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Physiological and Transcriptome Analysis Illuminates the Molecular Mechanisms of the Drought Resistance Improved by Alginate Oligosaccharides in *Triticum aestivum* L.

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ABSTRACT

Alginate oligosaccharides (AOS) enhance drought resistance in wheat (*Triticum aestivum* L.), but the definite mechanisms remain largely unknown. The physiological and transcriptome responses of wheat seedlings treated with AOS were analyzed under drought stress simulated with polyethylene glycol-6000. The results showed that AOS promoted the growth of wheat seedlings and reduced oxidative damage by improving peroxidase and superoxide dismutase activities under drought stress. A total of 10,064 and 15,208 differentially expressed unigenes (DEGs) obtained from the AOS treatment and control samples at 24 and 72 h after dehydration, respectively, were mainly enriched in the biosynthesis of secondary metabolites (phenylpropanoid biosynthesis, flavonoid biosynthesis), carbohydrate metabolism (starch and sucrose metabolism, carbon fixation in photosynthetic organisms), lipid metabolism (fatty acid elongation, biosynthesis of unsaturated fatty acids, alpha-linolenic acid metabolism, cutin, suberine and wax biosynthesis), and signaling transduction pathways. The up-regulated genes were related to, for example, chlorophyll a-b binding protein, amylosynthase, phosphotransferase, peroxidase, phenylalanine ammonia lyase, flavone synthase, glutathione synthetase. Signaling molecules (including MAPK, plant hormones, H₂O₂ and calcium) and transcription factors (mainly including NAC, MYB, MYB-related, WRKY, bZIP family members) were involved in the AOS-induced wheat drought resistance. The results obtained in this study help underpin the mechanisms of wheat drought resistance improved by AOS, and provides a theoretical basis for the application of AOS as an environmentally sustainable biological method to improve drought resistance in agriculture.

KEYWORDS

Alginate oligosaccharides; *Triticum aestivum* L.; drought resistance; transcriptomic; physiological analysis

1 Introduction

Wheat (*Triticum aestivum* L.) is one of the most important staple crops worldwide, accounting for 30% of the world's total harvested cereal area and approximately 26% of total cereal production [1], but its production in many regions is severely impacted by drought [2,3]. For example, a third of the UK wheat crop is grown on drought prone soil, resulting on average in a 10% to 20% loss in total production,



valued at £72 million [4]. The North China Plain, which accounts for more than 60% of China's wheat production, is usually affected by drought, and the intensity, frequency and extent of droughts have increased in recent decades [3,5]. Up to 60% of the wheat cultivation area will be impacted by severe water shortages by the end of the century without the adoption of climate adaptation or drought mitigation measures [6]. Wheat is readily damaged as a consequence of drought, owing to its poor drought resistance [7]. Continuous drought stress triggers various physiological responses, inhibits growth and photosynthesis, and results in increased plant death and reduced yield [8,9]. With global warming and increasingly variable precipitation, drought has become a major factor affecting wheat production [10], so it is important to improve wheat drought tolerance in order to ensure food security and sustainable agricultural development.

In agricultural production, measures that can be taken to improve wheat drought resistance include drought resistant breeding, the application of chemical agents, rational fertilization, and so on [11–13]. Chemical reagents to improve drought resistance are, relatively speaking, convenient to apply. At present, the drought resistance agents used are generally absorbents, exogenous abscisic acid (ABA), nutrient elements, fungicides, or rooting agents, which leave residues in the soil and lead to environmental pollution [14–16]. Hence, it is necessary to find an environmentally sustainable drought resistance agent, such as a biological compound, to replace existing chemical reagents.

Alginate oligosaccharides (AOS), which are produced from alginate, an abundant compound, is composed of β -D-mannuronic acid (M), α -L-guluronic acid (G) or both heterozygous segments [15,17–20]. Studies have shown that AOS with a low molecular weight and excellent solubility, promoted root growth, increased crop yield and enhanced the tolerance of crops to abiotic stress [17,18]. It was also shown that AOS enhanced the drought resistance of a wide variety of crops such as wheat, cucumber and *Arabidopsis*, which was reflected in the improvement of agronomic traits (including shoot and root length, biomass, etc.) and increases in the photosynthetic capacity and yield [15,19,20]. Therefore, AOS may be used as a new environmentally sustainable biological drought resistant agents in agriculture, in order to reduce the need to apply chemical agents, and to reduce the contamination of agricultural products and the environment. However, the application of AOS is currently limited due to a lack of a clear understanding of the mechanisms of action of AOS.

Plants respond and adapt to drought stress by perceiving the *stimulus*, receiving and transmitting a signal, and then initiating various defense mechanisms [21]. Reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide, and hydroxyl radical, will accumulate excessively in plants while plants are under drought stress, which causes the disorganization of cellular membranes, accompanied with an increase in malondialdehyde (MDA) content and photosynthetic apparatus [22,23]. However, the toxicity of ROS is balanced by integrated antioxidant systems, including enzymes such as superoxide dismutase (SOD), peroxidase, catalase and non-enzymatic antioxidants such as glutathione, ascorbate, flavonoids, and α -tocopherol [24]. Previous research showed that spraying AOS water solution (1 g L^{-1} , w/v) enhanced the activities of antioxidative enzymes, and reduced the MDA content in wheat leaves under drought stress simulated by adding polyethylene glycol-6000 (PEG-6000) water solution (150 g L^{-1} , w/v) [15]. Additionally, ABA as a major signaling molecule was involved in drought response by inducing antioxidant defenses and regulating stomatal closure [25,26]. Some carbohydrates and secondary metabolism products such flavonoids were also regulated by ABA signals in plant vegetative tissues [27,28]. The drought resistant related genes involved in the ABA signal pathway, such as the late embryogenesis abundant protein 1 gene (*LEA1*), *psbA* gene, sucrose non-fermenting 1-related protein kinase 2 gene (*SnRK2*) and pyrroline-5-carboxylate synthetase gene (*P5CS*) were up-regulated by AOS in wheat seedlings [15]. Further research showed that AOS induced ABA synthesis mainly by up-regulating the expression of ABA accumulation biosynthesis-related genes, i.e., *TaNCED*, *TaAOX* and *TaBG* [29]. These results preliminarily showed that ABA was involved in wheat drought resistance

enhanced by AOS. However, the molecular mechanisms of AOS-induced drought resistance in wheat are still unclear.

To date, studies on the mechanisms of plant tolerance to drought stress have shifted the focus from the physiological level to the molecular levels, especially at the omics levels. RNA sequencing (RNA-Seq) technology, as a tool to analyze gene functions and interactions at the omics level, has seen extensive usage in various fields of genetics and biology, including the analysis of growth characteristics and stress responses mechanism of plants [30,31]. Although the mechanisms of drought-tolerance have been reported recently [15,32], the transcriptional regulatory network of wheat response to drought stress has not been fully understood. PEG is a non-toxic and water soluble polymer, and can reduce the water potential, resulting in water deficiency in plants and causing physiological disorders. Hence, PEG has often been used to mimic drought, which is a common method to study plant responses to water stress [15,33]. On the basis of previous studies, transcriptome sequencing was performed on shoots and roots of wheat seedlings treated with or without AOS under drought stress simulated by PEG-6000, and the Illumina Novaseq 6000 platform was used to sequence the AOS-responsive genes. Gene ontology (GO) annotation and Kyoto encyclopedia of genes and genomes (KEGG) enrichment analysis were conducted to reveal the biological processes involved in the regulation of wheat drought resistance by AOS, which will enable a better understanding of the underlying mechanisms, and provide a scientific basis for the application of AOS as a new environmentally sustainable biological drought resistance agent in agriculture.

2 Materials and Methods

2.1 Materials

The alginate (sample No. 20100927004; viscosity, 1,010 mPas; molecular formula, $(C_6H_7O_6Na)_n$; molecular weight, 32,000–2,002,000; M/G ratio, 7:3) was obtained from Qingdao Bright Moon Seaweeds Group Co., Ltd. (Qingdao, Shandong, China) and degraded with an alginate lyase (purchased from Shanghai Nagase Trading Co., Ltd., Shanghai, China) from *Sphingomonas sp.* at 45°C for 10 h. Higher molecular weight components were precipitated by adding 80% (v/v) ethanol (4 volumes) and centrifuging at 8,000 rpm for 15 min. The supernatant was freeze-dried and identified by electrospray ionization mass spectrometry, and mainly composed of dimers, trimers and tetramers (the average molecular weight, 724; viscosity, 180 mPas) (Fig. 1). Other reagents were analytical grade (Sinopharm Chemical Reagent Beijing Co., Ltd., Beijing, China), and all solutions were prepared using deionized water (Resistivity > 16 MΩ cm at 25°C).

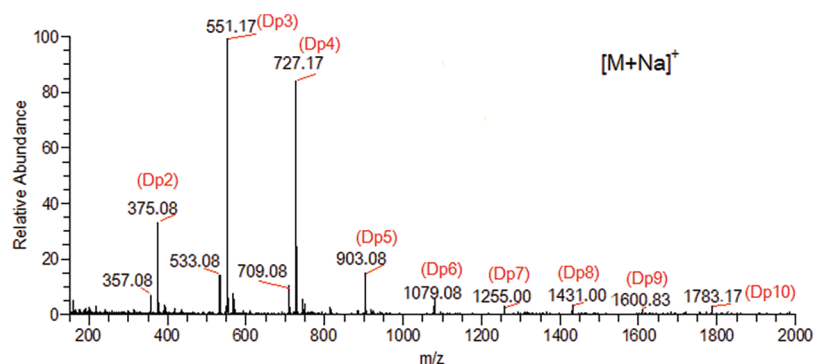


Figure 1: Electrospray ionizations mass spectrum(ESI-MS) of alginate oligosaccharides (AOS). AOS, alginate oligosaccharides; ESI-MS, electrospray ionization mass spectrum; Dp, degree of polymerization

Wheat seeds used in the experiment were of a drought sensitive wheat cultivar ‘Xinong979’, which was bred by Northwest A&F University (Shanxi, China) [15].

2.2 Plant Culture and Experimental Design

Wheat seeds were sterilized with 75% (v/v) ethanol for 5 min and washed extensively with deionized water. To obtain more uniformly germinated seeds, wheat seeds were soaked in deionized water for 8 h at 25°C in the dark, and then transferred to a 20 cm Petri dish containing one sheet of filter paper moistened with deionized water and cultured for 3 days at 25°C/20°C (12 h/12 h day/night cycle), with a relative humidity of 60% and a light intensity of 800 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Four independent experiments were conducted using 3-day-old wheat seedlings. In experiment 1, similar wheat seedlings were selected and transferred to 150 g L⁻¹ (w/v) PEG water solution (simulating drought stress). A preliminary study showed that spraying 1 g L⁻¹ (w/v) AOS water solution was optimal for enhancing wheat drought tolerance (Data not shown). Deionized water (the control) or AOS solution (the AOS treatment) was sprayed onto the seedlings leaf surfaces after dehydration for 1 h, respectively. Twice a day (morning and evening), 5 mL was sprayed once. The growth parameters and physiological characteristics of wheat seedlings were analyzed at 72 h after the treatment. In experiment 2, the above experiment was repeated, and samples of wheat shoots and roots were harvested for RNA-Sep analysis at 24 and 72 h after the treatment, respectively, and then quick-frozen in liquid nitrogen and stored at -80°C until processing. In experiment 3, the above experiment was repeated again, and samples of wheat shoots were collected at different time points (3, 5, 12, 24, 48, and 72 h) after treatment and analyzed with quantitative real-time RT-PCR (qRT-PCR). In experiment 4, mitogen-activated protein kinase (MAPK) inhibitor PD 98059 was used to study the role of the MAPK signal pathway in AOS-induced drought resistance in wheat. 3-day-old wheat seedlings were first root-dipped in 50 μM (w/v) PD 98059 for 6 h, and then cultured in 150 g L⁻¹ (w/v) PEG water solution and treated with AOS. Samples of wheat shoots and roots were collected at 3, 24, 48, and 72 h and analyzed by qRT-PCR. All the experiments were carried out in three independent repetitions (20 seedlings per biological replicate) in this study.

2.3 Growth Parameters and Physiology Characters

The lengths of shoots and roots of wheat seedlings were estimated using a ruler after harvesting. To determine the relative water content (RWC, %), fresh leaves were weighed immediately to obtain the fresh weight (FW), then these leaves were soaked in distilled water for 2 h and the saturated weight (SW) was determined, and finally these leaves were dried for 24 h at 75°C to determine the dry weight (DW). RWC was calculated as below: $\text{RWC}(\%) = (\text{FW} - \text{DW})/(\text{SW} - \text{DW}) \times 100\%$. Chlorophyll (Chl) was extracted from the fresh leaves using 95% (v/v) ethanol, and absorption of the filtered extract was measured at 665, 649 and 470 nm, respectively. For the enzyme analysis, samples of the leaves (0.5 g FW for each seedling) were homogenized in 50 mM phosphate buffer (pH 7.8) with ice-bath. The homogenate was then centrifuged at 4°C for 10,000 g 10 min, and the supernatant was used for enzyme activity determination. The SOD activity was measured with the nitroblue tetrazolium-reduction method. The POD activity was measured with the guaiacol method. The free proline content was measured with the acid ninhydrin method. The MDA content was measured with the thiobarbituric acid colourimetry method. The above determinations were performed according to the methods of Wang et al. [34]. The data obtained from the experiment were analyzed using SPSS software (version 17.0; IBM, USA). Duncan's pairwise comparison test was used to determine significant differences at the 5% confidence level ($p < 0.05$) and the different lowercase letters denote a significant difference.

2.4 Tissue RNA Extraction and Quality Inspection

Total RNA was extracted from three biological replicates of wheat shoots and roots using TRIZOL reagent (Trans GEN, Beijing, China), and its concentration and purity were detected with Nanodrop 2000 (Thermo Fisher Scientific Inc., Waltham, USA), and the RNA integrity value was evaluated using the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, USA). The total amount and concentration of RNA were

more than 1 μg and 35 $\text{ng } \mu\text{L}^{-1}$, respectively, and the values of OD260/280 and OD260/230 were more than 1.8 and 1.0, respectively, which were suitable for downstream processes.

2.5 Library Construction and Transcriptome Sequencing

To prepare the cDNA library, oligo-(dT) beads were used to collect poly (A) mRNA from total RNA. Fragmentation buffer was added to cut the long mRNA into short fragments of about 300 bp, and then these short mRNA fragments were used as templates to synthesize the first strand of cDNA. The second strand of cDNA was synthesized using DNA polymerase I (New England Biolabs), RNase H (Invitrogen), dNTPs and buffer. After purification with a QiaQuick polymerase chain reaction (PCR) kit (Qiagen, CA, USA) and washing with EB buffer (10 mM Tris-HCl, pH 8.5) for end repairing, poly(A) tails and adaptors were added to the fragments. The fragments of suitable size were selected by agarose gel electrophoresis and enriched by PCR amplification to construct the cDNA library. The constructed libraries were sequenced on Illumina Novaseq 6000 platform. The obtained raw image data were converted into sequence data by base calling to yield the raw reads, which were stored in FASTQ file format. In order to ensure the accuracy of subsequent bioinformatics analysis, low-quality and repetitive sequences were removed to obtain high-quality sequencing data (clean data) for downstream analysis.

2.6 Transcriptome Expression Analysis

The clean data were aligned to the wheat reference genome (http://plants.ensembl.org/Triticum_aestivum/Info/Index) using TopHat (v2.0.13). Gene expression levels were quantified using RNA-Sep by Expectation-Maximization (<http://www.biomedcentral.com/1471-2015/12/323>). The fragments per kilo base of transcript per million mapped reads (FPKM) was used to calculate the expression levels [35]. For the samples with biological replicates, differential expression analysis of two groups was performed using DESeq2 R package, and the screening threshold of the differentially expressed unigenes (DEGs) was $|\log_2 \text{fold-change}| \geq 1$ and $p\text{-value} < 0.001$. DEGs have been designated as “up-regulated” or “down-regulated” based on whether their expression levels were higher or lower than those of the untreated state.

2.7 GO Classification and KEGG Enrichment

The GO database (<http://www.geneontology.org/>) was used to understand the functions and mechanisms of differential genes, and the results were divided into three major categories, including biological processes (BP), cellular components (CC) and molecular functions (MF). GO annotation was performed for the DEGs in the two groups of wheat tissues. Taking one of the samples as a control, the results obtained from the other sample were drawn in a GO annotation histogram. KEGG pathway enrichment analyses were performed using the KEGG orthology based annotation system 2.0 (<http://bioinfo.org/kobas/genelist/>) with Fisher's exact test. In order to control the false positive rate, the false discovery rate method for multiple testing was used with a threshold of a corrected $p\text{-value} < 0.05$. The KEGG pathways meeting this condition were defined as significantly enriched pathways.

2.8 Real-Time Quantitative PCR Validation of Genes

The expression pattern of some DEGs related to signaling transduction pathway were obtained with qRT-PCR to validate the RNA-Sep data. 1 μg of RNA was reverse transcribed to cDNA using the PrimeScript RT reagent Kit (Takara Bio, Inc., Shiga, Japan). The qRT-PCR was performed using SYBR Green Premix Pro Taq HS qPCR Kit (Accurate Biology Co., Ltd., Hunan, China) on a real-time fluorescent quantitative PCR instrument (Bio-Rad Laboratories Inc., Hercules, CA, USA). All primers used in the qRT-PCR were designed using NCBI Primer-BLAST, and were listed in Table 1. Relative gene expression levels were calculated by the $2^{-\Delta\Delta\text{Ct}}$ method, using wheat GAPDH gene as the internal

reference gene. qRT-PCR analysis of each sample was performed in triplicate using three biological replicates per treatment.

Table 1: Primer sequences used in this study

Gene	Primer sequence for cDNA isolation
GAPDH	Forward 5'-ATCTGACTGTTAGACTTGCGAAGC-3'
	Reverse 5'-GGAAACAAGGTCCTCATCAACG-3'
<i>TaZEP</i>	Forward 5'-ATCCAGATCCAGAGCAACGCGCT-3'
	Reverse 5'-AAGAGATCACCTTCAAATTTCCGGC-3'
<i>TaERF1</i>	Forward 5'-GTGTCCGTGTCTGGCTTGGT-3'
	Reverse 5'-GCACCTTCACAGCAGTAGCG-3'
<i>TaNAC69</i>	Forward 5'-GCCAAGTCGTGCTCCCTCA-3'
	Reverse 5'-CGTAGCCGTTGTTGTTATTATAGTGT-3'
<i>TaPYR1</i>	Forward 5'-TGGGGCCAGGCCAATGCT-3'
	Reverse 5'-TCGAAGCGGCGGACGATG-3'
<i>TaPYR2</i>	Forward 5'-CTTCAGGCCCGTCCTACTGC-3'
	Reverse 5'-GCGAGTTTCTGGAGGTTGAGC-3'
<i>TaPYR3</i>	Forward 5'-CATCCTCAGCTTCAGCGTCGTG-3'
	Reverse 5'-CCTGGTGTCTCCTCGGTGTT-3'

3 Results

3.1 Analysis of the Growth Parameters and Physiological Characters of Wheat Seedlings

To examine the effects of AOS on the growth of wheat seedlings under drought stress, the growth parameters and physiological characters of wheat seedlings were analyzed (Table 2). Compared with the control, the length and FW of wheat shoots treated with AOS for 72 h were significantly increased by 7.6% and 14.6%, respectively ($p < 0.05$). RWC and total Chl contents in leaves were increased by 4.9% and 7.3%, and the activities of POD and SOD were also raised by 13.0% and 25.8%, but the contents of MDA and free proline were reduced by 32.0% and 31.9%. These results indicated that AOS reduced oxidative damage caused by drought stress and promoted the growth of wheat seedlings.

Table 2: Effects of alginate oligosaccharides (AOS) on the growth parameters and physiological characters of wheat seedlings under drought stress

Parameter	Control group	AOS group
Shoot length (cm)	8.13 ± 0.13 b	8.75 ± 0.26 a
Root length (cm)	7.25 ± 0.05 a	7.31 ± 0.04 a
Fresh weight of shoots (mg plant ⁻¹)	41.87 ± 0.60 b	47.98 ± 1.24 a
Fresh weight of roots (mg plant ⁻¹)	40.28 ± 2.23 a	40.20 ± 2.54 a
Relative water content (%)	77.90 ± 0.65 b	81.74 ± 1.04 a
Total chlorophyll content (mg g ⁻¹)	2.20 ± 0.04 b	2.36 ± 0.02 a

(Continued)

Table 2 (continued)

Parameter	Control group	AOS group
peroxidase (POD)activity (U mg ⁻¹ FW min ⁻¹)	10.56 ± 0.15 b	11.93 ± 0.24 a
superoxide dismutase (SOD)activity (U g ⁻¹ FW min ⁻¹)	225.13 ± 11.18 b	283.26 ± 16.90 a
Pro (proline) content (µg g ⁻¹ FW)	101.84 ± 4.14 a	69.22 ± 2.33 b
malondialdehyde (MDA) content (µmol g ⁻¹ FW)	11.45 ± 1.23 a	7.80 ± 1.65 b

Note: Data are presented as mean values ± SD from three independent experiments, and different lowercase letters indicate significant differences at $p < 0.05$, according to Duncan's pairwise comparison test. 150 g L⁻¹ PEG-6000 was used to simulate drought stress. Control group, wheat seedling sprayed with deionized water under drought stress for 72 h (3 d); AOS group, wheat seedlings sprayed with 1 g L⁻¹ AOS under drought stress for 72 h; AOS, alginate oligosaccharides; PEG-6000, polyethylene glycol-6000; SD, standard deviation.

3.2 Quality Evaluation of Transcriptome Sequencing Data

To further understand the molecular mechanisms of AOS-induced drought resistance in wheat, we examined the global gene expression profile of wheat seedlings treated with or without AOS using RNA-Seq analysis. RNA-Seq libraries of three biological replicates for the control and AOS group were prepared and sequenced. Firstly, the quality of the transcriptome sequencing data from all samples were evaluated (Table 3). After filtering out low quality data, the average number of total clean reads obtained from the control group of wheat seedlings were 74, 100, 790 and 57, 570, 146 (in shoots) as well as 74, 491, 194 and 59, 860, 560 (in roots) at 24 and 72 h after dehydration respectively, and those of the AOS treatment group were 59, 410, 160 and 60, 368, 632 (in shoots) as well as 65, 686, 468 and 61, 895, 548 (in roots). The error rates of all samples were less than 0.03%, and the Q20 and Q30 percentages were greater than 97% and 92%, respectively. The match ratio of the effective sequences obtained were more than 75% compared to the wheat reference genome. The results indicated that the quality of the sequencing results met the demands of the subsequent bioinformatics analysis.

Table 3: Statistics of RNA-seq data of samples

Treatment	Sample	Total raw reads	Total clean reads	Error rate (%)	Clean reads Q20 (%)	Clean reads Q30 (%)	Total mapping (%)
S-control 1	1	67 443 446	66 988 022	0.0234	98.60	95.87	93.17
	2	77 436 518	76 517 624	0.0246	98.20	95.04	90.16
	3	80 218 160	78 796 724	0.0240	98.40	94.66	92.31
S-control 3	1	57 645 386	57 364 522	0.0245	98.24	95.04	91.08
	2	57 667 514	57 218 262	0.0236	98.52	95.72	92.36
	3	59 624 018	58 127 654	0.0242	98.29	94.42	92.02
S-AOS 1	1	57 658 914	57 046 284	0.0245	98.04	94.56	88.98
	2	65 428 696	64 881 090	0.0236	98.49	95.63	92.93
	3	57 431 492	56 303 106	0.0245	98.46	94.78	88.78

(Continued)

Table 3 (continued)

Treatment	Sample	Total raw reads	Total clean reads	Error rate (%)	Clean reads Q20 (%)	Clean reads Q30 (%)	Total mapping (%)
S-AOS 3	1	58 365 452	57 980 241	0.0248	98.18	94.78	86.48
	2	67 680 866	67 113 014	0.0234	98.58	95.86	94.65
	3	57 535 718	56 012 641	0.0247	98.14	94.06	82.06
R-control 1	1	78 158 364	77 895 364	0.0254	98.12	94.56	89.78
	2	60 924 016	60 483 544	0.0237	98.48	95.57	92.53
	3	86 545 022	85 094 674	0.0238	98.36	94.84	90.21
R-control 3	1	58 985 134	57 875 135	0.0265	97.64	92.87	77.17
	2	63 306 942	62 879 630	0.0235	98.57	95.82	93.69
	3	58 931 102	58 826 915	0.0265	97.43	92.26	76.19
R-AOS1	1	68 876 157	67 985 345	0.0245	98.14	94.45	86.45
	2	53 926 650	53 463 406	0.0234	98.59	95.93	92.71
	3	76 198 227	75 610 653	0.0247	98.17	94.23	84.39
R-AOS 3	1	60 856 654	59 845 654	0.0245	98.18	94.84	75.04
	2	70 548 092	70 011 770	0.0234	98.59	95.93	92.71
	3	56 090 304	55 829 220	0.0244	98.31	94.41	69.46

Note: Q20 (bp) and Q30 (bp) represent the percentage of bases with Phred value more than 20 and 30 in the total base, respectively, indicating that the correct recognition rate of bases is 99.0% and 99.9%. 150 g L⁻¹ PEG-6000 was used to simulate drought stress. S-control 1 and S-control 3, wheat shoots of the control group under drought stress for 24 (1 d) and 72 h (3 d), respectively; S-AOS 1 and S-AOS 3, wheat shoots of the AOS treatment group under drought stress for 24 and 72 h, respectively; R-control 1 and R-control 3, wheat roots of the control group under drought stress for 24 and 72 h, respectively; R-AOS 1 and R-AOS 3, wheat roots of the AOS group under drought stress for 24 and 72 h, respectively. Three biological replicates were conducted for each group. 1, the sample repeats 1; 2, the sample repeats 2; 3, the sample repeats 3; AOS, alginate oligosaccharides; PEG-6000, polyethylene glycol-6000.

3.3 Comparative Analysis of DEGs

In order to analyze the mechanisms of wheat drought resistance enhanced by AOS, DEGs in shoots and roots of wheat seedlings treated with AOS were detected, with reference to the control (Figs. 2A–2D). A total of 5,185 (3,250 up-regulated genes and 1,935 down-regulated genes) and 4,827 DEGs (1,956 up-regulated genes and 2,871 down-regulated genes) in wheat shoots were screened out between the AOS treatment and control samples at 24 and 72 h after dehydration, respectively (Figs. 2A and 2B). However, a total of 4,879 (1,777 up-regulated genes and 3,102 down-regulated genes) and 10,381 DEGs (8,504 up-regulated genes and 1,877 down-regulated genes) in wheat roots were obtained (Figs. 2C and 2D). The results suggested that most DEGs were up-regulated in wheat shoots (at 24 h after dehydration) and roots (at 72 h after dehydration), compared to the control group.

3.4 GO Function Annotation Analysis of DEGs

GO enrichment analyses were conducted to describe the biological functions of DEGs between the AOS treatment and control groups (Figs. 3A–3D). These DEGs in wheat shoots were annotated to 20 BP, 14 CC, 13 MF categories and 21 BP, 14 CC and 14 MF categories at 24 and 72 h after dehydration, respectively. However, those in wheat roots were annotated to 19 BP, 14 CC, 14 MF categories and 20 BP, 14 CC,

15 MF categories, respectively. In terms of the BP category, the DEGs were mainly involved in metabolic process, cellular process, biological regulation, response to stimulus, cellular components organization or biogenesis, localization, developmental process, reproduction process, and multi-organism process. For the CC category, the DEGs were mainly associated with cell components, membrane components, organelle, membrane, organelle part, protein-containing complexes, extracellular regions and cell junction. The main terms of MF were binding, catalytic activity, transporter activity, transcription regulator activity, molecular function regulation and antioxidant activity.

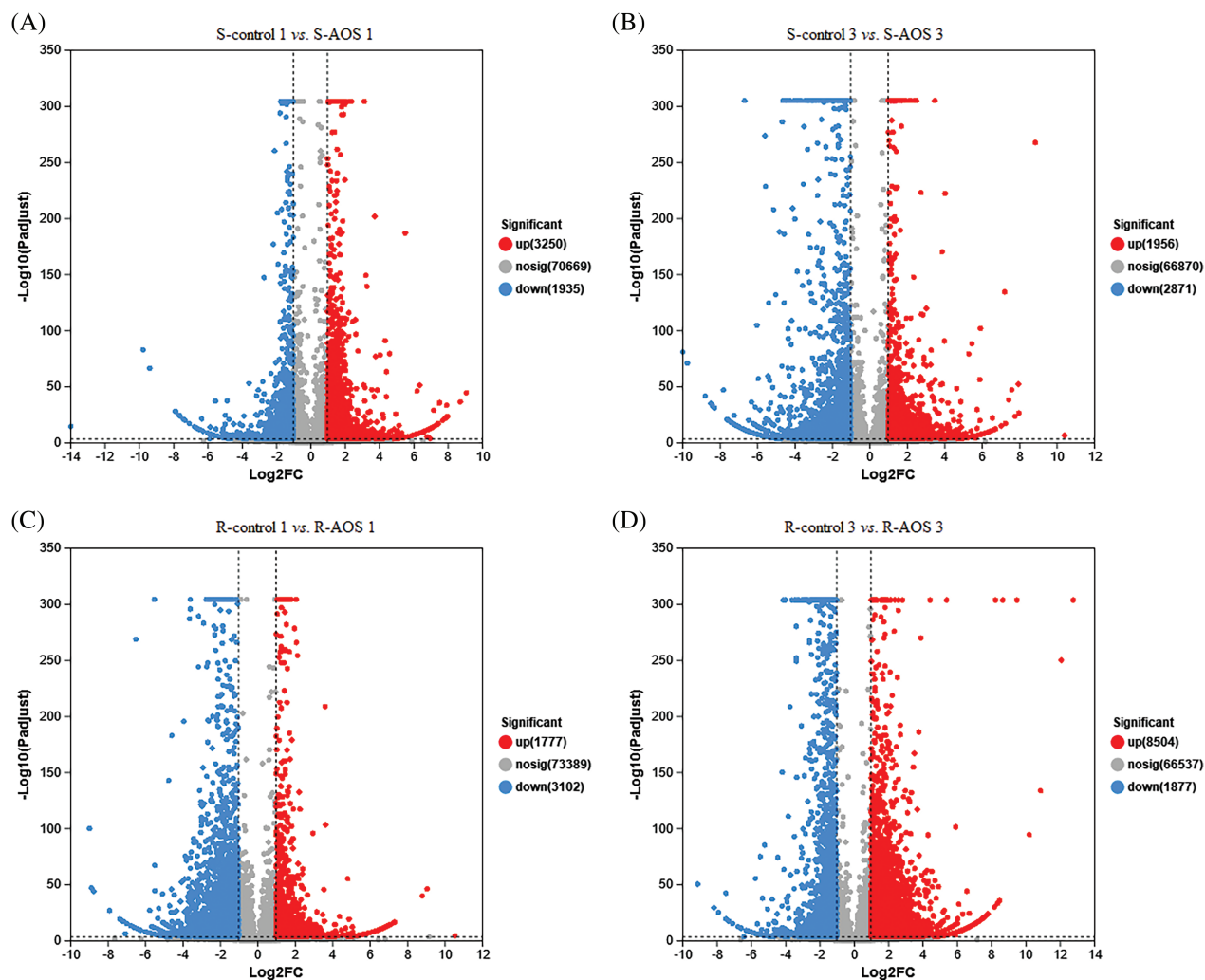


Figure 2: Volcano plot of differentially expressed genes (DEGs) between pairwise comparisons of alginate oligosaccharides (AOS) groups to control groups under drought stress. (A) S-control 1 vs. S-AOS 1; (B) S-control 3 vs. S-AOS 3; (C) R-control 1 vs. R-AOS 1; (D) R-control 3 vs. R-AOS 3. Gray dots indicate no significant difference sense, the red dots indicate significant difference up-regulated genes, the green dots indicate significant difference down-regulated genes. 150 g L^{-1} PEG-6000 was used to simulate drought stress. S-control 1 and S-control 3, wheat shoots of the control group under drought stress for 24 (1 d) and 72 h (3 d), respectively; S-AOS 1 and S-AOS 3, wheat shoots of the AOS treatment group under drought stress for 24 and 72 h, respectively; R-control 1 and R-control 3, wheat roots of the control group under drought stress for 24 and 72 h, respectively; R-AOS 1 and R-AOS 3, wheat roots of the AOS group under drought stress for 24 and 72 h, respectively; AOS, alginate oligosaccharides; DEGs, differentially expressed genes; PEG-6000, polyethylene glycol-6000

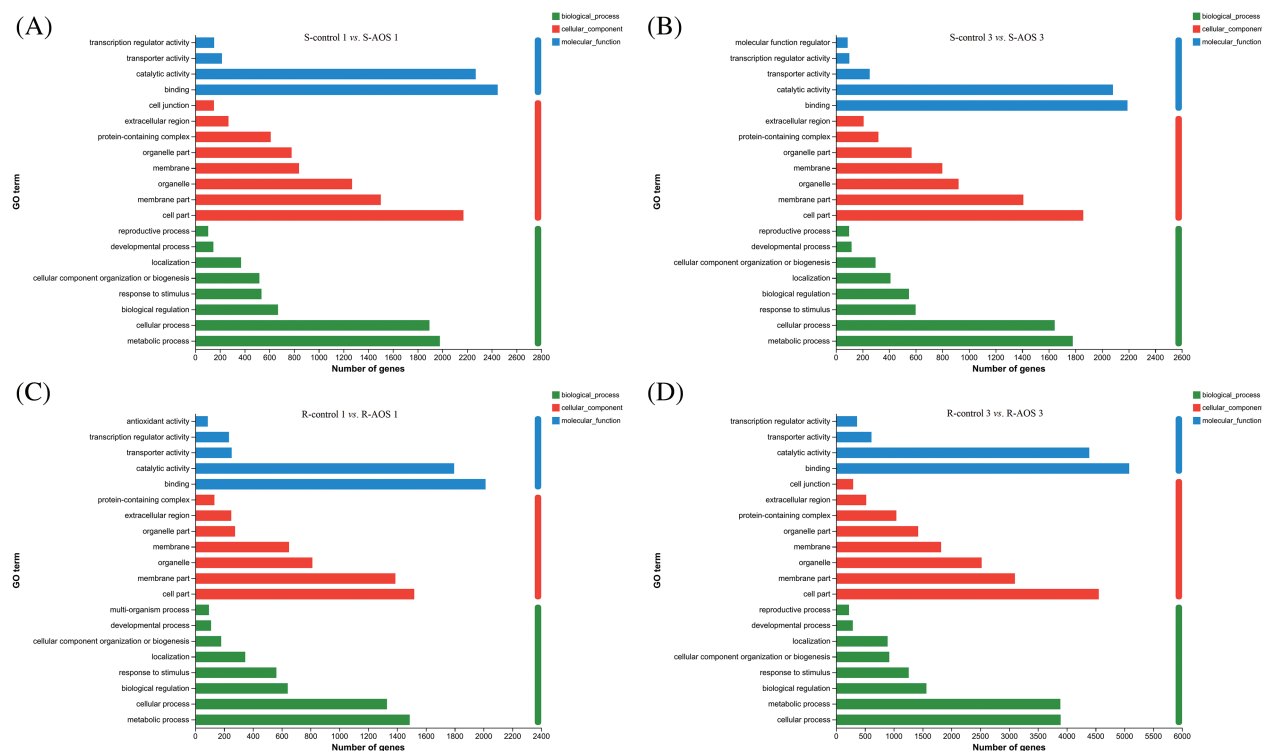


Figure 3: Gene ontology (GO) functional enrichment analysis of the differentially expressed genes (DEGs) between pairwise comparisons of alginate oligosaccharides (AOS) groups to control groups under drought stress. (A) S-control 1 vs. S-AOS 1; (B) S-control 3 vs. S-AOS 3; (C) R-control 1 vs. R-AOS 1; (D) R-control 3 vs. R-AOS 3. These DEGs were mainly involved in biological process, cellular component and molecular function. 150 g L^{-1} PEG-6000 was used to simulate drought stress. S-control 1 and S-control 3, wheat shoots of the control group under drought stress for 24 (1 d) and 72 h (3 d), respectively; S-AOS 1 and S-AOS 3, wheat shoots of the AOS treatment group under drought stress for 24 and 72 h, respectively; R-control 1 and R-control 3, wheat roots of the control group under drought stress for 24 and 72 h, respectively; R-AOS 1 and R-AOS 3, wheat roots of the AOS group under drought stress for 24 and 72 h, respectively; AOS, alginate oligosaccharides; DEGs, differentially expressed genes; GO, gene ontology; PEG-6000, polyethylene glycol-6000

3.5 The KEGG Pathway Annotation and Enrichment Analysis of DEGs

To further identify metabolic or signal transduction pathways in which the DEGs are likely to be involved in wheat drought resistance enhanced by AOS, KEGG pathway annotation and enrichment analysis were carried out (Table S1; Figs. 4A–4D). The results showed that the DEGs were mainly involved in carbohydrate metabolism, biosynthesis of other secondary metabolites, lipid metabolism, amino acid metabolism, energy metabolism and signal transduction (Table S1). The KEGG pathway enrichment analysis indicated that these pathways, including photosynthesis-antenna proteins and cutin, suberine and wax biosynthesis, were highly enriched in wheat shoots (Figs. 4A and 4B). Besides these, the enriched pathways of DEGs in wheat shoots included phenylpropanoid biosynthesis, DNA replication, flavonoid biosynthesis, fatty acid elongation, cyanoamino acid metabolism, biosynthesis of unsaturated fatty acids, flavone and flavonol biosynthesis and so on, at 24 h after dehydration (Fig. 4A). These pathways were enriched, including glycolysis/gluconeogenesis, glutathione metabolism, starch and sucrose metabolism, pyruvate metabolism, glyoxylate and dicarboxylate metabolism, carbon fixation in

photosynthetic organisms, peroxisome, glycine, serine and threonine acid metabolism, fatty acid degradation, alpha-linolenic acid metabolism and so on, at 72 h after dehydration (Fig. 4B).

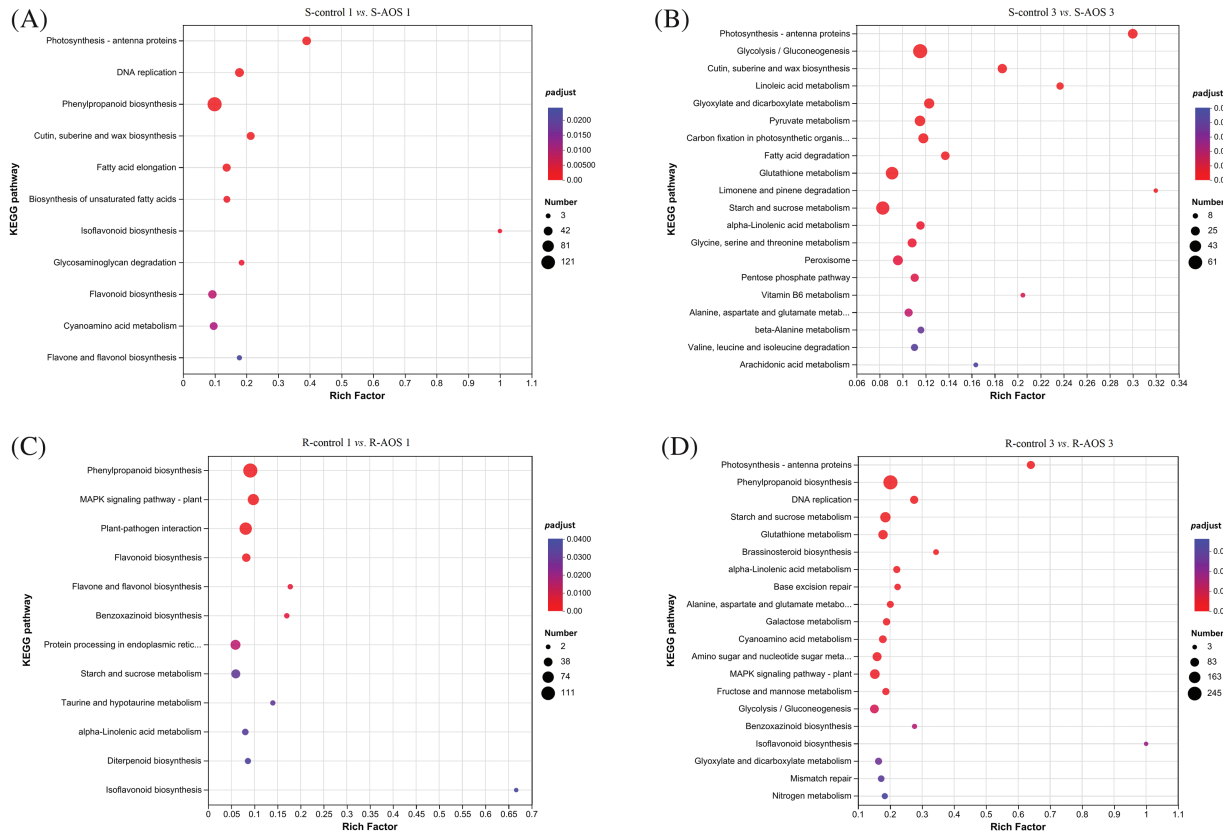


Figure 4: Kyoto encyclopedia of genes and genomes (KEGG) classification enrichment map of the differentially expressed genes (DEGs) between pairwise comparisons of alginate oligosaccharides (AOS) groups to control groups under drought stress. (A) S-control 1 vs. S-AOS 1; (B) S-control 3 vs. S-AOS 3; (C) R-control 1 vs. R-AOS 1; (D) R-control 3 vs. R-AOS 3. 150 g L⁻¹ PEG-6000 was used to simulate drought stress. S-control 1 and S-control 3, wheat shoots of the control group under drought stress for 24 (1 d) and 72 h (3 d), respectively; S-AOS 1 and S-AOS 3, wheat shoots of the AOS treatment group under drought stress for 24 and 72 h, respectively; R-control 1 and R-control 3, wheat roots of the control group under drought stress for 24 and 72 h, respectively; R-AOS 1 and R-AOS 3, wheat roots of the AOS group under drought stress for 24 and 72 h, respectively; AOS, alginate oligosaccharides; DEGs, differentially expressed genes; PEG-6000, polyethylene glycol-6000. The closer the *p*-value is to zero, the closer the color is to red, the more significant the enrichment is

In wheat roots, these pathways, including phenylpropanoid biosynthesis, starch and sucrose metabolism, alpha-linolenic acid metabolism, MAPK signaling pathway-plant, benzoxazinoid biosynthesis and isoflavonoid biosynthesis, were enriched (Figs. 4C and 4D). Besides these, the enriched pathways of DEGs in wheat roots included plant-pathogen interaction, protein processing in endoplasmic reticulum, flavonoid biosynthesis, and so on, at 24 h after dehydration (Fig. 4C). These pathways, including glycolysis/gluconeogenesis, DNA replication, photosynthesis-antenna proteins, cyanoamino acid metabolism, fructose and mannose metabolism, brassinosteroid biosynthesis, and so on, were enriched at

72 h after dehydration (Fig. 4D). The results indicated that carbohydrate metabolism, secondary metabolites synthesis, lipid metabolism, amino acid metabolism, energy metabolism and signal transduction were involved in the AOS-induced drought resistance in wheat.

3.6 Gene Analysis of the Enriched KEGG Pathways

DEGs in the main enriched KEGG pathways involved in carbohydrate metabolism, secondary metabolites synthesis, and lipid metabolism were analyzed (Tables 4 and 5). The results showed that the up-regulated genes in wheat shoots, which were involved in phenylpropanoid biosynthesis (mainly including peroxidase, phenylalanine ammonia lyase, β -glucosidase, 4-coumarate: coenzyme A ligase, phenylalanine deaminase and 4- α -glucanotransferase), photosynthesis-antenna proteins (chlorophyll a-b binding protein), fatty acid elongation (mainly including very-long-chain (3R)-3-hydroxyacyl-CoA dehydratase and 3-ketoacyl-CoA synthase), cutin, suberine and wax biosynthesis (mainly including fatty acyl-CoA reductase, caleosin and cytochrome P450), flavonoid biosynthesis (mainly including glycosyltransferase and flavone synthase), and biosynthesis of unsaturated fatty acids, were more than the down-regulated genes at 24 h after dehydration. Moreover, most DEGs involved in glycolysis /gluconeogenesis and glutathione metabolism in wheat shoots were up-regulated at 72 h after dehydration.

Table 4: Gene analysis of the main enriched Kyoto encyclopedia of genes and genomes (KEGG) pathways

Comparison	Pathway ID	KEGG pathway	Up gene	Down gene
S-control 1 vs. S-AOS 1	map00940	Phenylpropanoid biosynthesis	104	17
	map00196	Photosynthesis-antenna proteins	39	0
	map00062	Fatty acid elongation	29	4
	map00073	Cutin, suberine and wax biosynthesis	29	3
	map00941	Flavonoid biosynthesis	33	4
	map01040	Biosynthesis of unsaturated fatty acids	19	2
S-control 3 vs. S-AOS 3	map00010	Glycolysis/Gluconeogenesis	34	15
	map00500	Starch and sucrose metabolism	16	38
	map00480	Glutathione metabolism	34	15
	map00620	Pyruvate metabolism	2	33
	map00710	Carbon fixation in photosynthetic organisms	13	19
	map04146	Peroxisome	9	22
	Map00592	Alpha-linolenic acid metabolism	10	13
R-control 1 vs. R-AOS 1	map00940	Phenylpropanoid biosynthesis	64	47
	map04026	Plant-pathogen interaction	16	67
	map04016	MAPK signaling pathway-plant	13	53
	map00500	Starch and sucrose metabolism	13	26
	map04141	Protein processing in endoplasmic reticulum	5	45
	map00941	Flavonoid biosynthesis	27	6
	map00592	Alpha-linolenic acid metabolism	10	6

(Continued)

Table 4 (continued)				
Comparison	Pathway ID	KEGG pathway	Up gene	Down gene
R- control 3 vs. R-AOS 3	map00940	Phenylpropanoid biosynthesis	229	16
	map00500	Starch and sucrose metabolism	95	26
	map00010	Glucolysis/gluconeogenesis	60	20
	map04016	MAPK signaling pathway-plant	69	34
	map00480	Glutathione metabolism	88	8
	map00520	Amino sugar and nucleotide sugar metabolism	70	16
	map00196	Photosynthesis-antenna proteins	64	0
	map00592	Alpha-linolenic acid metabolism	40	4

Note: 150 g L⁻¹ PEG-6000 was used to simulate drought stress. S-control 1 and S-control 3, wheat shoots of the control group under drought stress for 24 (1 d) and 72 h (3 d), respectively; S-AOS 1 and S-AOS 3, wheat shoots of the AOS treatment group under drought stress for 24 and 72 h, respectively; R-control 1 and R-control 3, wheat roots of the control group under drought stress for 24 and 72 h, respectively; R-AOS 1 and R-AOS 3, wheat roots of the AOS group under drought stress for 24 and 72 h, respectively; AOS, alginate oligosaccharides; KEGG, Kyoto encyclopedia of genes and genomes; PEG-6000, polyethylene glycol-6000.

Table 5: Partial up-regulated genes involved in the main enriched KEGG pathways

Comparison	KEGG pathway	Name	Gene ID	
S-control 1 vs. S-AOS 1	Phenylpropanoid biosynthesis	Peroxidase	TraesCS7A02G424100, TraesCS4B02G176900, TraesCS4A02G136600, et al.	
		Phenylalanine ammonia lyase	TraesCS 6B02G258400	
		β-glucosidase	TraesCS7A02G450500, TraesCS3A02G398000	
		4-coumarate:coenzyme A ligase	TraesCS7A02G496200	
		Phenylalanine deaminase	TraesCS6D02G212200	
	Photosynthesis-antenna proteins	4-α-glucanotransferase	Chlorophyll a-b binding protein	TraesCS5D02G464700, TraesCS1D02G375100, TraesCS6A02G094100, et al.
			Glycosyltransferase	TraesCS2D02G462500, TraesCS6A02G327100, TraesCS3D02G534800, TraesCS3B02G313700
			Flavone synthase	TraesCS2D02G043500, TraesCS2A02G044900, TraesCS2B02G057100

(Continued)

Table 5 (continued)			
Comparison	KEGG pathway	Name	Gene ID
R-control 3 vs. R-AOS 3	Fatty acid elongation	Very-long-chain (3R)-3- hydroxyacyl-CoA dehydratase	TraesCSU02G174900, TraesCS4B02G268500, TraesCS3D02G074900, et al.
		3-ketoacyl-CoA synthase	TraesCS7A02G205700, TraesCS4A02G303900, TraesCS7B02G113200, et al.
	Cutin, suberine and wax biosynthesis	Fatty acyl-CoA reductase	TraesCS7A02G389300, TraesCS3A02G013800, TraesCS4B02G010100, et al.
		Caleosin	TraesCS2D02G364100, TraesCS3A02G280700
		Cytochrome P450	TraesCS4D02G283400
	Phenylpropanoid biosynthesis	Peroxidase	TraesCS1B02G330600, TraesCS2B02G615700, TraesCS7A02G424100, et al.
		Phenylalanine ammonia lyase	TraesCS2D02G204700, TraesCS2B02G398000, TraesCS2B02G224300, et al.
		4-coumarate:coenzyme A ligase	Traes CS2A02G145800, TraesCS2B02G171200
		S-adenosyl-l-methionine dependent methyltransferase superfamily protein	TraesCS7B02G027200
	Starch and sucrose metabolism	Cinnamate 4-hydroxylase	TraesCS3A02G342900
Amylosynthase		TraesCS7D02G064300, TraesCS7B02G093800, TraesCS4A02G418200	
Phosphotransferase		TraesCS1D02G355200, TraesCS3A02G276100, TraesCS3D02G276000, et al.	
Amylase		TraesCS4B02G393500, TraesCS6D02G313500, TraesCS3A02G248000	
Trehalose-6-phosphate phosphatase		TraesCS2B02G187100, TraesCS6A02G301800, TraesCS6A02G248400, et al.	
1, 4-alpha-glucan branching enzyme 3		TraesCS7D02G344000, TraesCS3B02G397900	

(Continued)

Table 5 (continued)			
Comparison	KEGG pathway	Name	Gene ID
		Endoglucanase	TraesCS1B02G377100, TraesCS4B02G104500, TraesCS1A02G389300, et al.
	Glycolysis/ gluconeogenesis	Aldose 1-epimerase	TraesCS2D02G321900, TraesCS7B02G067000
		Aldehyde dehydrogenase	TraesCS2B02G406900, TraesCS5D02G127300
		Glucose-6-phosphate 1-epimerase	TraesCS2A02G501600
		Fructose-1,6-bisphosphatase	TraesCS3B02G410400
		L-lactate dehydrogenase	TraesCS6A02G085300
		Fructose-bisphosphate aldolase	TraesCS5D02G122700, TraesCS7D02G377400
		ATP-dependent 6- phosphofructokinase	TraesCS7D02G101300
	MAPK signaling pathway	WRKY transcription factor	TraesCS7D02G336400, TraesCS1B02G088900
		Endochitinase	TraesCS3A02G260100
		Heavy metal P-type ATPase	TraesCS2D02G407800, TraesCS2A02G410400, TraesCS2B02G429200
		Mitogen-activated protein kinase	TraesCS1D02G422800, TraesCS4B02G197800
		Serine/threonine protein kinase	TraesCS2D02G170700, TraesCS2B02G630000, TraesCS7D02G525100
		Pathogen-associated protein	TraesCS7A02G198900, TraesCSU02G095300, TraesCS5B02G442900, et al.
		Catalase	TraesCS6D02G048300, TraesCS6A02G041700
		Calcium binding protein CP1	TraesCS4B02G182900, TraesCS4D02G182800, TraesCS4A02G123100
		Calmodulin TaCaM1-1	TraesCS2D02G097500
		Ribonucleoside diphosphate kinase	TraesCS1A02G444300
		Ethylene receptor	TraesCS6A02G399400, TraesCS6D02G 383600

(Continued)

Table 5 (continued)			
Comparison	KEGG pathway	Name	Gene ID
	Glutathione metabolism	TSO2	TraesCS7B02G112000, TraesCS7A02G204800, TraesCS7D02G207800
		Glutathione synthetase	TraesCS7D02G 431500
		Ribonucleoside diphosphate kinase reductase	TraesCS7B02G031500, TraesCS6B02G410800, TraesCS7D02G130500
		Hypothetical L-ascorbate peroxidase 6	TraesCS 7D02G249200
		R-glutamyl cyclotransferase	TraesCS2B02G334800
		Glucose-6-phosphate dehydrogenase	TraesCS3D02G491400
		Ascorbate peroxidase	TraesCS2A02G082100
		The assumed In2.1 protein	TraesCS4D02G200700
		Glutathione-S-transferase	TraesCS6A02G139000
		Amino sugar and nucleotide sugar metabolism	Hexosyltransferase
	NADH-cytochrome b5 reductase		TraesCS3A02G338700, TraesCS3B02G370400
	β -hexosaminidase		TraesCS1B02G270100, TraesCS1A02G259800, TraesCS1B02G058700, et al.
	Phosphomannomutase		TraesCS4B02G239500
	Similar to chitinase		TraesCS6D02G199500
	Bifunctional dTDP-4-dehydrorhamnose 3,5-epimerase/ dTDP-4-dehydrorhamnose reductase		TraesCS6D02G238800
	Chlorophyll a-b binding protein		TraesCS6B02G122800, TraesCS2A02G204800, TraesCSU02G250000, et al.
	Alpha-linolenic acid metabolism	Allene oxide synthase	TraesCS5B02G408000,
		Allene oxide cyclase	TraesCS6A02G334800, TraesCS6B02G365200, TraesCS6D02G314300
		Alcohol dehydrogenase	TraesCS4A02G202300

Note: 150 g L⁻¹ PEG-6000 was used to simulate drought stress. S-control 1 and S-AOS 1, wheat shoots of the control and AOS group under drought stress for 24 h (1 d), respectively; R-control 3 and R-AOS 3, wheat roots of the control and AOS group under drought stress for 72 h, respectively; AOS, alginate oligosaccharides; KEGG, Kyoto encyclopedia of genes and genomes; PEG-6000, polyethylene glycol-6000.

In wheat roots, the up-regulated genes, which were involved in phenylpropanoid biosynthesis, starch and sucrose metabolism (mainly including amylosynthase, phosphotransferase, amylase, trehalose-6-phosphate phosphatase, 1,4-alpha-glucan branching enzyme 3 and endoglucanase), glycolysis/gluconeogenesis, MAPK signaling pathway-plant (mainly including WRKY transcription factor, endochitinase, heavy metal P-type ATPase, mitogen-activated protein kinase, serine/threonine protein kinase, pathogen-associated protein, catalase, calcium binding protein, calmodulin, ribonucleoside diphosphate kinase and ethylene receptor), glutathione metabolism (mainly including TSO2, glutathione synthetase, ribonucleoside diphosphate kinase reductase, hypothetical L-ascorbate peroxidase 6, R-glutamyl cyclotransferase, glucose-6-phosphate dehydrogenase, ascorbate peroxidase, glutathione-S-transferase and hexosyltransferase), amino sugar and nucleotide sugar metabolism, photosynthesis-antenna protein, and alpha-linolenic acid metabolism (mainly including allene oxide synthase, allene oxide cyclase, and alcohol dehydrogenase) were more than the down-regulated genes at 72 h after dehydration. Moreover, most DEGs involved in the phenylpropanoid biosynthesis, flavonoid biosynthesis and alpha-linolenic acid metabolism were up-regulated at 24 h after dehydration.

The results indicated that, most DEGs in the main enriched KEGG pathways, including photosynthesis-antenna protein, starch and sucrose metabolism, phenylpropanoid biosynthesis, flavonoid biosynthesis, fatty acid elongation, cutin, suberine and wax biosynthesis, glutathione metabolism, were up-regulated by AOS, which appeared earlier in wheat shoots than in the roots. These genes were mainly related to chlorophyll a-b binding protein, amylosynthase, phosphotransferase, peroxidase, phenylalanine ammonia lyase, flavone synthase, glutathione synthetase. Signaling molecules, such as MAPK, plant hormones, H₂O₂ and calcium, were involved in the AOS-induced drought resistance in wheat.

3.7 Differential Expression Analysis of Transcription Factor (TF) Genes

TFs play important roles in response to drought stress, hence the expression of TFs genes were analyzed (Figs. 5A–5D and Table 6). A total of 80 and 68 differentially expressed TF genes in wheat shoots were obtained between the AOS treatment and control samples at 24 and 72 h after dehydration, and the top 10 TF families were *FARI*, *NAC*, *MYB*, *MYB-related*, *CATA*, *WRKY*, *bZIP*, *GRAS*, *CAMTA*, *C3H*, and *B3*, *bHLH*, *MYB*, *ERF*, *bZIP*, *HSF*, *ARF*, *WRKY*, *MYB-related*, *NAC*, respectively (Figs. 5A and 5B). Among which, 49 and 51 TF genes that regulated plant stress response (accounting for 31.25% and 75.0% of all differential TF genes) were obtained, mainly including *FARI*, *NAC*, *MYB*, *MYB-related*, *WRKY*, *bZIP*, *GRAS* family members (Table 6). In wheat roots, there were 88 and 78 differentially expressed TF genes at 24 and 72 h after dehydration, and the top 10 families were *WRKY*, *MYB*, *bHLH*, *HSF*, *ERF*, *MYB-related*, *B3*, *bZIP*, *NAC*, *GRAS* and *MYB*, *bHLH*, *bZIP*, *HB-other*, *MYB-related*, *WRKY*, *ERF*, *C3H*, *HSF*, *B3*, respectively (Figs. 5C and 5D). Among which, 68 and 51 TF genes that regulated plant stress response (accounting for 77.27% and 65.38% of all differential TF genes) were obtained, mainly including *WRKY*, *MYB*, *bHLH*, *MYB-related*, *NAC*, *bZIP*, *B3*, *ERF*, *HSF* family members (Table 6). These results indicated that many TFs were involved in the regulation of AOS on wheat drought resistance, mainly the *NAC*, *MYB*, *MYB-related*, *WRKY*, *bZIP* families.

3.8 Verification of Some Key Genes Related to Signaling Transduction

To further clarify the signal transduction mechanism of AOS in regulating wheat drought resistance, the key genes related to signaling transduction were tested by qRT-PCR in view of the key roles of ABA in drought resistance. The results showed that, at 72 h after dehydration, the expression levels of ABA biosynthesis genes *TaZEP*, and ABA receptor genes *TaPYR1*, *TaPYR2* and *TaPYR3* in wheat shoots treated with AOS were significantly higher than those in the control (Fig. 6), indicating that AOS could positively regulate ABA synthesis and signaling in wheat responding to drought stress.

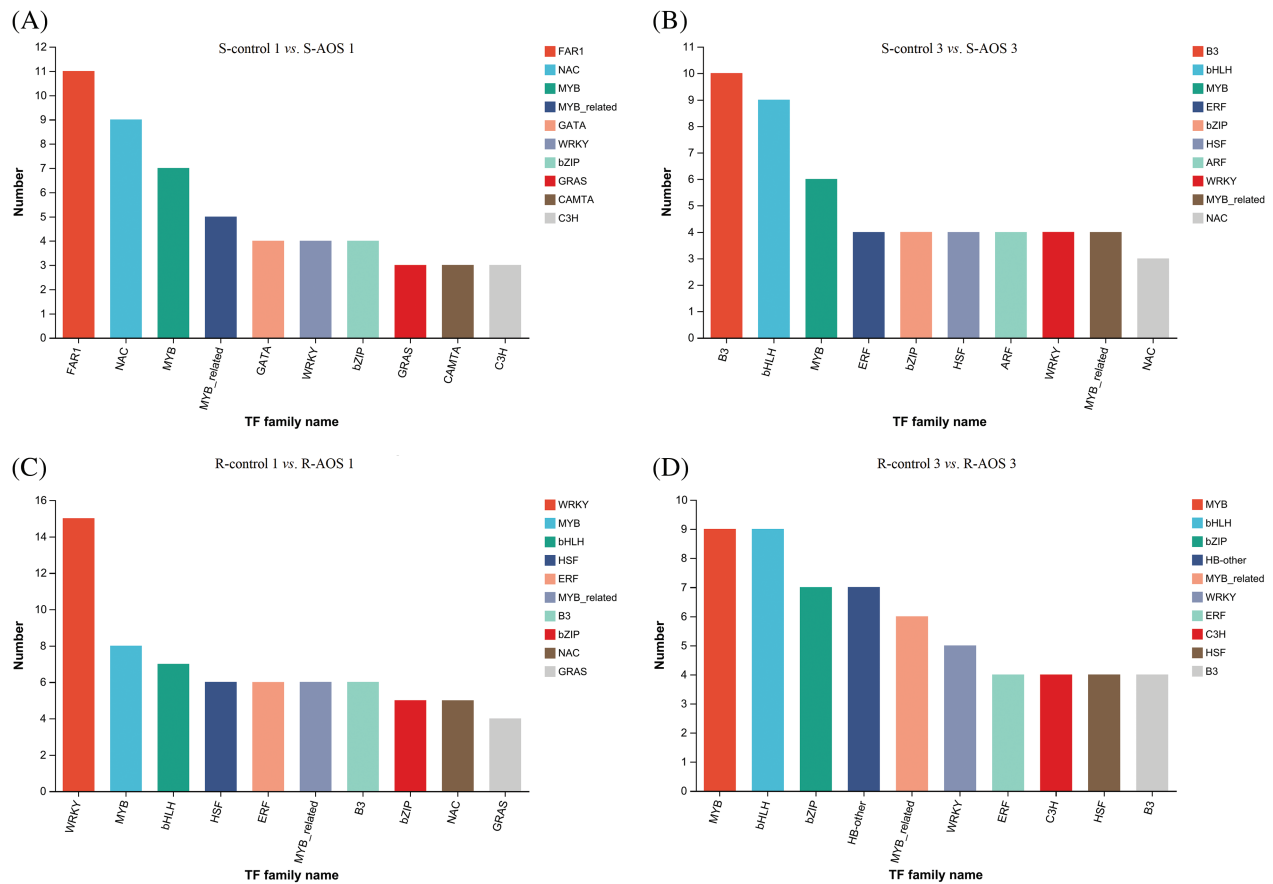


Figure 5: Top 10 transcription factor (TF) families of differentially expressed genes between pairwise comparisons of alginate oligosaccharides (AOS) groups to control groups under drought stress. (A) S-control 1 vs. S-AOS 1; (B) S-control 3 vs. S-AOS 3; (C) R-control 1 vs. R-AOS 1; (D) R-control 3 vs. R-AOS 3. 150 g L⁻¹ PEG-6000 was used to simulate drought stress. S-control 1 and S-control 3, wheat shoots of the control group under drought stress for 24 (1 d) and 72 h (3 d), respectively; S-AOS 1 and S-AOS 3, wheat shoots of the AOS treatment group under drought stress for 24 and 72 h respectively; R-control 1 and R-control 3, wheat roots of the control group under drought stress for 24 and 72 h, respectively; R-AOS 1 and R-AOS 3, wheat roots of the AOS group under drought stress for 24 and 72 h, respectively; AOS, alginate oligosaccharides; PEG-6000, polyethylene glycol-6000. TF: transcription factor

Then, after the MAPK signaling pathway was blocked by MAPK inhibitor PD 98059, the expression levels of ABA-responsive genes in wheat seedlings were again analyzed. The results showed that, at 24 and 48 h of treatment, the expression levels of *TaPYR1*, *TaPYR2*, *TaPYR3*, *TaERF1* and *TaNAC69* in wheat shoots under the PD 98059+PEG+AOS treatment were lower than those of the PD 98059+PEG treatment (Fig. 7), while the opposite trend was observed in wheat roots at 24 h. At 72 h of treatment, the expression levels of *TaPYR1*, *TaPYR2*, *TaPYR3*, and *TaNAC69* in wheat shoots under the PD 98059+PEG+AOS treatment were higher than those of the PD 98059+PEG treatment, while the opposite trend was observed in wheat roots. Additionally, the expression levels of *TaERF1* and *TaNAC69* in wheat root treated with PD 98059+PEG+AOS were also lower than those of the PD 98059 PEG treatment at 48 h

of treatment. These results further indicated that MAPK cascades were involved in AOS-induced drought resistance mediated by plant hormones in wheat, showing a time-effect relationship in different wheat tissues.

Table 6: Numbers of transcription factor (TF) genes differentially expressed between alginate oligosaccharides (AOS) groups and control groups under drought stress

TF family	S-control 1 vs. S-AOS 1	S-control 3 vs. S-AOS 3	R-control 1 vs. R-AOS 1	R-control 3 vs. R-AOS 3
AP2	1	0	4	1
ARF	2	4	1	2
B3	3	10	6	4
C2H2	2	0	0	0
3H	3	3	1	4
CAMTA	3	0	0	0
CPP	1	0	0	0
DBB	1	1	2	1
EIL	1	0	0	1
ERF	1	4	6	4
FAR1	11	2	0	2
GATA	4	1	3	1
GRAS	3	1	4	0
GRF	1	0	0	0
GeBP	1	0	0	0
HB-other	2	1	3	7
HSF	1	4	6	4
LSD	0	1	0	1
MIKC	2	1	0	0
MYB	7	6	8	9
MYB_related	5	4	6	6
M_type	2	1	0	0
NAC	9	3	5	1
SBP	0	2	2	1
NF-YA	2	0	0	0
Nin-like	1	0	2	0
S1Fa-like	1	0	0	0
TCP	0	2	1	1
WRKY	4	4	15	5

(Continued)

Table 6 (continued)				
TF family	S-control 1 vs. S-AOS 1	S-control 3 vs. S-AOS 3	R-control 1 vs. R-AOS 1	R-control 3 vs. R-AOS 3
bHLH	2	9	7	9
bZIP	4	4	5	7
Dof	0	0	0	1
HD-ZIP	0	0	0	2
LBD	0	0	1	3

(AS2/LOB)

Note: 150 g L⁻¹ PEG-6000 was used to simulate drought stress. S-control 1 and S-control 3, wheat shoots of the control group under drought stress for 24 (1 d) and 72 h (3 d), respectively; S-AOS 1 and S-AOS 3, wheat shoots of the AOS treatment group under drought stress for 24 and 72 h, respectively; R-control 1 and R-control 3, wheat roots of the control group under drought stress for 24 and 72 h, respectively; R-AOS 1 and R-AOS 3, wheat roots of the AOS group under drought stress for 24 and 72 h, respectively; AOS, alginate oligosaccharides; PEG-6000, polyethylene glycol-6000; TF, transcription factor.

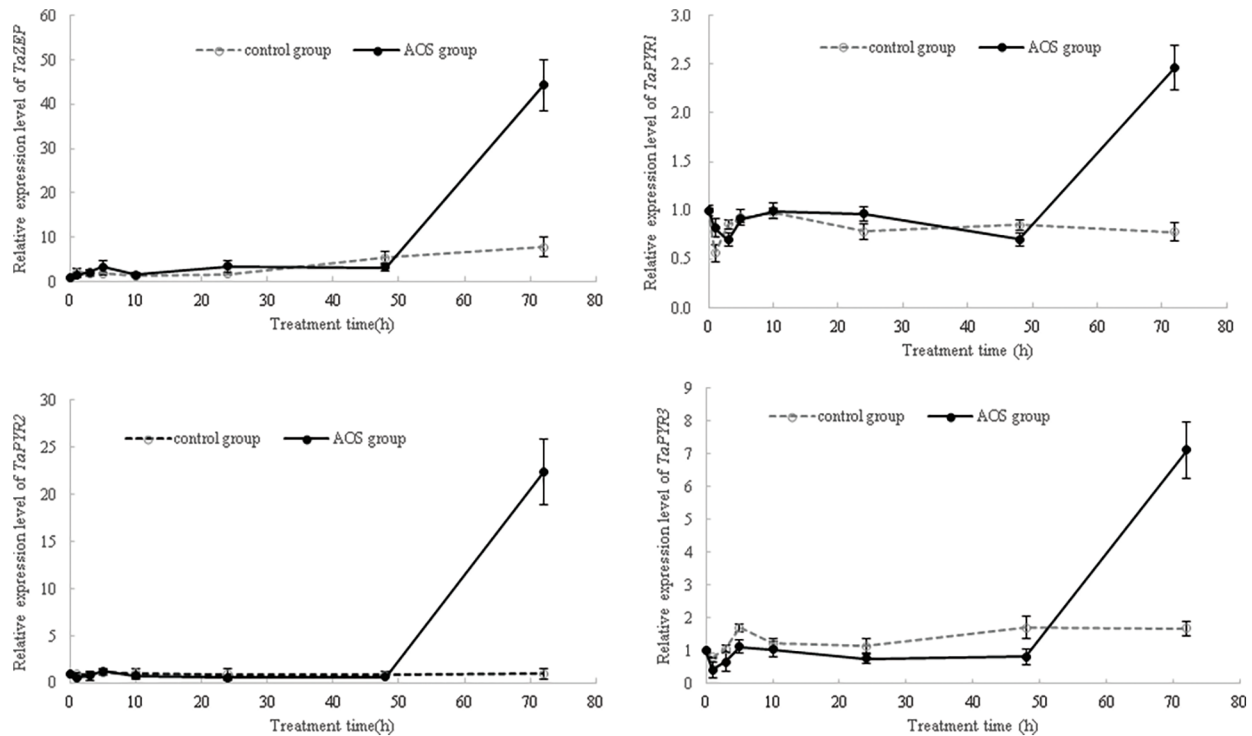


Figure 6: Effects of alginate oligosaccharides (AOS) on the expression of key genes involved in ABA signaling in wheat seedlings under drought stress. 150 g L⁻¹ PEG-6000 was used to simulate drought stress. Control group, wheat seedling sprayed with deionized water under drought stress for 72 h (3 d); AOS group, wheat seedlings sprayed with 1 g L⁻¹ AOS under drought stress for 72 h; AOS, alginate oligosaccharides

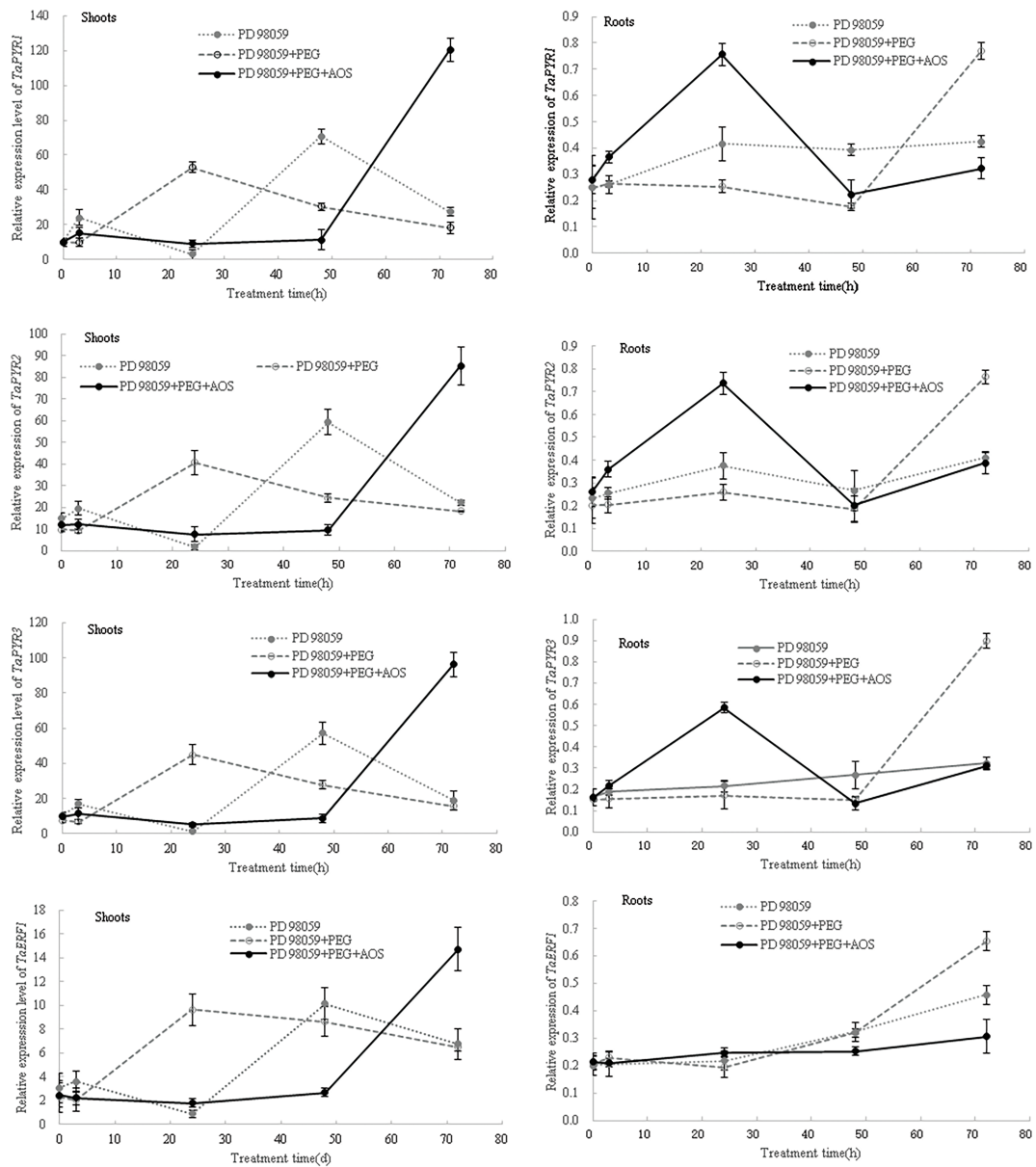


Figure 7: (Continued)

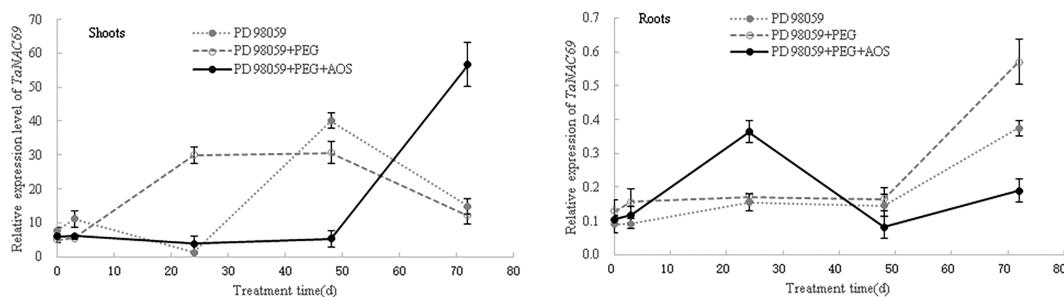


Figure 7: Effects of mitogen-activated protein kinases (MAPK) inhibitor PD 98059 on the expression of ABA-responsive genes induced by alginate oligosaccharides (AOS) in wheat seedlings under drought stress. 150 g L⁻¹ PEG-6000 was used to simulate drought stress. PD 98059 group, wheat seedlings sprayed with deionized water for 72 h (3 d) after being root -dipped in 50 μM PD 98059 for 6 h; PD98059+PEG group, wheat seedling sprayed with deionized water under drought stress for 72 h after being root-dipped in 50 μM PD 98059 for 6 h; PD 98059+PEG+AOS group, wheat seedlings sprayed with 1 g L⁻¹ AOS under drought stress for 72 h after being root-dipped in 50 μM PD 98059 for 6 h. AOS, alginate oligosaccharides; MAPK, mitogen-activated protein kinases; PD 98059, a specific inhibitor of the MAPK/extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathway, without significant inhibitory activity of MAPK itself; PEG-6000, polyethylene glycol-6000

4 Discussion

In this study, the wheat seedlings were treated with 1 g L⁻¹ AOS for 72 h, based on previous studies. The morphology of the wheat seedlings cultured was dramatically changed by AOS under drought stress, which was reflected in the increase of the length and FW of wheat shoots. Total Chl content and the activities of POD and SOD in leaves were also improved by AOS under drought stress, but the MDA content was reduced, compared with the only PEG treatment (Table 2). These results indicated that AOS reduced oxidative damage induced by drought stress, and therefore promoted the growth of wheat seedlings, in agreement with previous studies [15]. Similar results were also obtained for cucumber [18,19].

The fundamental means by which AOS affected wheat morphology and physiology under drought stress were by inducing gene changes at the molecular level. The transcriptome analysis of wheat seedlings reflected on the dynamic changes in AOS-induced genes and proteins directly. Compared with the control, a total of 10,064 (including 5,185 DEGs in shoots and 4,879 DEGs in roots) and 15,208 DEGs (including 4,827 DEGs in shoots and 10,381 DEGs in roots) in AOS-treated wheat seedlings were obtained at 24 and 72 h after dehydration, respectively (Figs. 2A–2D). GO and KEGG pathway enrichment analysis showed that these DEGs, which were annotated to BP (metabolic process, cellular processes, biological regulation and response to stimulus), CC (cell components, membrane components, organelle and membrane) and MF categories (binding, catalytic activity, transporter activity and transcription regulator activity), and were mainly involved in carbohydrate metabolism (starch and sucrose metabolism, carbon fixation in photosynthetic organisms, amino sugar and nucleotide sugar metabolism, glycolysis/gluconeogenesis, pyruvate metabolism, glyoxylate and dicarboxylate metabolism), secondary metabolites synthesis (phenylpropanoid biosynthesis, flavonoid biosynthesis) and lipid metabolism (fatty acid elongation, biosynthesis of unsaturated fatty acids, alpha-linolenic acid metabolism, cutin, suberine and wax biosynthesis), and signaling transduction (Table S1; Figs. 4A–4D). Further analysis showed that most DEGs in the main enriched KEGG pathways were up-regulated by AOS in wheat shoots (at 24 h after dehydration) and roots (at 72 h after dehydration). These key genes were related to chlorophyll a-b binding protein, amylosynthase, phosphotransferase, peroxidase,

phenylalanine ammonia lyase, flavone synthase, glutathione synthetase and so on. Signaling molecules, such as MAPK, plant hormones, H₂O₂ and calcium, were involved in the AOS -induced wheat drought resistance (Tables 4 and 5).

Photosynthesis is the primary metabolic sink for plant growth and the basis of crop yield formation; however, it is a complex metabolic process that includes light absorption, energy conversion, electron transfer, adenosine triphosphate synthesis, and carbon dioxide fixation [36]. In this study, most DEGs related to photosynthesis–antenna proteins (chlorophyll a-b binding protein) in wheat shoots (at 24 h after dehydration) and roots (at 72 h after dehydration) were up-regulated by AOS (Table 5). The key genes involved in starch and sucrose metabolism, including amylosynthase, phosphotransferase and trehalose-6-phosphate phosphatase, were also up-regulated in wheat roots at 72 h (Table 5). These results suggested that AOS enhanced the photosynthetic ability of wheat seedlings and accumulated more photosynthate under drought stress, which was confirmed by the increase in total Chl content and FW of wheat shoots (Table 2). Other findings for flowering Chinese cabbage showed that AOS raised the net photosynthetic rate, carboxylation efficiency of carbon dioxide and light saturation point in functional leaves, but reduced the light compensation point, resulting in increases in sucrose and starch accumulation and yield [37]. Leaf photosynthesis and above-ground growth of maize seedlings were improved by AOS under drought conditions, which was reflected in increases to the net photosynthetic rate, relative Chl content (SPAD value) and dry matter accumulation [38]. Similar results were also obtained for *Catharanthus roseus* L [39]. Our results therefore agreed with the findings of the previous studies at the transcriptional level.

The biomembrane system is the initial site of injury, particularly in terms of its structure, function, stability, and enzyme activity, resulting in substantial metabolic imbalance, which affects plant growth and development [40]. Phospholipids, as major components of the cell membrane, participate in many cellular biological processes by controlling and regulating cellular function [41]. Glycerolipid metabolism of plants responds to the changing environments, leading to the modification of membrane lipid composition to ensure optimal biochemical and physical properties [42]. In our study, most DEGs enriched in the biosynthesis of unsaturated fatty acids and alpha-linolenic acid metabolism were up-regulated (Fig. 4 and Tables 4, 5), suggesting that AOS altered the membrane lipid composition of wheat seedlings under drought stress. The results of flowering Chinese cabbage showed that AOS raised linoleic acid (C18:2) content while reduced the contents of palmitic acid (C16:1) and stearic acid (C18:0), resulting in an increase in the index of unsaturated fatty acid (IUFA) of thylakoid membrane lipids [43]. Furthermore, IUFA was positively correlated with photosystem II electron transport activity, affecting thylakoid membrane fluidity and membrane-bound enzyme activity [44]. Therefore, the AOS-induced changes in membrane lipid composition were beneficial for improving the photosynthetic capacity of wheat leaves under drought stress (Figs. 4A, 4B and Tables 2, 4). Moreover, cutin, suberine and wax, a family of complex biopolyesters composed of fatty acids and aromatic compounds, limited water loss through non-stomatal transpiration under drought stress, and their biosynthesis began with fatty acid synthesis and elongation [45,46]. Therefore, most DEGs enriched in fatty acid elongation and cutin, suberine and wax biosynthesis were up-regulated in wheat shoots (Fig. 4A and Tables 4, 5), helping to improve RWC and FW of wheat shoots under drought stress (Table 2).

Secondary metabolites of plants perform a vital role in response to drought stress. For example, phenylpropanoid metabolism yields more than 8,000 metabolites (including flavonoids, hydroxycinnamic acid esters, hydroxycinnamic acid amides, and the precursors of lignin and tannins) contributing to plant development and plant–environment interplay [47]. Among them, flavonoids function as antioxidants that reduce the oxidative damage caused by ROS accumulation under abiotic stresses such as drought [48]. In our study, most DEGs involved in phenylpropanoid biosynthesis and flavonoid biosynthesis were up-regulated, mainly including peroxidase, phenylalanine ammonia lyase, β-glucosidase, 4-coumarate:

coenzyme A ligase, phenylalanine deaminase, 4- α -glucanotransferase, glycosyltransferase and flavone synthase in wheat shoots at 24 h after dehydration (Tables 4 and 5), which was beneficial for alleviating the oxidative damage caused by drought stress, and improving the growth of wheat seedlings. Additionally, glutathione, a small intracellular thiol molecule considered to be a strong non-enzymatic antioxidant, plays an important role in antioxidant defense in plants [49]. Most DEGs involved in glutathione metabolism were up-regulated, mainly including glutathione synthetase, ascorbate peroxidase and glutathione-S-transferase in wheat roots at 72 h after dehydration (Tables 4 and 5), helping to reduce the oxidative damage caused by drought, which was also confirmed by the decrease of the MDA content in AOS-treated wheat leaves under drought stress (Table 2).

ABA acts as a major signaling molecule in response to drought, by inducing antioxidant defense and regulating stomatal closure, in conjunction with ROS, Ca²⁺, and sugars [25,26,50,51]. MAPK cascades also played important roles in transducing signals initiated by diverse internal and external cues involving in growth, development, and responses to biotic and abiotic stresses [52]. Our results confirmed that signaling molecules, such as MAPK, plant hormones, H₂O₂ and calcium, were involved in the AOS-induced drought resistance in wheat (Fig. 4 and Tables 4, 5). AOS induced ABA synthesis and signaling in wheat responding to drought stress, by up-regulating the expression levels of *TaZEP*, *TaPYR1*, *TaPYR2* and *TaPYR3* (Fig. 6). However, after blocking the MAPK signaling pathway, the ABA-responsive genes were inhibited first and then induced in wheat shoots treated with AOS under drought stress, while the opposite trend was observed in wheat roots (Fig. 7), which further indicated that MAPK cascades were involved in AOS-induced drought resistance mediated by ABA in wheat, showing a time-effect relationship in different wheat tissues. These results were basically consistent with the results of transcriptome data; however, the detailed signal transduction mechanisms still need further investigation.

Among all the stress-resistance systems in plants, many TFs related to plant tolerance to stresses, including *bZIP*, *NAC*, *AP2*, *WRKY*, *PHD*, *DREB*, *ERF* and *MYB* families, have been identified as playing important roles in abiotic stress by binding specific cis-acting elements to form a complex regulatory network [32,53,54]. In our study, differentially expressed TF genes that regulated plant stress response mainly include the *NAC*, *MYB*, *MYB-related*, *WRKY*, *bZIP*, *bHLH* families (Table 5). *MYB* and *bHLH* TFs played a central regulatory role in phenylpropanoid metabolism, especially the regulation of the structural genes of lignin and flavonoid biosynthesis [54,55]. *TaNAC5D-2*, *TaNAC48* and *TaNAC69* acted in the ABA-mediated transcriptional regulation in response to drought stress in wheat [32,53]. The expression levels of *TaNAC2*, *TabZIP15* and *TaSIM* were negatively correlated with MDA content, but positively correlated with RWC under drought stress [56]. *TaERF1* induced by exogenous hormones (ABA, ethylene, auxin and salicylic acid) increased multiple stresses tolerance including drought [57]. AOS changed the expression profile of the above TFs genes in our study (Table 5 and Fig. 7), which may regulate the morphology and physiology of wheat seedlings in adapting to drought stress. However, not much is known about the complex molecular network that brings about drought resistance induced by AOS, thus it is necessary to study the interactions of the different signaling pathways induced by AOS, for example using the gene chip and laser scanning confocal microscope technique in a follow-up study.

5 Conclusion

In summary, physiological and transcriptome analysis preliminarily confirmed that AOS reduced oxidative damage caused by drought stress, and thereby promoted the growth of wheat seedlings. This appeared to be a cumulative outcome of DEGs mainly involved in the biosynthesis of secondary metabolites, carbohydrate metabolism, lipid metabolism, amino acid metabolism, and signaling transduction. Signaling molecules (MAPK, plant hormones, H₂O₂ and calcium) and transcription factors (*NAC*, *MYB*, *MYB-related*, *WRKY*, *bZIP* family members) were involved in the AOS-induced drought resistance in wheat. These results help us to understand the mechanisms of the improved drought

resistance by AOS at the transcriptome level in wheat, and provide a theoretical basis for the exploitation and application of AOS as a type of environmentally sustainable biological agent for improving the drought resistance of agricultural crops.

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