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Maize 4-coumarate coenzyme A ligase *Zm4CL-like9* gene positively regulates drought stress response in *Arabidopsis thaliana*

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ABSTRACT

Maize is a major food crop in China, and drought is one of the major abiotic stresses that threaten the growth and development of the crop, seriously affecting the crop yield. 4-coumaric acid coenzyme A ligase (*4CL*) is a key enzyme in the phenylpropane metabolic pathway, which can regulate the lignin content of the plant and play an important role in the plant's resistance to drought stress, plays an important role in plant resistance to drought stress. In the present study, we screened the differentially expressed up-regulated gene *Zm4CL-like9* under drought stress by pre-transcriptome sequencing data (PRJNA793522) in the laboratory, and analyzed the significant up-regulation of *Zm4CL-like9* gene in roots under drought stress by qRT-PCR(Real-Time Quantitative Reverse Transcription PCR). The results of prokaryotic expression experiments showed that the protein encoded by the *Zm4CL-like9* gene was able to be expressed in prokaryotic cells and could effectively improve the drought tolerance of E. coli. Phenotypic analysis of transgenic *Arabidopsis* plants under drought stress revealed that seed germination rate, root length, and plant survival after drought rehydration were significantly higher in transgenic *Zm4CL-like9 Arabidopsis* compared with wild-type *Arabidopsis*; physiological and biochemical indexes revealed that peroxidase activity, proline (Pro) content, and chlorophyll content were significantly higher in transgenic *Arabidopsis* compared with wild-type *Arabidopsis*. Under drought stress, the expression of drought-related genes was significantly up-regulated in transgenic *Arabidopsis* compared with wild-type *Arabidopsis*. Taken together, the *Zm4CL-like9* gene enhances plant resistance to drought stress by reducing reactive oxygen species accumulation in plants.

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4-coumarate coenzyme A ligase; *Arabidopsis thaliana*; drought tolerance; phenylpropane pathway

1. Introduction

As the world's number one crop in terms of production, maize is grown by people all over the world. However, plants are subjected to many abiotic stresses during growth, and abiotic stresses can have a huge impact on plant yield, and maize is no exception.[1]

Abiotic stresses mainly include drought, salinity, high and low temperatures, heavy metals and mechanical damage. It has been estimated that abiotic stresses can cause annual crop yield losses of up to 60%.[2] Of these, drought stress has a severe impact on maize growth and development, leading to lower yields and reduced quality. In recent years, the frequency and intensity of droughts have increased dramatically due to the reduction in global precipitation caused by extreme weather events.[2] Under drought conditions, the balance of reactive oxygen species (ROS) that maintain normal metabolism is disrupted in the plant, and the excess ROS cause damage to the plant because they are not properly removed.

Elevated ROS levels will lead to metabolic disorders in the plant, and further cause membrane peroxidation, electrolyte imbalance and other phenomena. In order to balance the level of reactive oxygen species in the metabolism, plants will increase the metabolism by increasing the activity of relevant metabolic enzymes, hormone content and the level of expression of relevant genes. For example, reactive oxygen radicals (O_2^-) and H_2O_2 are converted to H_2O and O_2 in plants by increasing the activities of superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD), which in turn play a role in scavenging reactive oxygen

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species (ROS).[3] Overexpression of the desert shrub SpABR1 gene in *Arabidopsis thaliana* promotes ROS scavenging through the ABA pathway, which in turn enhances ROS scavenging in transgenic *Arabidopsis thaliana*. which in turn enhances the drought resistance of transgenic *Arabidopsis*.[4] Meanwhile, the occurrence of drought stress reduces the utilization of water and minerals by the plant root system, leading to water loss and crumpling of plant cells, and ultimately wilting the plant, which seriously affects the growth and development of the plant. In addition, under drought conditions, plants close their stomata out of self-regulatory mechanisms, which in turn reduces the intensity of photosynthesis, leading to slow growth, reduced nutrient accumulation, and severe quality damage. If rice is subjected to drought during the reproductive period, its respiration and photosynthesis are affected by the opening and closing of stomata, which in turn can lead to the consequences of poor pollen development and reduced fruit set.[5] Therefore, as one of the abiotic stresses, drought stress has gradually become one of the factors that seriously affect the growth and development of maize. However, there is a lack of drought-tolerant maize inbred varieties in China, therefore, it is of great significance to improve the original varieties of maize at the molecular level through transgenic technology, and to create and cultivate new germplasm of drought-tolerant maize to safeguard the yield and quality of maize in China.

The 4-Coumaric acid coenzyme A ligase (4CL) family is a family of proteins that are widely present in plants and have a variety of functions. Depending on the monocotyledonous and dicotyledonous plants, the *4CL* family of genes can be classified into different types. In dicotyledons, the *4CL* genes are classified into *types I and II*, and in monocotyledons, the *4CL* genes are classified into *types III* and *IV*. *Type I* is responsible for the synthesis of lignin monomers, *type II* is responsible for the production of phytoflavonoids, *type III* regulates the synthesis of lignin, and *type IV* regulates the synthesis of lignin.[6] The *4CLs* genes are associated with the synthesis of phytoalexins and regulate downstream synthesis via the phenylpropane pathway, which is involved in lignin synthesis. The phenylpropane pathway regulates downstream synthesis, which in turn affects biotic and abiotic stresses.[7] Lignin is an important component of the plant cell wall, which enhances the hardness of the cell wall, and plays an important role in plant growth and development, physiological metabolism, and resistance to biotic and abiotic stresses, e.g., the accumulation of lignin promotes drought resistance in peonies, rice, and other plants.[8,9] In addition, the *4CL* genes may have different stress-resistant functions in different species, such as water plants. In addition, *4CL* genes may have different antistress functions in different species, for example, salicylic acid induces an increase in the expression of *4CL* genes in potato and regulates the synthesis of chlorogenic acid (CGA), which in turn improves potato resistance to pests and diseases;[10] the *OsAAE3* gene encoding a 4CL-like protein in rice negatively regulates the resistance of rice to rice blast by decreasing the activity of peroxidase;[11] and the use of viral-induced gene silencing in *Arabidopsis* and viral-induced gene silencing to negatively regulate water stress is not possible in *Arabidopsis*, but rather in *Arabidopsis*. in *Arabidopsis thaliana* to negatively regulate rice resistance to rice blast.[12] Virus-induced gene silencing and overexpression of the *Gh4CL7* gene in *Arabidopsis thaliana* have been shown to be associated with lignin synthesis and increased drought resistance in transgenic cotton.[13] All these studies suggest that the *4CL* family of genes indirectly influence the tolerance and resistance of different plants to biotic and abiotic stresses by affecting flavonoid and lignin content in potato, rice, cotton and other plants.

In this paper, a *4CL* gene, *Zm4CL-like9*, was screened by pre-laboratory maize drought tolerance transcriptome data (PRJNA793522). In this study, we constructed plant overexpression vectors and transformed them into *Arabidopsis thaliana*, performed phenotypic identification and physiological and biochemical indexes of transgenic *Arabidopsis thaliana*, and detected the expression of drought-resistant related genes, so as to clarify the response of Zm4CL-like9 to drought stress, and preliminarily explored the biological function and drought-regulating mechanism of Zm4CL-like9, which laid the foundation for the cultivation of drought-resistant germplasm for maize, and

was helpful for the improvement of drought-resistant maize. This will lay the foundation for cultivating new drought-resistant germ plasm of maize, which is of great significance for improving the drought resistance of maize.

2. Materials and Methods

2.1. Plant Material and Growing Conditions

Maize was grown in an artificial culture room at 25°C, 16 h light, 8 h dark, 70% relative humidity, and 450 μmol/(m^2 -s) light intensity, using the inbred line H8186 as the material. When the seedlings grew to the three-leaf stage, they were placed in 10% PEG6000 solution to simulate drought stress treatment. Maize root tissue parts were collected at 0, 2, 4, 8, 12, 24, and 48 h, respectively, frozen in liquid nitrogen, and stored in a −80°C refrigerator for subsequent RNA extraction experiments.

The seeds were sterilized with 75% ethanol and 1% sodium hypochlorite, and then rinsed 3–4 times with sterile water. The sterilized seeds were spotted into 1/2 MS medium and incubated at 4°C in the dark for 72 h. Then they were incubated at 22°C, 16 h of light and 8 h of darkness for 7 d. Finally, the grown *Arabidopsis* seedlings were transferred to mixed soil (normal soil: vermiculite = 3:8) and watered and irrigated for the subsequent experiments.

2.2. RNA Extraction and qRT-PCR Analysis

In this study, the differentially expressed up-regulated gene *Zm4CL-like9* (Zm00001d003702) was screened for drought stress based on the pre-transcriptome sequencing data (PRJNA793522) in the laboratory. *The Zm4CL-like9* gene was cloned from the maize inbred line "H8186." The CDS sequence of this gene is 1692 bp in length, encoding 610 amino acids with a relative molecular weight of 65.7 kDa.

Total RNA was extracted from the maize root samples using the Trizol method. qRT-PCR (Real-Time Quantitative Reverse Transcription PCR) was performed using the TransScript® Uni All-in-One First-Strand cDNA Synthesis SuperMix for qPCR (Quantitative Real-time PCR) Reverse Transcription Kit to reverse- transcribe the RNA into first-strand cDNA. the reaction program was 94°C 30s, 94°C 5s, 60°C 30s, 45 cycles. Expression calculation was analyzed by the $2^{-\Delta\Delta CT}$ method.[14] Primer 5.0 was used to design the qRT-PCR primer qZm*4CL*-like9-F/R, and the primer sequences are shown in Table 1. *The ZmActin1* gene was used as the internal reference gene in this study. Three replicates were performed for each sample.

2.3. Analysis of Prokaryotic Expression of the Zm4cl-Like9 Gene

In this study we cloned the full-length CDS sequence of maize *Zm4CL-like9* gene. The CDS

Table 1. Primer sequences.

Primers Name	Primer sequence(5′—3′)
Zm*4CL*-like9-F	ATGGGCGACGCGGCTATTGCCGTCG
Zm*4CL*-like9-R	CTAGGCTGCCGCCGTTTTGGTCCTG
3301-Zm*4CL*-like9-F	ACTCTTGACCATGGTAGATCTCACTTCCACCAATTCACTAC
3301-Zm*4CL*-like9-R	GGGGAAATTCGAGCTGGTCACCTGCAATGTAAGTATAACT
22b-Zm*4CL*-like9-F	TCGAGCTCCGTCGACAAGCTTCACTTCCACCAATTCACTAC
22b-Zm*4CL*-like9-R	GTGGTGGTGGTGGTGCTCGAGGGCTGCCGCCGTTTTGGTCCTG
qZm*4CL*-like9-F	ACTACAGGAAGAAGGAGGAGACGG
qZm*4CL*-like9-R	ACGGGTGGGACAGGAGAATG
qZmActin1-F	AAAGGTTTAGGTGCCCCGAG
qZmActin1-R	AGATCCCCCACTGAGGACAA
qAtActin1-F	GGCTCCTCTTAACCCAAAGG
qAtActin1-R	CCCTCGTAGATTGGCACAGT
qAtDREB2A-F	TGACCTAAATGGCGACGATGT
qAtDREB2A-R	TCCAAGTAACTCAAGTCGTCG
qAtRD26-F	GAAGGTGAGGCGGAGAGTG
qAtRD26-R	CCCGAAACTCTGAGTCAACCT
qAtRD29A-F	CTTGATGGTCAACGGAAGGT
qAtRD29A-R	CAATCTCCGGTACTCCTCCA
qAtRD29B-F	AGAAGGAATGGTGGGGAAAG
qAtRD29B-R	CAACTCACTTCCGGAAT

region of *Zm4CL-like9* gene (Zm00001d003702) was inserted into pET-22b prokaryotic expression vector by seamless cloning method, and the recombinant vector plasmid was transferred into Escherichia coli strain BL21 (DE3), and the positive recombinant plasmid was added with 0.1 mm isopropyl β- d -thiogalactopy- ranoside (IPTG) for induced expression of proteins, and the bacterial liquid was collected for sampling at 1 h intervals when the concentration of bacterial liquid reached the $OD_{600} = 0.8$. The bacterial fluid was collected for sampling.

2.4. Analysis of Drought Resistance of Zm4cl-Like9 Gene in Escherichia coli BL21

E. coli bacterial fluids containing vector pET-22b and recombinant vector pET-22b-Zm*4CL*-like9 were subjected to activation culture on LB solid medium containing ampicillin. The activated bacterial fluids were cultured overnight in 5 mL of LB liquid medium containing 5 μL of ampicillin. The bacterial fluids were incubated in LB+Amp liquid medium as well as LB+Amp +300 mm mannitol liquid medium, and the bacterial fluids were induced by adding 1.0 mm IPTG. The OD_{600} value of the bacterial solution was measured every 2 h and the growth curve was plotted, and this experiment was repeated three times.

2.5. Overexpression Vector Construction and Genetic Transformation of Transgenic Arabidopsis thaliana

The complete CDS sequence of the *Zm4CL-like9* gene was amplified using primers *Zm4CL-like9-F/R* (Table 1) with cDNA as template. Enzymatic cleavage sites BglII and BstEII were selected on the overexpression vector pCAMBIA3301. The recombinant vector *pCAMBIA3301-Zm4CL-like9* was constructed by double cleavage of the overexpression vector pCAMBIA3301 and insertion of *the Zm4CL-like9* gene into the polyclonal site region of pCAMBIA3301 vector. The sequences of the seamless cloning primers 3301-Zm*4CL*-like9-F/R, which were required for the construction of the overexpression vector, are shown in Table 1. 1. Subsequently, the recombinant vector plasmid was transferred into Agrobacterium tumefaciens EHA105 receptor cells. Genetic transformation of *Arabidopsis* was performed by Agrobacterium-mediated method. Positive plants were screened by Basta solution at 0.1% concentration, and finally eight transgenic *Arabidopsis* lines were obtained in the T_3 generation. The transgenic *Arabidopsis* lines were analyzed for the expression of the *Zm4CL-like9* gene, and the sequences of the primers used, qZm*4CL*-like9-F/R, are shown in Table 1, and the three transgenic *Arabidopsis* lines with the highest expression, OE-6, OE-7, and OE-8, were selected for the subsequent phenotypic experiments.[15]

2.6. Phenotypic Analysis of Maize Zm4cl-Like9 Gene for Drought Tolerance in Transgenic Arabidopsis thaliana

In this study, wild-type *Arabidopsis thaliana* and transgenic *Arabidopsis thaliana* seeds were inoculated on 1/2 MS medium containing different concentrations of mannitol (0, 100, 200, and 300 mm), and incubated at 4°C for 3 d. Subsequently, they were placed in an artificial climate chamber for both flat and vertical cultures, and the germination rate of *Arabidopsis thaliana* in flat culture was counted after 14 d of incubation, and the root length of *Arabidopsis thaliana* in vertical culture was examined. The root length of *Arabidopsis thaliana* in vertical culture was examined. The experiments were repeated three times.

The vernalized wild-type *Arabidopsis* as well as the transgenic *Arabidopsis* were cultured in pots, and the *Arabidopsis* plants were subjected to a natural drought treatment for 21 d when the seedlings reached the age of four leaves and the soil moisture content was about 30%, followed by rehydration for 5 d. The survival of *Arabidopsis*was observed.

2.7. Staining Experiments with P-Nitroblue Tetrazolium Chloride (NBT) and 3,3′-Diaminobenzidine (DAB)

In this study, drought simulation experiments were carried out on wild-type *Arabidopsis thaliana* (WT) as well as transgenic *Arabidopsis thaliana* (OE-6, OE-7, and OE-8) using 0% and 10% PEG 6000 solutions. The accumulation of reactive

oxygen species in plants under drought stress was detected by p-nitroblue tetrazolium chloride (NBT) assay and 3,3'-diaminobenzidine (DAB) assay, respectively. Leaves of *Arabidopsis thaliana* plants of different lines were immersed in NBT and DAB staining solution for 16 h. The chlorophyll in the leaves was discolored with 95% ethanol in a water bath at 80°C.[15]

2.8. Transgenic Arabidopsis Thaliana Plants O_2^-, H_2O_2 and Physiological and Biochemical Indexes Detection

The Boxbio bio-engineering kit was used (https://www.boxbio.cn/.) for the measurement of O_2^-, H_2O_2, malondialdehyde (MDA), proline (Pro), chlorophyll content, and SOD, POD, and CAT antioxidant enzyme activities.

2.9. Expression Analysis of Drought Tolerance Related Genes Under Drought Stress

The *DREB2A*(Dehydration-responsive element-binding protein 2A), *RD26*(NAC transcription factor Rd26 gene), *RD29A*(Responsive to Dehydration 29A), and *RD29B*(Responsive to Dehydration 29B) genes are known to be involved in stress defense responses and are often used as stress marker genes[16–19] Wild-type *Arabidopsis thaliana* (WT) and transgenic *Arabidopsis thaliana* (OE-6, OE-7, OE-8) were planted in soil and subjected to natural drought stress for 21 d at a soil moisture content of about 30%. *Arabidopsis thaliana* rosette leaves were taken before and after the stress treatment, and total RNA was extracted from the leaves using the Trizol method, and was reverse transcribed into cDNA for qRT-PCR. The marker gene-specific primers qZmDREB2A-F/R, qZmRD26-F/R, qZmRD29A-F/R, and qZmRD29B-F/R were used for qRT-PCR analysis, and the expression of four drought-related genes was calculated using *AtActin* as the internal reference gene, and the sequences of the primers are shown in Table 1.

2.10. Statistical Analysis

Differences between data were determined by analysis of variance using one-way ANOVA with SPSS (SPSS Inc., Chicago, IL, USA). Significant differences were tested at $p < .05$ (*) or $p < .01$ (**).

3. Results and Analyses

3.1. Expression Analysis of the Zm4cl-Like9 Gene

The relative expression of *Zm4CL-like9* gene was analyzed in maize roots under drought stress, as shown in Figure 1. The results showed that *the Zm4CL-like9* gene responded significantly to drought stress, with the highest expression at 4 h, and showed a trend of increasing and then decreasing with the increase of drought stress time. Therefore, this study speculates that *Zm4CL-like9* gene plays an important role in resisting drought stress.

3.2. Expression Analysis of the Zm4cl-Like9 Gene in Prokaryotic Systems

According to the previous operation Finally we examined the recombinant protein expression by sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE), as shown in Figure 2a. The results showed that a single protein band appeared at the position of 63kDa ~75kDa, which was consistent with the predicted relative molecular weight size of Zm*4CL*-like9 protein, indicating that the pET-22b-Zm*4CL*-like9 recombinant protein could be successfully expressed in prokaryotic cells.

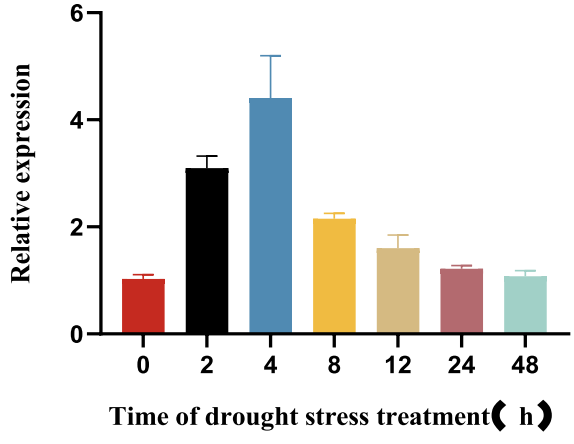

Figure 1. Relative expression analysis of *Zm4CL-like9* gene in maize roots under drought stress conditions.

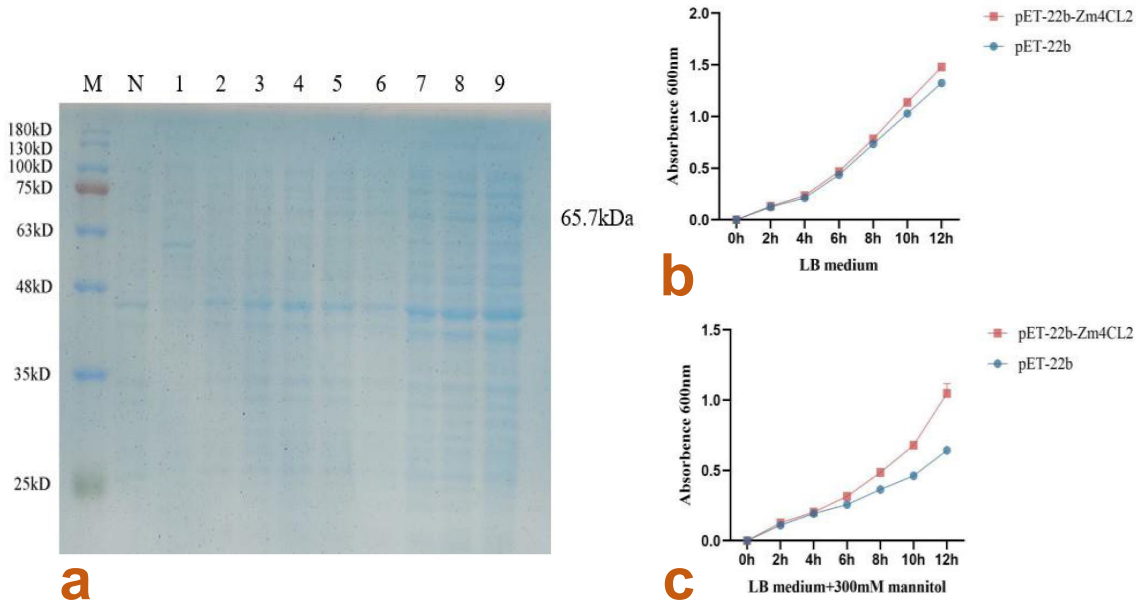

Figure 2. Expression analysis of the *Zm4CL-like9* gene in the prokaryotic system and its growth curve in e.coli. a: Analysis of *Zm4CL-like9*gene expression in the prokaryotic system (M: color pre-stained protein Marker, P6110M; N: negative control; 1-9. corresponding time (h) sample). b: Growth curve of E. coli with *Zm4CL-like9*gene when using LB + Amp liquid medium. c: Growth curve of E. coli with *Zm4CL-like9*gene when using liquid medium with LB + Amp +300 mm mannitol.

3.3. Drought Tolerance Analysis of the Zm4cl-Like9 Gene in Escherichia coli

The pET-22b-Zm*4CL*-like9 recombinant vector bacteriophage as well as pET-22b empty vector bacteriophage were cultured in LB+Amp liquid medium as well as LB+Amp +300 mm mannitol liquid medium, respectively. Under the induction of 1.0 mm IPTG, the OD_{600} value of the bacterial solution was recorded every 2 h and plotted as a growth curve. The results were shown in Figure 2b,c. In the LB liquid medium with 300 mm mannitol added, the inhibition of the growth of BL21 (pET-22b- Zm*4CL*-like9) bacterial fluids was significantly lower than that of BL21 (pET-22b) bacterial fluids, thus, indicating that, the expression of *Zm4CL-like9* could effectively improve the drought tolerance of E. coli.

3.4. Zm4cl-Like9 Gene Enhances Drought Tolerance in Arabidopsis thaliana

In this study, we obtained eight *Arabidopsis thaliana* lines transgenic *for the Zm4CL-like9* gene in the T_3 generation. The expression of *Zm4CL-like9* gene was measured in transgenic *Arabidopsis thaliana* grown to the three-leaf stage, and three transgenic *Arabidopsis thaliana* plants (OE-6, OE-7, and OE-8) with high expression were selected for subsequent drought response experiments, as shown in Figure 3.

After that, four 1/2 MS media containing different concentration gradients of mannitol (0 mm, 100 mm, 200 mm, and 300 mm) were set up, and the wild-type *Arabidopsis* seeds and transgenic strain seeds were inoculated on the four different concentrations of the media, respectively. The results are shown in Figure 4a,b, where we observed that there was no significant difference

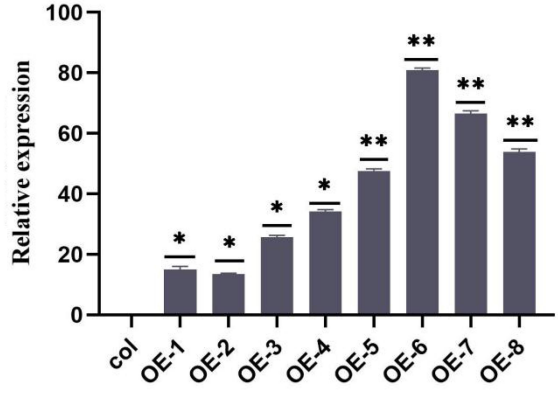

Figure 3. Relative expression levels of *Zm4CL-like9* gene in transgenic *Arabidopsis* from T_3 generation.

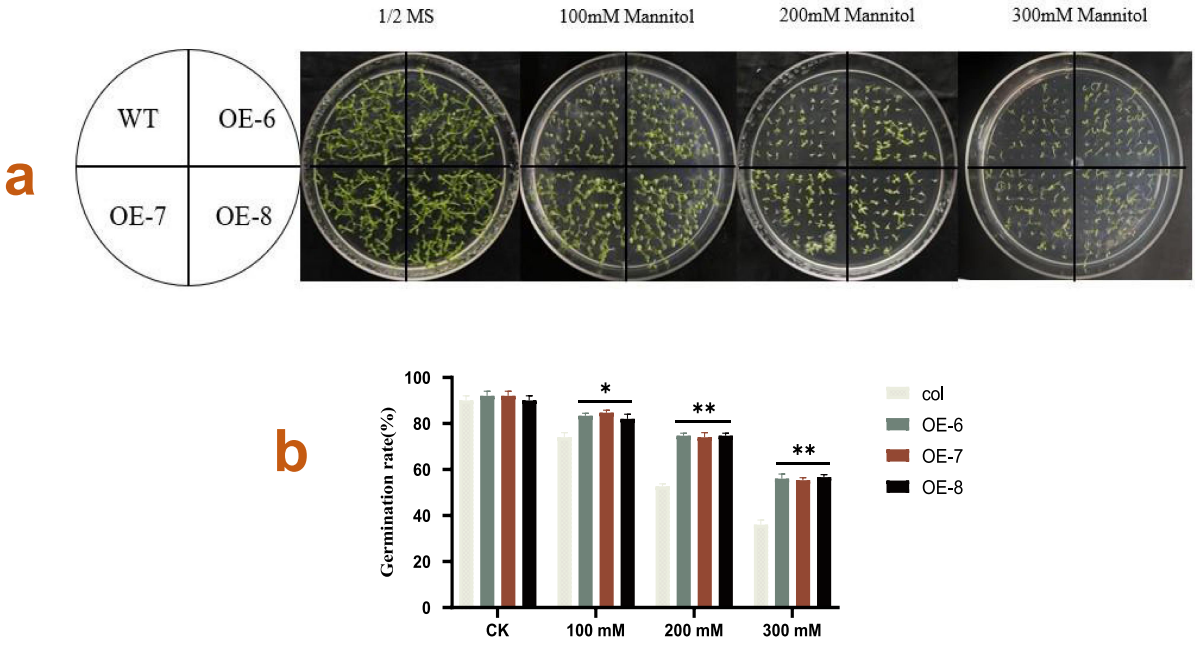

Figure 4. T_3 generation of transgenic *Arabidopsis* on 1/2 MS medium containing different concentrations of mannitol growth status and germination rate growth status and germination rate. a: Growth of T_3 generation transgenic *Arabidopsis* on 1/2 MS medium containing different concentrations of mannitol. b: germination rate statistics of T_3 transgenic *Arabidopsis* on 1/2 MS medium containing different concentrations of mannitol.

in the germination rates of wild-type *Arabidopsis* and transgenic *Arabidopsis* seeds on 1/2 MS medium without mannitol. At 100 mm, 200 mm, and 300 mm of 1/2 MS medium, the growth of transgenic *Arabidopsis* seeds and the germination rate were significantly better than those of wild-type *Arabidopsis* seeds.

In order to further observe the growth of root length, the seeds of wild-type *Arabidopsis* and transgenic *Arabidopsis* were still sown onto four types of 1/2 MS medium containing different concentration gradients of mannitol (0 mm, 100 mm, 200 mm, and 300 mm), and then placed in the vertical incubation under light for 10 d to observe the growth. The results, as shown in Figure 5a,b, showed that there was no significant difference between the root growth of transgenic *Arabidopsis* and wild-type *Arabidopsis* on 1/2 MS medium

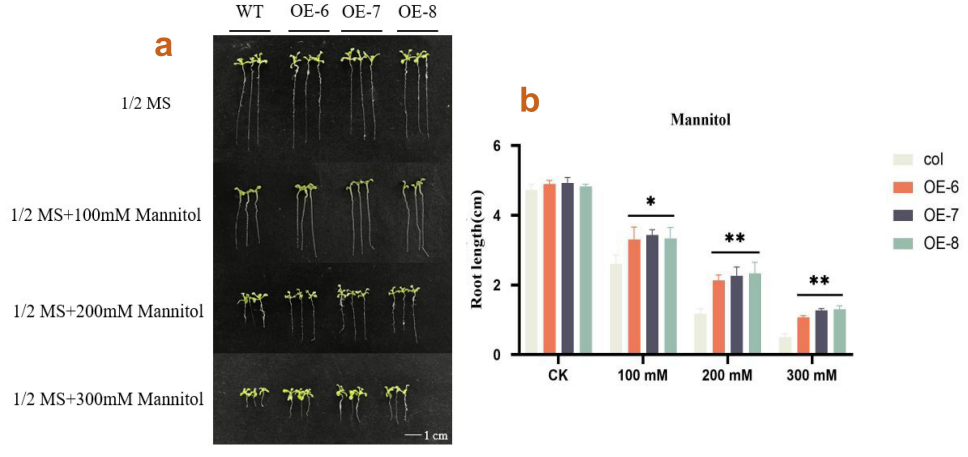

Figure 5. Root growth and root length statistics of T_3 generation transgenic *Arabidopsis* on 1/2 MS media containing different concentrations of mannitol. a: Root growth of the T_3 generation of transgenic *Arabidopsis* plants on 1/2 MS medium containing different concentrations of mannitol. b: Root length statistics of T_3 transgenic *Arabidopsis* on 1/2 MS medium containing different concentrations of mannitol.

without mannitol. On 1/2 MS medium containing 100, 200, and 300 mm, the root lengths of the three transgenic *Arabidopsis* strains were significantly longer than those of wild-type *Arabidopsis*.

To further verify the response of the *Zm4CL-like9* gene to drought stress, we performed drought rehydration experiments on wild-type *Arabidopsis* and transgenic *Arabidopsis*. Under the same environment, wild-type *Arabidopsis* and transgenic *Arabidopsis* were grown in pots and water supply was stopped at the same time for natural drought stress, and water supply was resumed after 10 days, and the growth of *Arabidopsis* was observed after 3 days of rewatering. The results were shown in Figure 6a,b, and the survival rate of transgenic *Arabidopsis* was significantly higher than that of wild-type *Arabidopsis*. Therefore, the expression of *Zm4CL-like9* gene in *Arabidopsis* enhanced the ability of plants to resist drought stress.

3.5. *Expression of the* Zm4cl-Like9 *Gene in* Arabidopsis Thaliana *Increases Plant Sensitivity to Drought*

When plants are subjected to adversity stress, a large amount of reactive oxygen species (ROS) will be generated, which will break the original reactive oxygen species balance in the plant body and cause damage to plant cell membrane lipids.[20–22] In order to avoid the damage caused by the high concentration of reactive oxygen species, the plant will increase the enzymatic reaction system to remove the excessive reactive oxygen species, thus protecting the plant and reducing the damage.[21] Therefore, we can determine the resistance of plants to abiotic stresses by detecting the reactive oxygen species (ROS) content in plants. Meanwhile, we learned that nitrogen blue

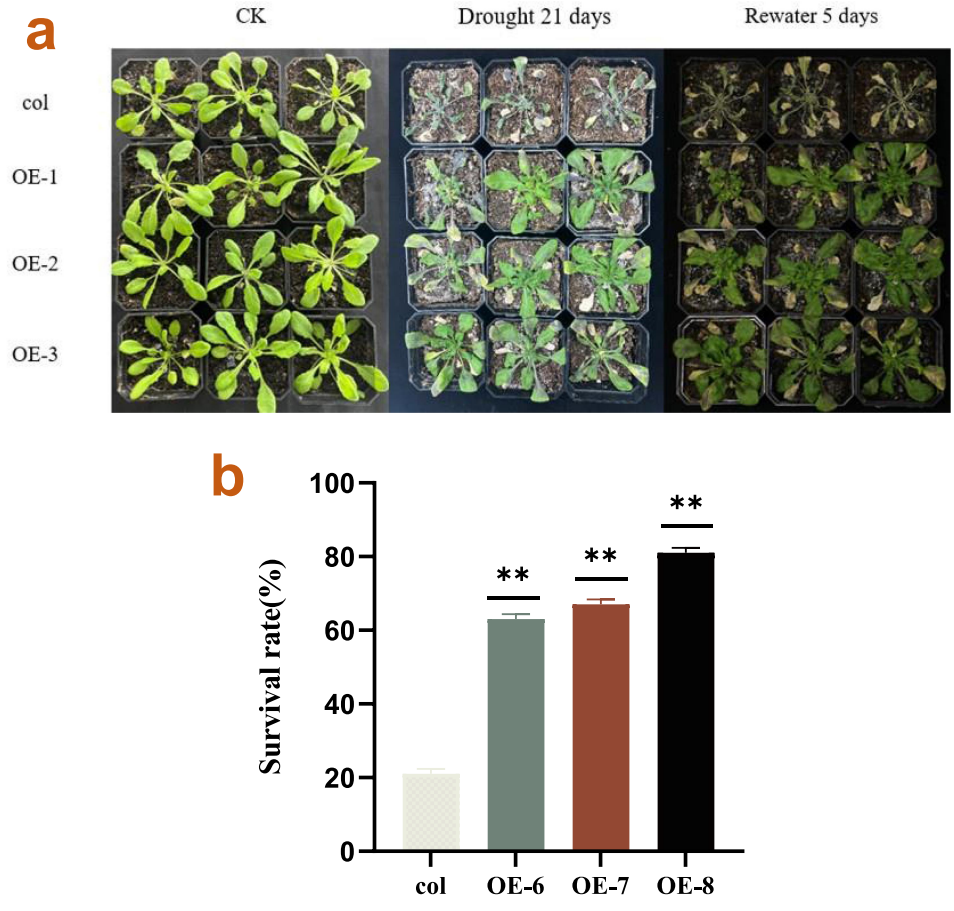

Figure 6. Drought rehydration experiments in wild-type and transgenic *Arabidopsis*. a: Growth of wild-type and transgenic *Arabidopsis* plants after drought stress and rehydration. b: Statistics on survival of wild type and transgenomes in the same batch of *Arabidopsis* after rehydration.

tetrazolium (NBT) can react with superoxide anion (O_2^-) to produce a blue precipitated methylene filth, and during the staining process, the darker the staining of *Arabidopsis thaliana* leaves, the more the content of superoxide anion (O_2^-), and the weaker the resistance of the plant.[23] 3,3-diaminobenzidine (DAB) reacts with hydrogen peroxide (H_2O_2) to produce a yellowish-brown product that is insoluble in water and alcohol, and during the staining process the The darker the staining of *Arabidopsis* leaves, the more hydrogen peroxide (H_2O_2) content, the weaker the plant resistance to abiotic stress.[23]

In this study, *Arabidopsis* leaves were stained by nitrogen blue tetrazolium (NBT) and 3,3-diaminobenzidine (DAB) staining, and the ability of *Arabidopsis* plants to resist drought stress was inferred by observing the degree of staining depth of leaves. The results are shown in Figure 7a,b. Under normal unstressed treatment conditions, there was no significant difference in the degree of staining between wild-type *Arabidopsis* and transgenic *Arabidopsis*. After drought stress treatment, the transgenic *Arabidopsis* was lighter stained compared with the wild-type *Arabidopsis*, indicating that the transgenic plants contained less reactive oxygen species (ROS) than the wild-type plants, which was speculated to be possibly due to the overexpression of the Zm*4CL2* gene that reduced the production of substances, such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), which in turn reduced the accumulation of reactive oxygen species. Subsequently, we

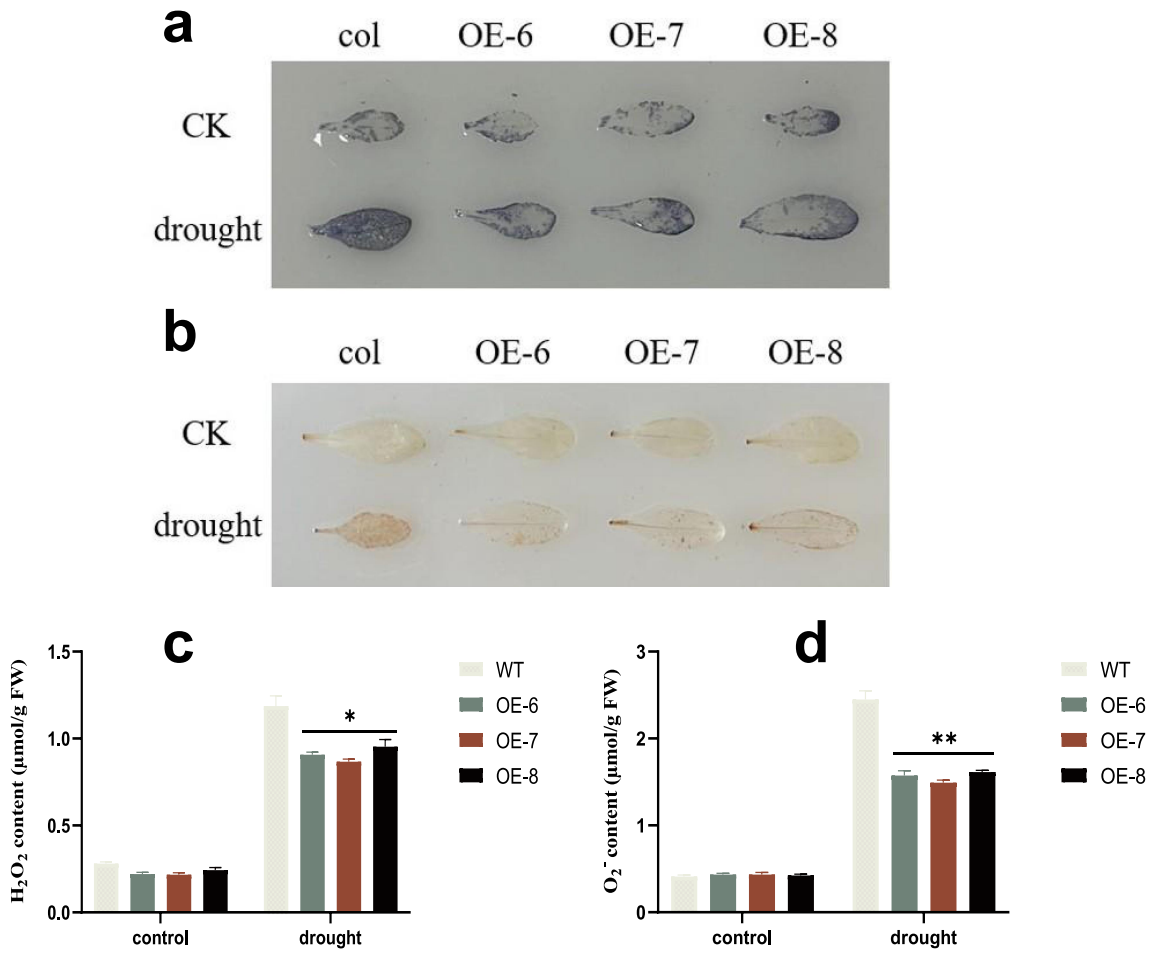

Figure 7. NBT and DAB staining in wild-type, transgenic *Arabidopsis* plants after drought stress, and the measurement of superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) content. a: Nitroblue tetrazole (NBT) staining; B: 3,3-diaminobenzidine (DAB) staining b: Content of hydrogen peroxide (H_2O_2); D: Content of superoxide anion (O_2^-).

measured the content of superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) in transgenic *Arabidopsis thaliana*, and the results are shown in Figure 7c,d. This is consistent with the staining results that superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) accumulation was reduced in transgenic *Arabidopsis*.

We initially concluded *that the Zm4CL2* gene is regulating the antioxidant response capacity of plants, and that the peroxidation of cellular membrane plasma produces malondialdehyde (MDA) after plants are subjected to abiotic stresses. Therefore, it is possible to determine the degree of cellular damage in plants after exposure to stress by detecting malondialdehyde (MDA) content. To a certain extent, superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) can protect cells from damage by scavenging reactive oxygen species (ROS).[24,25] Therefore, we can determine the ability of plants to respond to drought stress by detecting the three enzyme activities. In order to further analyze the effect of overexpression of *Zm4CL2* gene on the accumulation of reactive oxygen species (ROS) content in plants, in this study, we measured malondialdehyde (MDA), proline (Pro) and chlorophyll content in *Arabidopsis thaliana* with overexpression of *Zm4CL2* after drought stress and completed the determination of antioxidant enzymes activities such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and other antioxidant enzyme activities. The results, as shown in Figure 8, showed that the malondialdehyde (MDA) content was lower than that of wild-type *Arabidopsis* in the overexpression lines after drought stress, whereas the superoxide dismutase (SOD) activity, peroxidase (POD) activity, catalase (CAT) activity, proline (Pro) content,

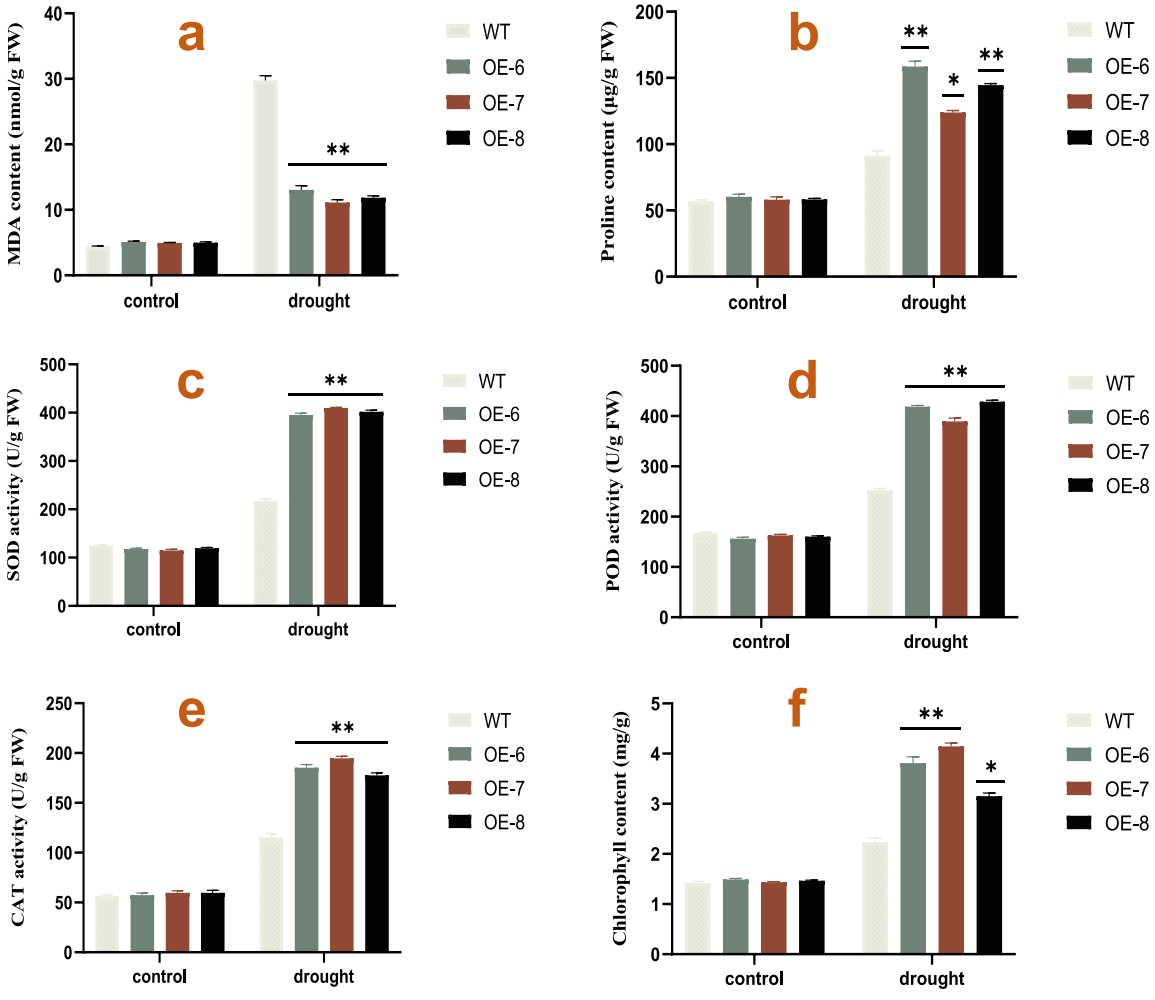

Figure 8. Determination of physiological and biochemical indicators related to transgenic *Arabidopsis thaliana*.

and chlorophyll content were higher than those of wild-type *Arabidopsis* in the transgenic *Arabidopsis*. Taken together, it further indicated that overexpression of Zm*4CL2* gene increased antioxidant enzyme activities, decreased superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) contents, and reduced the accumulation of reactive oxygen species (ROS) in the plants after drought stress, which in turn enhanced the tolerance of transgenic *Arabidopsis thaliana* to drought stress.

3.6. Expression Analysis of Drought Stress Related Genes in Transgenic Arabidopsis thaliana Under Drought Stress

To further verify the response of *Zm4CL2* gene to drought stress, we measured the expression levels of stress-related response genes by qRT-PCR. The *DREB2A*, *RD26*, *RD29A*, and *RD29B* genes are known to be involved in stress defense responses and are often used as stress marker genes.[16–19] Therefore, the expression levels of the four stress marker genes were measured in this study and the results of qRT-PCR analysis are shown in Figure 9. Under drought stress, the expression levels of both wild-type *Arabidopsis* and transgenic *Arabidopsis* were up-regulated, and the expression levels of the stress marker genes in transgenic *Arabidopsis* were significantly higher than those in wild-type *Arabidopsis*; therefore, under drought stress conditions, the *Zm4CL2* gene could affect the stress response by influencing the stress response genes, which in turn positively regulate plant sensitivity to drought stress.

4. Discussion

As one of the abiotic stresses, drought stress is affecting the production of many crops, and maize is no exception. Therefore, it is desirable to improve existing maize varieties through genetic engineering and inheritance to create new drought-resistant germplasm.[26] Currently, genes for drought resistance in maize have been discovered, and the relationship between the lignin synthesis pathway and drought resistance has been clarified. In cotton, for example, the *4CL* protein has been shown to be associated with abiotic stress resistance.[13] Therefore, in this study, we used transcriptome data (PRJNA793522) to screen for the maize *4CL* gene *Zm4CL-like9*, which was significantly up-regulated under drought conditions.We found that the relative expression of this gene in

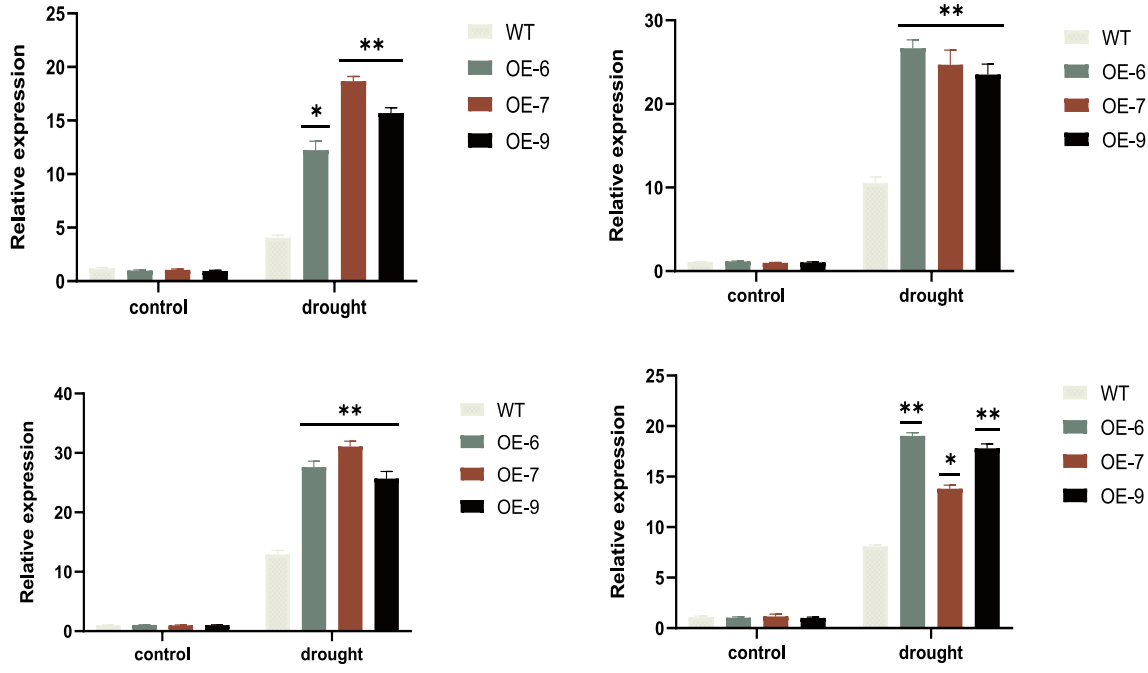

Figure 9. Expression statistics of transgenic *Arabidopsis thaliana* drought resistance related genes.

maize changed significantly with the imposition of drought conditions, which is in agreement with the previous results of transcriptome data (Figure 1). The use of the single-celled prokaryote *E. coli* BL21 to express target proteins and to verify resistance in transgenic *E. coli* has become one of the tools for gene function studies.[27–30] For example, overexpression of a gene and a gene has been shown to affect drought tolerance in *E. coli*. In this paper, the tolerance function of *Zm4CL-like9* to drought stress was investigated using a prokaryotic expression system. The results showed that *E. coli* transfected with the *Zm4CL-like9* gene, grew better under simulated drought conditions, and therefore were more tolerant to drought stress (Figure 2a–c).

Drought stress causes changes in the apparent morphology of plants as well as in the level of physiological metabolism within the plant.[31] Morphological changes in the plant root system are particularly evident under drought stress.[32] Plants respond to drought conditions by increasing the length of the root system.[33–35] After we infested wild-type *Arabidopsis* with *Agrobacterium* with corresponding vectors, we identified and selected three positive strains OE-6, OE-7, and OE-8 with the highest expression levels (Figure 3). Afterward, the changes in germination rate and root length of *Arabidopsis* under drought conditions simulated by mannitol were investigated for each strain.[36] The results showed that the three overexpression strains of *Arabidopsis*, OE-6, OE-7, and OE-8, had greater germination rates and root lengths than those of wild-type *Arabidopsis* under drought conditions (Figures 4a,b and 5a,b). Afterward, this study used the mature *Arabidopsis* of each strain to perform a drought-rehydration experiment as a test of drought resistance in adult *Arabidopsis* plants.[37] The results showed that the three overexpression *Arabidopsis* lines, OE-6, OE-7, and OE-8, had significantly higher survival rates after drought-rehydration than wild-type *Arabidopsis* (Figures 6a,b). Thus, overexpression of the Zm*4CL*-like9 gene could effectively improve the drought tolerance of transgenic *Arabidopsis*.

Under drought conditions, the life activities of plants cause them to produce and accumulate more reactive oxygen species (ROS). And the accumulation of ROS will cause damage to the DNA and cell membrane of plants.[38] Therefore, plants will increase the activity of related enzymes and the expression of related genes to regulate the metabolism of ROS.[39] By determining the changes of relevant physiological and biochemical indicators and related gene expression in plants under drought conditions, the strength of plant resistance to drought can be effectively reflected.[40] It has been reported that overexpression of *OsAHL1* gene in rice under drought stress promotes the development of rice root system under drought stress, whereas the root volume of RNAi plants carrying the *OsAHL1* gene was significantly reduced whereas RdreB1BI transgenic plants improved the drought resistance of the plants by increasing the activities of peroxidase (POD) and superoxide dismutase (SOD) and by decreasing the malondialdehyde (MDA) content.[41] Therefore, in this study, we measured the changes in MDA content as well as peroxidase (POD) activity, superoxide dismutase (SOD) activity, and catalase (CAT) activity in transgenic *Arabidopsis thaliana* and wild-type *Arabidopsis thaliana* under drought stress. The results showed that the activity, peroxidase (POD) activity, superoxide dismutase (SOD), and catalase (CAT) activity of the transgenic lines overexpressing *Zm4CL-like9* were significantly higher than those of the wild type after drought stress (Figure 8). These results indicated that the *Zm4CL- like9* gene enhanced drought tolerance in *Arabidopsis*.

Plants under drought stress produce large amounts of reactive oxygen molecules, such as peroxides and superoxide radicals, which disrupt the reactive oxygen species (ROS) balance in plants it causes cellular damage and leads to impaired plant development.[42] Therefore, it is crucial to investigate the mechanism of scavenging reactive oxygen species in plants. It has been shown that the gene in Fm*4CL*2 water hyacinth improves the ability of plants to scavenge reactive oxygen species (ROS) by down-regulating hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-), thereby improving drought tolerance in transgenic plants.[43] The maize senescence gene, *ZmSAG39*, accelerates senescence by decreasing the activities of antioxidant enzymes and peroxidases, which increases ROS accumulation and reduces plant drought tolerance.[44] In this study, we preliminarily determined the accumulation of reactive oxygen species

(ROS) in transgenic *Arabidopsis thaliana* stained with nitroblue tetrazolium (NBT) and 3,3'-diaminobenzidine (DAB) and measured physiological and biochemical indices of *Arabidopsis thaliana* in each strain.[45] The results showed that overexpression of *Arabidopsis* after drought stress had shallower DAB and NBT staining than wild-type *Arabidopsis* (Figure 7); and hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) were lower in overexpression of *Arabidopsis* after drought stress than in wild-type *Arabidopsis*, which was in agreement with the implication of the staining results. Subsequently, we used typical metabolism-related genes (*DREB2A*, *RD26*, *RD29A*, and *RD29B*) in drought to determine the degree of relevant metabolism by examining their expression profiles, and then verified the drought resistance of transgenic *Arabidopsis*.[46–49] The results showed that the relative expressions of the four related genes were up-regulated (Figure 9). Therefore, it indicates that the *Zm4CL-like9* gene can reduce the accumulation of reactive oxygen species (ROS) in *Arabidopsis*, which in turn improves the drought tolerance of *Arabidopsis*.

4-Coumarate coenzyme A ligase (*4CL*) is involved in the phenylpropane metabolism pathway during lignin biosynthesis, is the final enzyme in the downstream reactions of the pathway, and is a key rate-limiting enzyme for lignin production.[50] Lignin is an important phenolic product of phenylpropane metabolism that regulates the composition and mechanical properties of cell walls to enhance plant resistance to adversity stress.[51] For example, genes such as *DRE2* and *UF3GT* regulate lignin and anthocyanin synthesis, which in turn affects drought and cold tolerance in *Arabidopsis*.[52–54] In addition, *4CL* family member genes play indirect roles in resistance to various abiotic stresses and also enrich the synthesis and distribution of biochemicals in plants. For example, willow *4CL* family genes are involved in cell wall and lignin synthesis in water hyacinth;[44] Fm*4CL*-like1 genes play a role in plant resistance to osmotic stress by affecting lignin synthesis, cell wall development and thus plant resistance to osmotic stress[10]; longan *4CL* family genes are involved in early embryonic pigment synthesis and tissue- and organ-specific expression whereas tomato *4CL* family genes are involved in nitrogen distribution.[55,56] In addition to abiotic stress and biochemical partitioning, *4CL* family genes also play a role in plant disease resistance by participating in the phenylpropane pathway and regulating downstream lignin synthesis. For example, treatment of peaches with methyl jasmonate increased PAL and *4CL* activities and promoted the accumulation of the total phenolics, total flavonoids, and lignin content of the, thereby increasing the resistance of peaches to root mold.[57] In addition, many studies have validated gene function through crop phenotypes in *Arabidopsis*.Wang et al. transfected the maize *ZmWRKY40* gene into *Arabidopsis* and found that *ZmWRKY40* reduced reactive oxygen species accumulation by increasing antioxidant enzyme activity and increased drought tolerance in resident transgenic *Arabidopsis* by regulating the expression of stress genes.[58] Mao et al. found that *ZmNAC55* gene in *Arabidopsis thaliana* led to hypersensitivity to abscisic acid (ABA) at the germination stage, which in turn led to enhanced drought tolerance in transgenic *Arabidopsis thaliana* [59]; and the maize senescence gene *ZmSAG39* reduced drought resistance in plants by decreasing the activities of antioxidant enzymes and peroxidases in order to increase the accumulation of ROS.[44] *4CL* genes have been cloned and characterized in rice, soybeans, and cotton but have not been investigated for drought tolerance in maize. Therefore, in this study, *Arabidopsis thaliana* was used as the main subject to verify the function of the maize *4CL* family gene *Zm4CL-like9* in enhancing drought resistance in plants, and the transgenic *Arabidopsis thaliana* was functionally verified by using genetic engineering technology and methods such as physiological index measurements under simulated drought conditions and relative gene expression analyses, and a preliminary preliminary conclusion that overexpression of *Zm4CL-like9* in *Arabidopsis thaliana* enhances plant The preliminary conclusion that *Zm4CL-like9* overexpression in *Arabidopsis thaliana* enhances the drought resistance of the plant provides a theoretical basis for further exploration of the specific function and mechanism of action of the *Zm4CL-like9* gene in the drought resistance process.

5. Conclusions

In this study, we demonstrated that overexpression of *Zm4CL-like9* gene in *Arabidopsis thaliana* could improve the drought tolerance of transgenic *Arabidopsis thaliana* through expression assay, prokaryotic induction, drought rehydration, and physiological and biochemical indexes, etc. *The Zm4CL-like9* gene belongs to the family of 4-coumarate coenzyme A ligase and regulates drought tolerance of *Arabidopsis thaliana* by participating in the synthesis of lignin. The experimental results showed that the expression of *Zm4CL-like9* gene was up-regulated under drought stress and improved the survival rate of transgenic *Arabidopsis thaliana* after drought rehydration, and its transgenic Escherichia coli also showed better mannitol tolerance. Physiological and biochemical indexes showed that after drought stress, the activities of SOD, POD and CAT in *Arabidopsis thaliana* overexpressing *Zm4CL-like9* gene were higher than those in common *Arabidopsis thaliana*, and the chlorophyll content of transgenic *Arabidopsis thaliana* was higher than, and the content of MDA and ROS was lower than that of common *Arabidopsis thaliana*, which indicated that overexpression of Zm*4CL*-like9 gene improved the drought tolerance of *Arabidopsis thaliana* by altering the enzyme activities and the content of related substances. drought resistance. Under drought conditions, the expression of drought-related genes showed that the expression of drought-related genes (antioxidant and stress defense) was significantly increased in *Arabidopsis thaliana* overexpressing *the Zm4CL-like9* gene compared with that of common *Arabidopsis*, which also suggests *that the Zm4CL-like9* gene in *Arabidopsis* may improve the drought resistance of *Arabidopsis thaliana* by affecting the expression of antioxidant and stress-responsive genes.[60] In this paper, we have analyzed the role of 4CL in plant resistance to drought stress, but the role of Zm4CL-like9 in the lignin synthesis pathway is yet to be investigated in the laboratory.All these data and experiments lay the foundation for further exploring the role position and mechanism of action of *Zm4CL-like9* gene in drought resistance.

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Jiayi Fan conceived the study design. Jiayi Fan and Zhipeng Luo collected data. Jiayi Fan, Zhipeng Luo, Yuankai Wang, Peng Jiao, Siyan Liu and Shuyan Guan performed the statistical analyses, interpreted the results, and drafted the manuscript. Qingxu Wang, Yuntao Dai, Yiyong Ma and Huiwei Yu performed a critical revision of the manuscript. All authors approved the version to be published and agreed to be accountable for all aspects of the work.

Author contributions

CRediT: **Jiayi Fan:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Writing – original draft; **Zhipeng Luo:** Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing; **Yuankai Wang:** Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – review & editing; **Peng Jiao:** Investigation, Methodology, Resources, Validation; **Qingxu Wang:** Investigation, Validation, Writing – review & editing; **Yuntao Dai:** Investigation, Validation, Writing – review & editing; **Shuyan Guan:** Formal analysis, Project administration, Resources, Software, Supervision; **Yiyong Ma:** Project administration, Supervision, Writing – review & editing; **Huiwei Yu:** Investigation, Validation, Writing – review & editing; **Siyan Liu:** Conceptualization, Data curation, Funding acquisition, Project administration, Resources, Supervision.

Disclosure Statement

No potential conflict of interest was reported by the author(s).

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Data Availability Statement

Data will be made available on request.

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