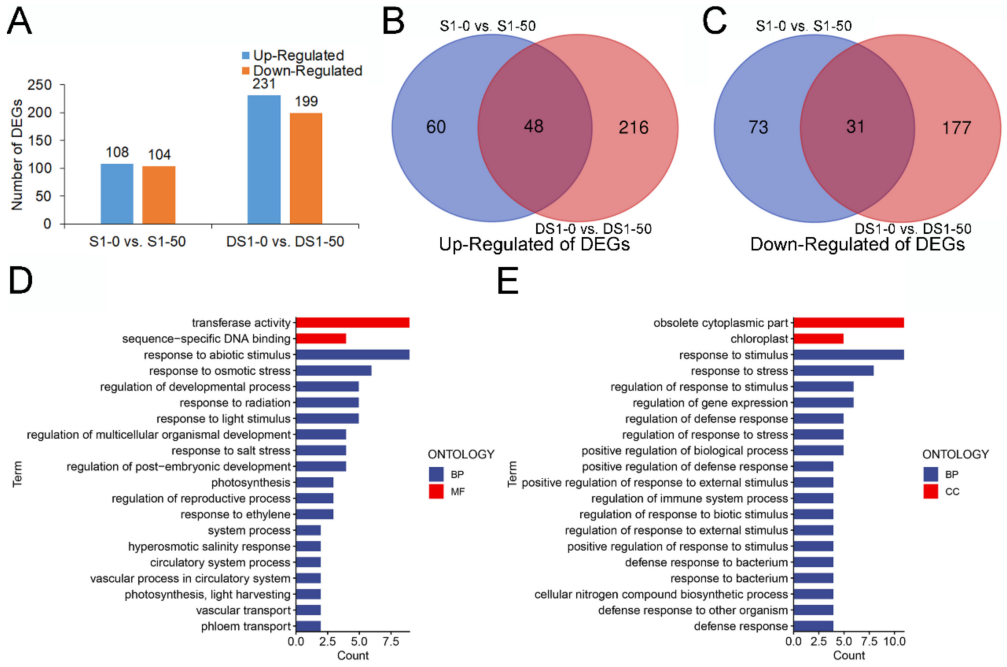

Figure 4. Differentially expressed genes (DEGs) that were jointly up- and down-regulated before and after drought treatment between S1-0 and S1-50. (**A**) Number of differentially expressed genes between S1-0 and S1-50 before and after drought treatment. (**B**,**C**) Venn diagram of DEGs detected in S1-0 and S1-50 before and after drought treatment, with the number at each intersection indicating the number of genes co-up-regulated for expression or co-down-regulated for expression. (**B**) Venn diagram of up-regulated genes. (**C**) Venn diagram of down-regulated gene expression. (**D**) GO enrichment histogram of up-regulated genes. In the figure, BP represents biological processes, and MF represents molecular functions. (**E**) GO enrichment histogram of down-regulated genes. In the figure, BP represents biological processes, and CC represents cellular components.

3.6. Integrative Analysis of Drought Tolerance Enhancement through the ABA and Waxes Biosynthesis Pathways

A further analysis of the jointly up-regulated genes revealed that genes related to the ABA pathway play an important role in this process (Figure 5A). We found that the expression trends of genes related to ABA synthesis, such as *PYS*, *NCED*, and *ABA2*, were similar, with a high expression level in S1-50 plants without drought treatment. When drought stress appeared, the genes responding to ABA synthesis in the S1-0 plants appeared to be up-regulated, with a gradual expression level similar to or even higher than that of the S1-50 material, but this regulation may have been relatively slow in plants that were tolerant to drought stress. At the same time, we observed that S1-50 had higher *AOG* expression levels, which may also predict that S1-50 is able to store ABA to avoid higher ABA levels from affecting growth, as well as to enhance its drought tolerance. Analyzing the genes of ABA signaling, we found that the expression of *PYR/PYL* in S1-50 species

was higher than that in S1-0 after drought stress, while *PP2C*, which is the downstream gene of PYLs, showed a high expression in S1-0. In addition, some DEGs between S1-0 and S1-50 were targeted into the wax synthesis pathway (Figure 5B). The analysis of the KEGG annotations revealed that the key genes *WSD* and *CER* regulated wax synthesis, were highly expressed in S1-50, which was also able to shed light on our results in the water loss experiments in isolated leaves. To verify the reliability of the RNA-seq data, six genes related to the ABA metabolic pathway were randomly selected and validated by qRT-PCR (Figure 5C). The results showed that the qRT-PCR results were similar to the trend of FPKM changes, confirming the reliability of the RNA-seq data. Therefore, we summarized a flowchart for accelerating the selection of new drought-tolerant alfalfa varieties via ABA screening at seed germination and combining it with the traditional breeding method (Figure 6).

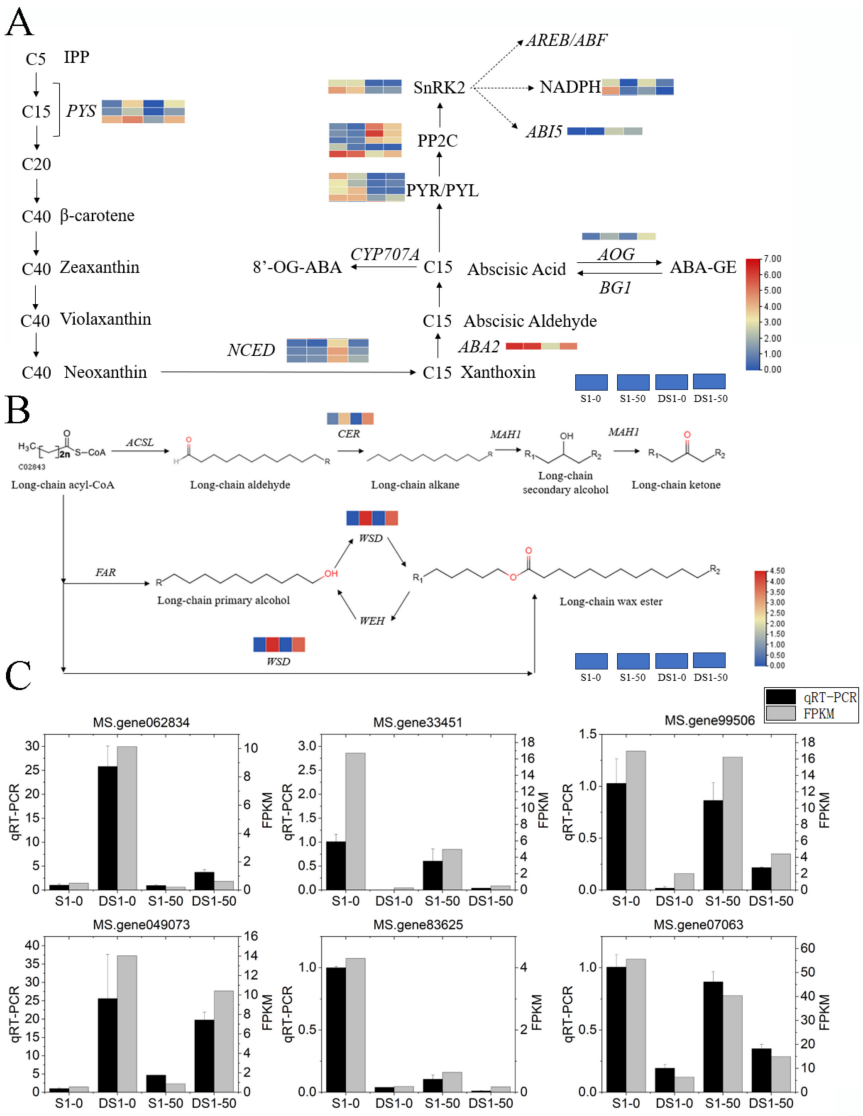

Figure 5. DEGs associated with ABA and wax synthesis before and after drought treatment on S1-0 and S1-50 plant. (**A**) Map of genes and their pathways associated with ABA synthesis and signaling. (**B**) Map of genes associated with wax synthesis and their pathways. Colored squares next to each gene are heat maps of the expression of the associated key genes. (**C**) Verification of the expression patterns of the DEGs before and after a drought treatment by qRT-PCR test. Left and right *y*-axis represent the relative expression level of the genes detected by qRT-PCR and the FPKM value of the RNA-seq genes, respectively.

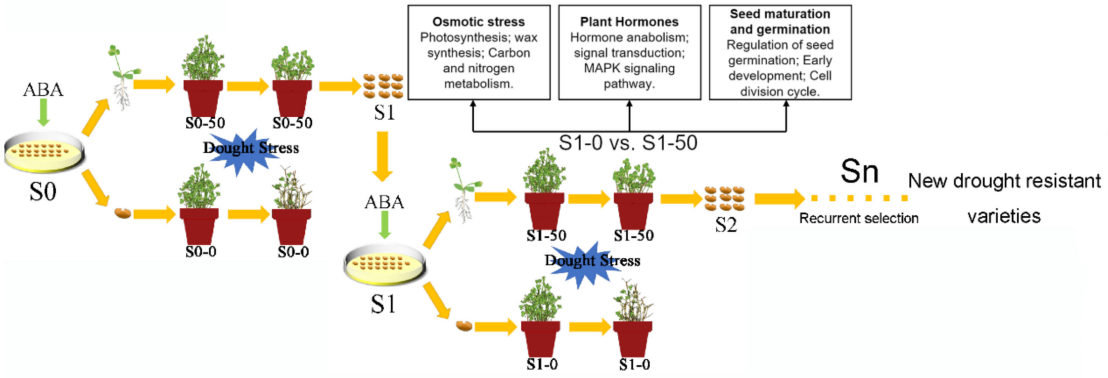

Figure 6. Processes and their molecular mechanisms for accelerating drought-tolerant alfalfa breeding through ABA screening at seed germination.

4. Discussion

ABA plays an important role in the drought tolerance of plants as a key stress hormone. The inheritance of seed sensitivity to ABA has rarely been reported to date. Our previous study found that plants with ABA insensitivity during the seed germination stage had higher drought tolerance, which was well inherited into the next generation [22]. In this study, we further verified the relationship between ABA-insensitive plants during the seed germination stage and the drought tolerance of adult plants, with the ABA-sensitive and -insensitive plants isolated from two half-sib family lines (ZMAD2 and ZMAD3). Subsequently, RNA-seq analyses were performed on the drought-tolerant line S1-50 and the drought-susceptible line S1-0 to uncover key drought-tolerant genes.

When exogenously applied, ABA significantly inhibited seed germination and post-emergence growth. In our previous study, we verified isolate differences in response to ABA in different populations [22]. In this study, we found that seeds from ABA-insensitive plants have higher germination rates compared to the wild type under ABA treatment, which indicates that the sensitivity to ABA can be inherited by the next generation. However, this phenomenon was not always positively correlated with drought tolerance in plants. For example, in rice, the *ospin1b* mutant is sensitive to ABA during seed germination, but plants show a significant decrease in drought tolerance [26]. In contrast, the mutant in *Arabidopsis* is less sensitive to ABA treatment, and the plants also show greater drought tolerance [27]. In addition, hypersensitivity to ABA was reported in Arabidopsis overexpressing *BG757*, but the plants showed significantly enhanced osmotic stress tolerance [28]. More interestingly, the attlp2 mutant, although insensitive to ABA, showed completely opposite results in response to drought stress at the seedling stage and at the late growth stage [29]. The expression of some genes is not consistently induced by ABA and drought treatment; therefore, the difference in drought tolerance between S1-0 and S1-50 plants may not be due to the pretreatment with ABA [19,30,31]. However, experimental verification of the correlation between ABA sensitivity and plant drought tolerance in other species is still needed.

It has been reported that drought limits photosynthesis in plants and may affect the biological clock [32,33]. Under drought stress, pathways related to plant signaling, photosynthesis, and circadian rhythms appeared significantly enriched in S1-0, while genes related to amino acid metabolism appeared more frequently in S1-50. A rice metabolome analysis revealed that genes and proteins related to aromatic amino acids, especially L-phenylalanine, were associated with drought tolerance [34]. The exogenous application of GABA can increase drought tolerance in plants by regulating photosynthesis and maintaining osmotic pressure [35], in addition to the application of proline, which induces stress tolerance and improves cell membrane homeostasis [36]. This suggests that when drought occurs, it has a greater impact on photosynthesis in S1-0, while S1-50 avoids disruption of the light and system due to its greater drought tolerance and has the potential to regulate

its drought tolerance through amino acid metabolism; however, the specific mechanism is less studied and needs to be further researched.

To further understand the segregation that occurs between S1-0 and S1-50, we hypothesized that segregation of the two materials had occurred and that genes that differed between the two materials before drought treatment continued to differ after the onset of drought stress, meaning that there is a possibility that there are key genes that continue to regulate drought tolerance in plants. We compared the differential genes shared between S1-0 and S1-50 before and after the drought treatment. We believe that if a gene has a regulatory effect on drought tolerance, then it will remain different between the two plants. We found a total of 79 consistently differentially expressed genes, in which their GOs were mainly focused on response to stress, regulation of post-embryonic development, and response to external stimuli. LEA (Late Embryonic Enrichment Protein) plays an important function during seed germination, which can influence the dehydration response [37]. Moreover, chloroplast-targeted LEAs regulate Rubisco activity and stomatal movement in alfalfa [38]. We also found many genes related to ABA biosynthesis and signaling, such as PYL and PP2C, through this analytical occurrence. The functions of these co-differentially expressed genes indicate the reliability of our analytical approach and also reveal that the differences between the two plants are likely to be ABA-related. Further analyses of the ABA metabolic pathway with genes related to ABA synthesis and catabolism, such as *NCED*, *ABA2*, and *AOG*, differed between the two lines. *NCED*, as a key gene for ABA synthesis, was extremely rapid in responding to drought stress and, at the same time, was able to improve plant drought tolerance by increasing POD activity and reducing reactive oxygen species [39]. *ABA2* can regulate the NADP/NADPH ratio at low water potential to protect plants from drought-stress damage [40]. However, we did not observe a differential expression of BG1-related genes, which may be due to the fact that we sampled at a later stage of drought stress, when the plants would more often store the synthesized ABA. PP2C, which is associated with ABA signaling, was up-regulated, and ABA binding to PYR/PYL could deregulate the inhibition of SnRK2 activity by PP2C to promote the plant's response to adversity [41], but the function of PP2C in the regulation of drought tolerance was not conserved. In wheat, PP2C was found to interact with and dephosphorylate SnRK1.1, thereby negatively regulating drought tolerance [42]. However, PP2C19 was found to positively regulate drought tolerance in plants in *Cyperus esculentus* [43]. So, the specific function of PP2C for the difference between S1-0 and S1-50 needs to be further investigated. In addition, it has been reported that PYLs can improve the expression of inhibited PP2C and further improve the drought tolerance of plants. However, unlike what we previously reported, AREB/ABF did not show a difference, which could also be due to the timing of our sampling. However, it is also possible that genes responsive to seed germination, such as ABI5, showed higher expression in the S1-0 material, which could be responsible for the different sensitivities shown by the two materials under ABA treatment.

Interestingly, in addition to this, we found that genes related to wax synthesis were differentially expressed between S1-0 and S1-50. Waxes reduce water dissipation and protect the light and system, so they can protect plants against drought stress [44,45]. WSD is a key enzyme involved in wax synthesis. Heterologous expression of sunflower *HaWSD9* in Arabidopsis resulted in a significant increase in total wax esters and enhanced plant drought tolerance and salt tolerance [46]. At the same time, CER1, which encodes the formation of alkanes, can be induced by ABA and increase the content of C25-C33 alkanes, thereby increasing the water loss rate of plant leaves [47].

This study further confirmed that ABA treatment during alfalfa seed germination can screen a group of plants with a higher drought-tolerance ability. In this way, the alfalfa breeding process can be accelerated, and it is expected that new highly drought-tolerant alfalfa varieties can be obtained throughout six-to-eight generations of selection (Figure 6). Meanwhile, this study provides a new perspective for understanding the molecular mechanism of drought tolerance in alfalfa and lays a solid foundation for subsequent gene-function studies.

5. Conclusions

In this study, we proved that the ABA-sensitivity phenotype of alfalfa could be inherited by the next generation in S1 generation seeds. Moreover, the plants of S1-50 were determined to be more drought tolerant by observing the water loss of isolated leaves and determining the leaf water content and electrolyte leakage after drought treatment, which provided a theoretical basis for the rapid screening of drought-tolerant alfalfa. And combined with the RNA-seq analysis, we analyzed the genes that were co-differently expressed before and after drought treatment in S1-0 and S1-50 and found that there were some similarities between the two lines in response to drought stress. In addition, the DEGs in response to drought stress between S1-0 and S1-50 were explored, and they were mainly clustered in the ABA pathway and the wax synthesis pathway. These results provide new insights into the study of drought tolerance in alfalfa and even some genes that are not functionally conserved; thus, further functional validation is still needed to provide more molecular tools for alfalfa drought-tolerance breeding.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14030406/s1, Supplementary Data S1 and Supplementary Data S2.

Author Contributions: M.X., Y.L. (Yanrong Liu) and W.Z. conceived and designed the research; M.X. and Z.X. conducted experiments; M.X., Z.X., Y.L. (Yaling Liu) and Y.L. (Yanrong Liu) analyzed the data; M.X., Z.X., J.L. and W.Z. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the National Center of Pratacultural Technology Innovation (under preparation) (CCPTZX2023B01) and the Ministry of Science and Technology of the People's Republic of China (2022ZD040120301).

Data Availability Statement: The original contributions presented in the study are included in the article/Supplementary Materials, further inquiries can be directed to the corresponding authors.

Acknowledgments: We are grateful to Dr. Qingchuan Yang of The Animal Husbandry Institute of CAAS for giving us Zhongmu No.1 seeds. We also give thanks to the support from the people at the Shangzhuang experimental station of CAU for their help with field-plant caring.

Conflicts of Interest: The authors declare no conflict of interest.

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