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Overexpression of Maize Glutathione S-Transferase *ZmGST26* Decreases Drought Resistance of *Arabidopsis*

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Abstract: Drought stress critically endangers the growth and development of crops. Glutathione S-transferase plays a vital role in response to abiotic stress. However, there are few studies on the role of glutathione S-transferase in maize drought stress. In this study, the significantly downregulated expression of *ZmGST26* in roots under drought stress was analyzed by qRT-PCR. Promoter analyses showed that there were several cis-acting elements related to drought stress and that were involved in oxidative response in the promoter region of *ZmGST26*. Subcellular localization results showed that *ZmGST26* was localized in the nucleus. The transgenic lines of the *Arabidopsis* over-expressing *ZmGST26* were more sensitive to drought stress and ABA in seed germination and inhibited ABA-mediated stomatal closure. Under drought stress, phenotypic analyses showed that the germination rate, root length and survival rate of *ZmGST26* overexpressing lines were significantly lower than those of wild-type lines. The determination of physiological and biochemical indexes showed that the water loss rate, malondialdehyde, O_2^- and H_2O_2 of the overexpression lines significantly increased compared with wild-type *Arabidopsis*, but the antioxidant enzyme activities (CAT, SOD and POD), and proline and chlorophyll contents were significantly reduced. Subsequently, the qRT-PCR analysis of drought stress-related gene expression showed that, under drought stress conditions, the expression levels of *DREB2A*, *RD29A*, *RD29B* and *PP2CA* genes in *ZmGST26* overexpression lines were significantly lower than those in wild-type *Arabidopsis*. In summary, *ZmGST26* reduced the drought resistance of plants by aggravating the accumulation of reactive oxygen species in *Arabidopsis*.

Keywords: drought resistant; ROS; glutathione S-transferase

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1. Introduction

Plants often suffer from some abiotic stresses during growth and development, among which drought and water shortages are the most serious abiotic stresses that threaten plant production [1]. The process of plants resisting drought is very complex. When subjected to drought stress, morphological changes, physiological changes and molecular changes will occur to resist the effects of drought and water shortage. Among them, regulating the balance of intracellular reactive oxygen species (ROS) content is essential [2]. Under normal growth conditions, ROS is necessary for plant growth and also acts as a signal transduction molecule in order to positively guide plant growth [3]. Under drought stress conditions, excessive ROS can cause different degrees of peroxidation with respect to cell membrane lipids, damage membrane stability and change the cell membrane's permeability [4]. In order to effectively resist drought stress, the enzymatic antioxidant system in plants is activated, which can effectively help plants to remove excessive ROS and free radicals in vivo, protect cells from damage, reduce the degree of peroxidation of cell membrane and improve their drought resistance [5]. The enzymatic active oxygen-scavenging systems mainly include peroxidase (POD), catalase (CAT), superoxide dismutase (SOD) and so on. Among them, superoxide dismutase (SOD) is the first line of defense against ROS

and free radical damage to the cell membrane, and it reacts to remove excess O_2^- [6]. The antioxidant function of POD is mainly reflected in the early stages of drought stress, scavenging excessive hydrogen peroxide (H_2O_2). When plants suffer from drought stress for too long or severe drought stress, POD will be directly involved in the process of reactive oxygen species production, directly leading to plant senescence [7].

Glutathione S-transferase (GST) is a family of enzymes with a wide range of functions in plants. It has important functions in abiotic stress [8], antioxidant [9], plant detoxification [10] and resistance to pathogen infections [11]. At the same time, GSTs, as one of the main members of the plant ROS-scavenging enzymes, catalyze the reaction of electrophilic substrates to remove ROS and exogenous compounds, thereby protecting plants from damage. Plant GST plays an antioxidant role by repairing membrane phospholipid damage caused by free radicals and inhibiting microsomal peroxidation [12]. GSTs are classified into eight classes: Phi, Tau, Theta, Zeta, Lambda, EF1G, DHAR and TCHQD, based on protein homology and genome structure [13]. In recent years, several plant-specific GSTs, such as Tau and Phi, received extensive attention in regulating the removal of reactive oxygen species in response to abiotic stress. Some studies have shown that, under the osmotic stress of ABA, in *Arabidopsis*, glutathione S-transferase *AtGSTU17* knockout mutants show enhanced drought and salt stress tolerance [14]. The overexpression of the *Arabidopsis* glutathione S-transferase *AtGSTU7* gene reduces the germination rate of seeds and reduces the tolerance to droughts [15]. Soybean glutathione S-transferase *GsGSTU42* enhances the antioxidant capacity of leaves by enhancing ROS detoxification or homeostasis reconstruction [16]. The jasmonic acid-responsive glutathione S-transferase gene *SlGSTU24* can reduce cold-induced oxidative stress in tomato plants [17]. The overexpression of rice Tau-like glutathione S-transferase *OsGSTU30* in *Arabidopsis* enhances its tolerance to heavy metals and drought stress by biochemical and physiological analyses [18]. The overexpression of cotton glutathione S-transferase *GhGSTF12* gene causes anthocyanin accumulation in cotton [19].

Many glutathione S-transferases have been identified in previous studies. However, there are relatively few studies on the function of glutathione s-transferase in maize [20,21]. In this study, the *ZmGST26* gene with significantly downregulated expressions under drought stress was screened from the transcriptome sequencing data of maize H8186 [22]. We first cloned and characterized the *ZmGST26* gene, and we analyzed its expression pattern in roots, stems and leaves under drought stress by real-time PCR and determined its subcellular localization. The overexpression of *ZmGST26* in *Arabidopsis* decreased the antioxidant enzyme activity of transgenic *Arabidopsis*, inhibited stomatal closure and increased the accumulation of ROS, thereby reducing its tolerance to drought stress. These results suggest that *ZmGST26* may be involved in the negative regulation of maize responses to drought stress.

2. Results

2.1. Drought Stress Induced the Downregulated Expression of ZmGST26 Gene

In maize, we obtained a *ZmGST26* gene, which is a member of the glutathione S-transferase family and belongs to the Tau class. To further understand the evolutionary relationship between *ZmGST26* and other reported tau proteins, we performed a phylogenetic analysis of the amino acid sequences of 20 Tau genes, and the results showed that *ZmGST26* was relatively close to these proteins as the highest homology to OsGSTU7 protein; meanwhile, because the expression level of the gene was regulated by the upstream promoter, when analyzed by a 2000 bp promoter element from the upstream promoter of the *ZmGST26* gene, it showed that the upstream promoter of the *ZmGST26* gene contained several active oxygen-, drought-, ABA and light-responsive elements (Table S1). Therefore, the expression of the *ZmGST26* gene in different tissues of maize plants under drought stress was verified by qRT-PCR. The results showed that the expression level of the *ZmGST26* gene was significantly downregulated in roots, stems and leaves under drought stress (Figure 1B), and in roots, it reached the lowest level at 8 h and then stabilized. In

leaves and stems, the lowest levels were reached at 4 h and 8 h, and subsequently, the expression increased again, but it was lower than the control level. These results suggest that *ZmGST26* is likely involved in the drought response in maize.

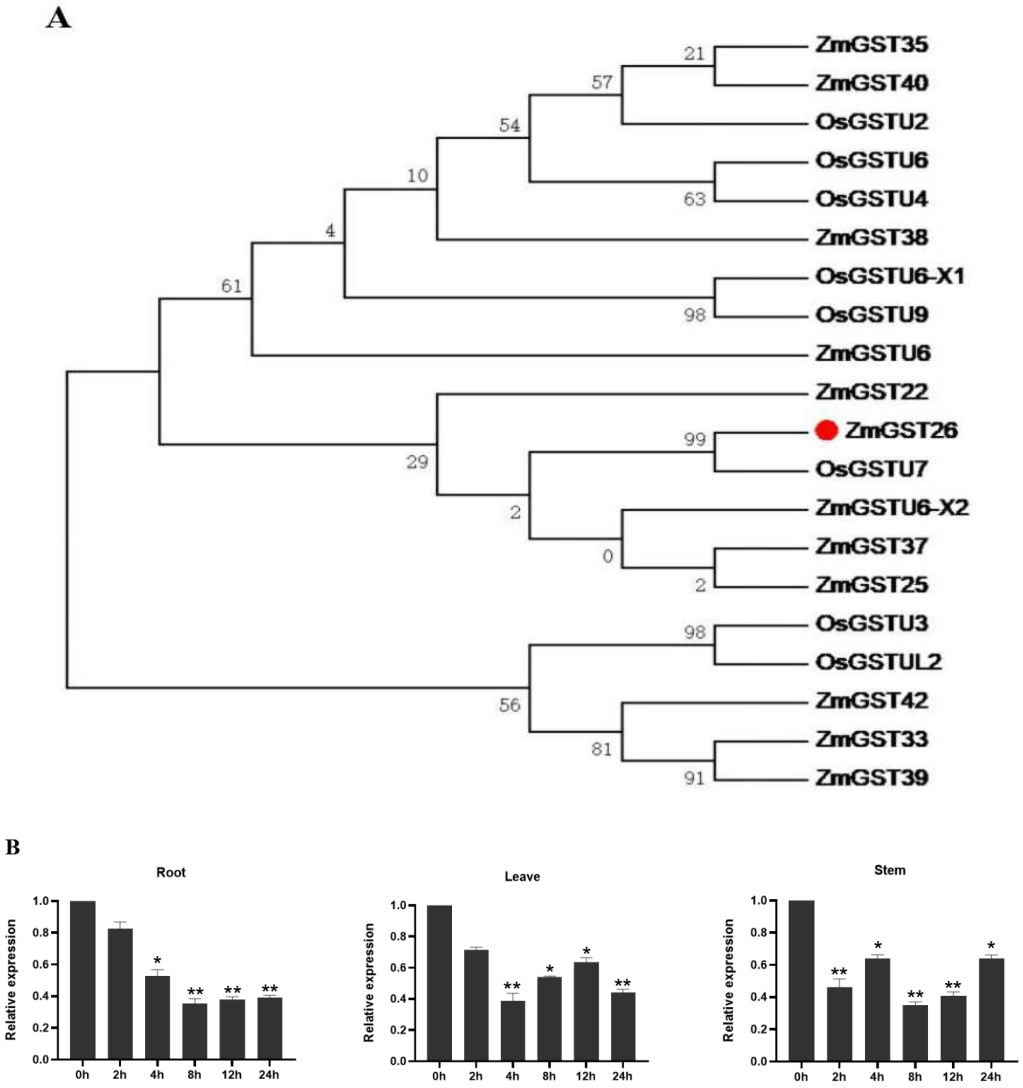

Figure 1. Phylogenetic analysis and expression pattern analysis of *ZmGST26*. (**A**) Phylogenetic analysis of the Tau class of Glutathione S-transferases. (**B**) Expression of *ZmGST26* in roots, leaves and stem of maize at different time under drought stress. (*ZmActin1* was used as an internal reference gene). * $p < 0.05$; ** $p < 0.01$. All bars represent means ± SD, (n ≥ 3).

2.2. Subcellular Localization

Understanding the subcellular localization of gene expression products is of great significance for the functional analyses of genes. To determine the subcellular localization of *ZmGST26*, p35S::GFP and p35S::-ZmGST26-GFP were transiently expressed in tobacco leaves. As shown in Figure 2, the unfused GFP green fluorescence signal in tobacco leaves was distributed in the nucleus and cytoplasm, while the green fluorescence signal of ZmGST26-GFP was only distributed in the nucleus. It was further confirmed that *ZmGST26* acts in the nucleus.

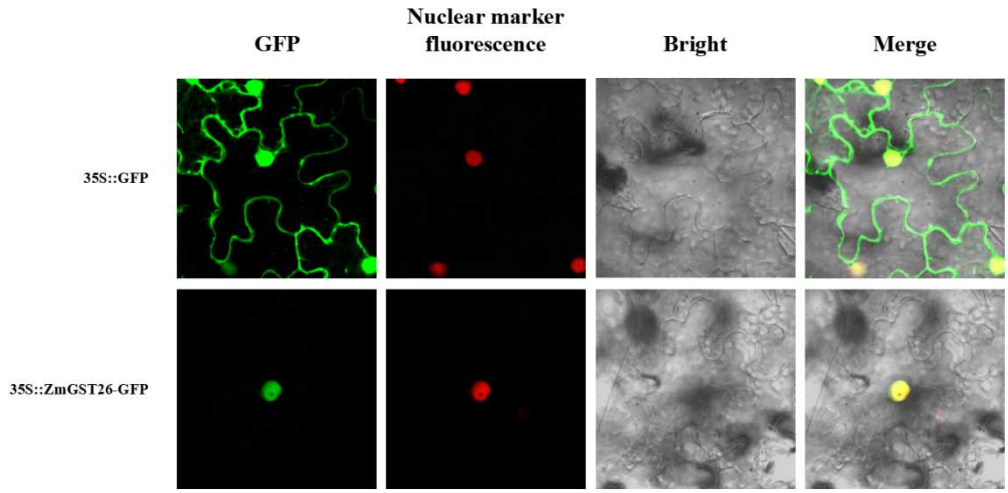

Figure 2. Subcellular localization of *ZmGST26* in tobacco epidermal cells. (Bars = 25 μm).

2.3. Overexpression of ZmGST26 Reduced the Drought Tolerance of Transgenic Arabidopsis

To better define the role of *ZmGST26* under abiotic stress, we employed an overexpression strategy. Transgenic *Arabidopsis* plants were obtained by the overexpression of *ZmGST26* under the control of the 35S promoter. We verified and screened 15 lines of *ZmGST26* overexpression in T_3 generation. Finally, three lines OE-4, OE-9 and OE-13 with obvious overexpression effects were selected for subsequent phenotypic experiments (Figure 3A). The seeds of the overexpression lines and wild-type *Arabidopsis* thaliana were inoculated on a 1/2 MS medium containing different concentrations of mannitol (0, 100 and 200 mM) and different concentrations of ABA (0.100 and 200 μM), as shown in the figure (Figure 3B–E). Under normal conditions, there were no noticeable changes during germination among the transgenic and WT plants, and germination was about 95% in all cases. However, seed germination was inhibited under mannitol and ABA stress conditions to a more pronounced extent for the overexpression lines relative to the WT. Under 100 mM mannitol stress, the germination rate of the WT seeds reached 72.3%; however, the overexpression lines showed 53.4%, 51.2% and 49.3% germination. This gap was more significant at 200 mm mannitol stress, with 50.2% germination in the wild-type, but only 29.6%, 25.3% and 30.2% in the overexpression lines. At 100 μM ABA stress, the germination rate of wild-type *Arabidopsis* was 75.7%, whereas that of the overexpression line *Arabidopsis* was 37.6%, 36.9% and 37.2%. At 200 μM ABA stress, the germination rates of the overexpression line for *Arabidopsis* were 24.6%, 22.8% and 15.9% in the overexpression lines, whereas it was 38.9% in wild-type *Arabidopsis*, indicating that the overexpression of *ZmGST26* decreased the germination rate of *Arabidopsis*.

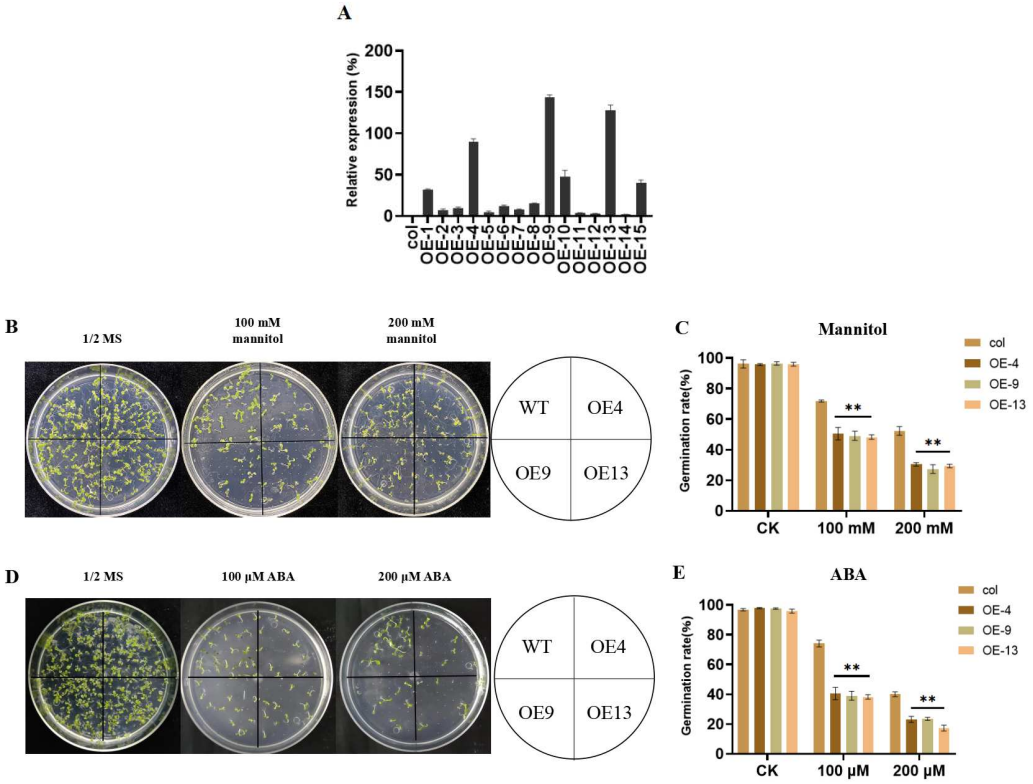

Figure 3. Drought resistance of wild-type and *ZmGST26*-overexpressing *Arabidopsis* plants. (**A**) *ZmGST26* expression in leaves of wild-type and overexpression lines *Arabidopsis*. (**B**,**C**) Seedling growth and germination rate in transgenic and wild-type *Arabidopsis* under mannitol treatment. (**D**,**E**) Seedling growth and germination rate in transgenic and wild-type *Arabidopsis* under ABA treatment. ** $p < 0.01$. All bars represent means ± SD, (n ≥ 3).

Similarly, the seeds of the overexpression lines and wild-type *Arabidopsis* were sown on a 1/2 MS medium with different concentrations of mannitol and cultured in light and vertical conditions to observe the root's length. After 10 days of culture, as shown in the figure (Figure 4A,E), there were no significant changes in the root length of the overexpression lines and the wild-type line under the control treatment without mannitol. Under the treatment of 100 mM and 200 mM mannitol, the root length of the three overexpression lines was significantly shorter than that of the wild-type *Arabidopsis* thaliana. In order to further verify the role of the *ZmGST26* gene in drought stress, the overexpression lines grown for 21 days and wild-type *Arabidopsis* plants were treated with drought stress for 10 days and then re-watered for 3 days. The results showed that the survival rate of the overexpression lines was significantly lower than that of wild-type *Arabidopsis*. The survival rate after rehydration was 67.6% for the wild-type, but only 27.3%, 29.2% and 25% for the overexpression lines (Figure 4B,C).

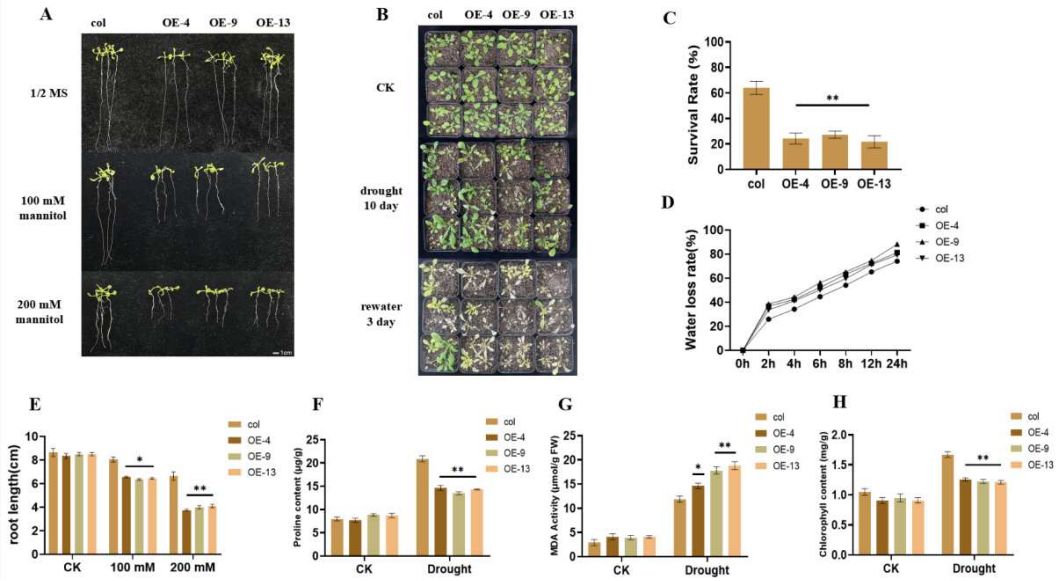

Figure 4. Drought resistance of wild-type and *ZmGST26*-overexpressing *Arabidopsis* plants. (**A**,**E**) Roots length in transgenic and wild-type *Arabidopsis* treated with mannitol. (**B**) Phenotypes of transgenic and wild-type *Arabidopsis* plants under drought conditions. (**C**) Survival rates of *ZmGST26*-overexpressing transgenic plants and wild-type *Arabidopsis* plants after re-watering. (**D**) Water loss rate. (**F**) Proline content. (**G**) MDA content. (**H**) Chlorophyll content. * $p < 0.05$; ** $p < 0.01$. All bars represent means ± SD, (n ≥ 3).

The determination of physiological and biochemical parameters such as water loss rate, proline, malondialdehyde and chlorophyll content can well reflect the physiological changes exhibited by plants subjected to drought stress and thus demonstrate whether plants are tolerant to drought stress. After drought stress treatments, compared to the wild-type line, all three overexpression lines showed significantly lower levels of proline (Figure 4F) and chlorophyll (Figure 4H). However, the water loss rate (Figure 4D) and malondialdehyde content (Figure 4G) were significantly elevated. These results indicated that the overexpression of *ZmGST26* reduced the drought tolerance of transgenic *Arabidopsis*.

2.4. Overexpression of ZmGST26 Increased the Sensitivity of Transgenic Arabidopsis to Drought

Plants produce more ROS in adversity. In order to avoid the damage of excess ROS, plants initiate feedback mechanisms to increase the enzymatic scavenging system and non-enzymatic scavenging system activity; this eliminates excess ROS in vivo. The activity of ROS-scavenging enzymes in plants is significantly different due to the different species. The activity of ROS-scavenging enzymes is reflected by the effects of O_2^- production rates, H_2O_2 content, membrane lipid peroxidation and antioxidant enzyme activity in plants.

Firstly, NBT and DAB staining were used to observe the color of leaves to identify the changes in the ROS content in leaves. The results (Figure 5A,B) showed that, with the increase in drought stress time, the color of overexpressed *ZmGST26* lines was significantly darker than that of wild-type lines, indicating that the ROS content in overexpressed *Arabidopsis* leaves increased. Then, we measured the content of H_2O_2 and O_2^-, and the results are shown in Figure 5C,D. Under normal conditions, there is no significant difference between the two; after drought stress treatments, the content of O_2^- and H_2O_2 in overexpression lines was significantly higher than that in wild-type *Arabidopsis*, resulting in a decrease in drought resistance.

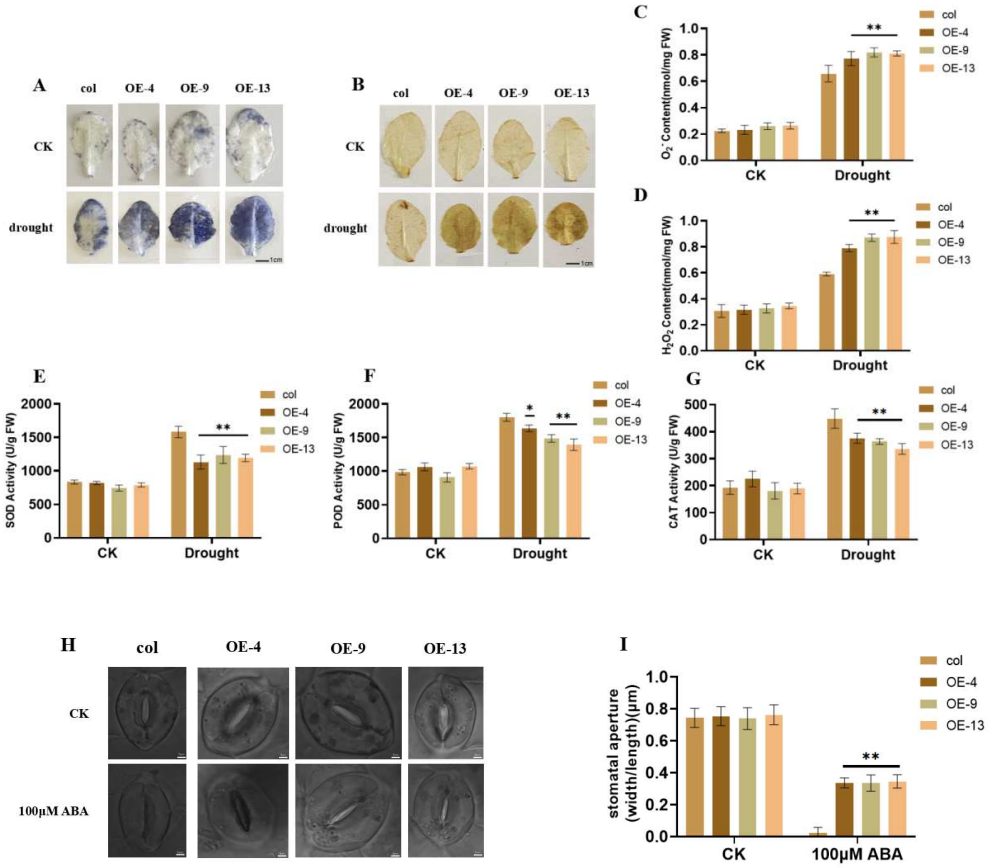

Figure 5. Reactive oxygen species staining and physiological indices in ZmGST26-overexpressing Arabidopsis plants. (**A**,**B**) NBT and DAB staining of leaves for H2O2 from ZmGST26 transgenic seedlings and wild-type plants under normal conditions and drought stress. (**C**,**D**) Accumulation of H_2O_2 and O_2^- in leaves of diferent lines. (**E**) SOD content. (**F**) POD content. (**G**) CAT content. (**H**,**I**) Stomatal aperture of transgenic Arabidopsis under 100 μM ABA treatment. * $p < 0.05$; ** $p < 0.01$. All bars represent means ± SD, (n ≥ 3).

The high and low activities of antioxidant enzymes can well reflect the amount of ROS in plants; the antioxidant enzyme activities, such as SOD, POD and CAT, were measured in the overexpression lines of *Arabidopsis* and the wild-type *Arabidopsis*. The results showed that after drought stress treatments, the activities of SOD, POD and CAT antioxidant enzymes (Figure 5E–G) were significantly lower in all three overexpression lines compared with the wild-type *Arabidopsis*. Thus, this further illustrated that overexpressing the *ZmGST26* gene after drought stresses decreased antioxidant enzyme contents and elevated O_2^- and H_2O_2 contents in the body of the overexpression lines, which led to the accumulation of ROS content in vivo; this then caused the overexpression lines to cope with reduced drought-stress response tolerance.

Under a normal culture environment, the stomatal aperture of the leaves of each line was almost the same. However, under 100 μM ABA stress, the stomatal apertures in overexpressing *Arabidopsis* lines were 0.358 μm, 0.299 μm and 0.375 μm. However, wild-type *Arabidopsis* stomata tended to close completely, thus indicating that stomatal pore sizes differ significantly between the overexpression lines and wild-type *Arabidopsis* (Figure 5H,I). We speculated that overexpressing *ZmGST26* reduced plant tolerances to drought stress by regulating the stomatal opening and thus causing water loss.

2.5. ZmGST26 Negatively Regulates the Expression of Drought-Related Genes in Overexpressed Arabidopsis

The response of plants to drought stress can be evaluated by the expression level of known drought-related genes. In this study, the genes such as *DREB2A*, *RD29A*, *RD29B* and *PP2CA*, which were significantly upregulated under drought stress, were selected. In this experiment, the relative expression levels of these four genes in wild-type and overexpressed *Arabidopsis* were detected. Via an analysis of qRT-PCR results (Figure 6), the expression levels of *DREB2A*, *RD29A*, *RD29B* and *PP2CA* were upregulated in wild-type and overexpression lines under drought stress treatments, but the expression levels of *DREB2A*, *RD29A*, *RD29B* and *PP2CA* in the *ZmGST26* overexpression lines were significantly lower than those in wild-type plants. Therefore, *ZmGST26* may regulate plant drought stress-related genes, thereby negatively regulating plant responses to drought stress.

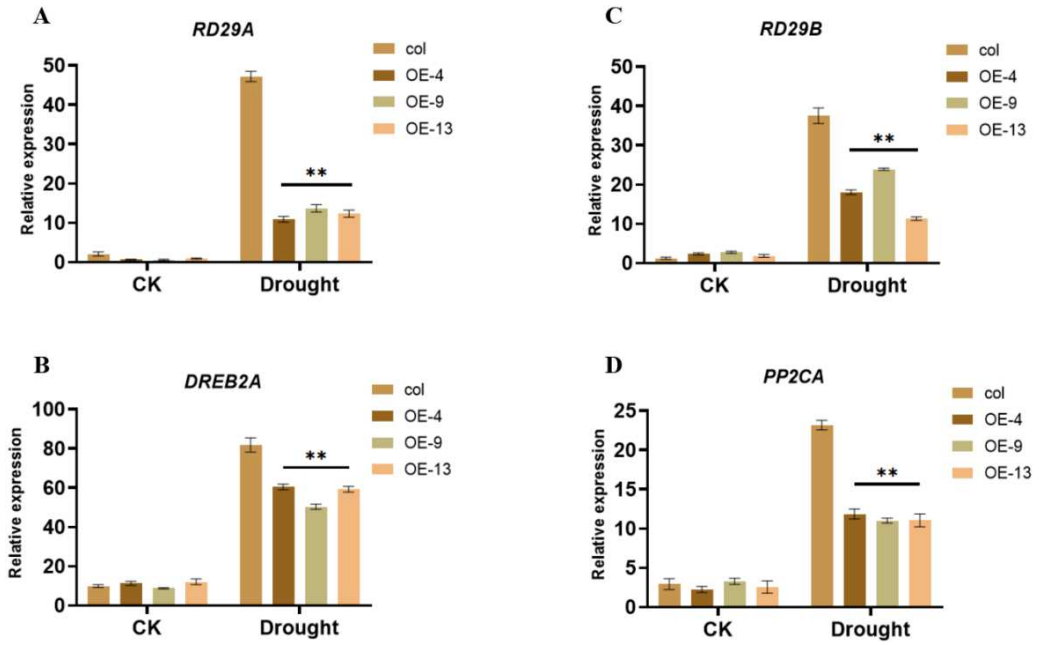

Figure 6. Expression of drought stress response genes in wild-type and *ZmGST26*-overexpressing transgenic *Arabidopsis* leaves. Quantitative real-time PCR analysis was conducted to detect the relative expression levels of (**A**) *RD29A*, (**B**) *RD29B*, (**C**) *DREB2A* and (**D**) *PP2CA* in three-week-old plants after not watering for 10 days. The *AtActin2* gene was used as an internal reference. ** $p < 0.01$. All bars represent means ± SD, ($n \geq 3$).

3. Materials and Methods

3.1. Plant Materials and Growth Conditions

The H8186 maize inbred line was used as the material. Maize seeds with full and well-developed kernels were sown in soil and cultivated in an artificial culture room (25 °C, 16 h light/8 h dark).

Arabidopsis thaliana takes the Colombian ecotype as the wild-type material. The seeds were first disinfected with 75% ethanol for 1 min and then sterilized with 1% sodium hypochlorite for 10 min; finally, they were rinsed with sterile water 3–5 times. Sterilized *Arabidopsis* seeds were cultured in a 1/2 MS medium at 4 °C in darkness for 72 h and then transferred to the tissue culture room (22 °C, 16 h light/8 h dark) for germination and growth. After 7 days of incubation, the seedlings were transplanted into soil (22 °C, 16 h light/8 h dark).

3.2. Sequence Analysis

The cDNA sequence of the *ZmGST26* gene was derived from NCBI (https://www.ncbi.nlm.nih.gov/ (accessed on 11 November 2022)). The phylogenetic tree was constructed

by the neighbor-joining method using MEGA7 software. The parameter of boot strap repetitions was set to 1000 [23]. The 2000 bp promoter upstream of the *ZmGST26* gene was analyzed by Plant CARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/ (accessed on 11 November 2022)) [24].

3.3. RNA Extraction and qRT-PCR Analysis

Total RNA was isolated from maize tissues under drought stress by the TRIzol method. The seedlings at the three-leaf stage were treated with 200 mmol mannitol. The tissues were collected at 0, 2, 4, 8, 12 and 24 h after treatment. The harvested samples were immediately frozen in liquid nitrogen for subsequent RNA extraction, and then the first strand of cDNA was generated by using the M-MLV reverse transcription kit (Takara, Kusatsu, Japan). qRT-PCR was applied using SYBR green and the Light Cycler ® 96 PCR detection system (Roche, Switzerland). The reaction procedure was 95 °C 30 s, 95 °C 10 s and 60 °C 30 s at 40 cycles. The melting point curve and data analysis were performed using the $2^{-\Delta\Delta CT}$ method. *ZmActin1* was used as an internal reference gene for calculating the Ct value.

The rosette leaves of *Arabidopsis* thaliana with normal growth and drought stress for 10 days were selected. The total RNA was extracted and the first strand cDNA was synthesized for qRT-PCR detection. The marker gene-specific primers *DREB2A*-F/R, *RD29A*-F/R, *RD29B*-F/R and *PP2CA*-F/R were used for qRT-PCR analyses. *AtActin2* was used as an internal reference gene to calculate the expression of four stress-related marker genes.

Each sample was repeated three times. The primers used in this study are shown in Supplementary Materials Table S2.

3.4. Subcellular Localization

The full-length coding sequence of the *ZmGST26* gene without a stop codon was amplified by PCR using specific primers. Then, the *Nco*I and *Spe*I sites of pCAMBIA1302-35S-GFP were selected to generate the pCAMBIA1302-35S-ZmGST26-GFP recombinant vector. The recombinant vector was transferred into the *Agrobacterium tumefaciens* EHA105 strain. It was infiltrated and co-injected into the tobacco leaf and cultured in an incubator at 25 °C for 48 h. It was observed using an FV1000 laser scanning confocal microscope (LSCM IX83-FV1200, Olympus, Hamburg, Germany).

3.5. Acquisition and Phenotypic Analysis of Overexpressed Arabidopsis

The full-length cDNA sequence of the *ZmGST26* gene was cloned into the pCAMBIA3301-35S-Bar vector. Subsequently, the pCAMBIA3301-35S-ZmGST26-Bar vector was introduced into the *Agrobacterium strain* EHA105 and transformed into *Arabidopsis* plants by the floral dip method. Positive plants were screened by Basta until homozygous *Arabidopsis* plants were obtained in T_3 generation. Three overexpression lines were selected for subsequent research.

After disinfection, the seeds were sown on a 1/2 MS medium containing 0 mM, 100 mM, 200 mM mannitol, 100 μM ABA and 200 μM ABA; these seeds grew in a greenhouse. After 8 days of culture, the germination rate of the seedlings was recorded: germination rate = germinated seeds/total seeds × 100% [25]. Each sample contains three biological replicates. A one-way analysis of variance was used for statistical analyses, and the average value of each overexpression line was compared with the wild-type line.

The method of root length determination was first performed by sanitizing the seeds sown on a 1/2 MS medium containing 0 mm, 100 mm and 200 mm mannitol, and then growing them vertically for 10 days. The main root lengths of *Arabidopsis* thaliana under different mannitol concentrations were measured. All the experiments were repeated three times. Each biological replicate contained at least 12 seedlings [26].

The *Arabidopsis* thaliana that grew normally during the four-leaf stage was subjected to drought stress, with watering stopped for 10 days, before it was watered for 3 days to investigate its survival rate. Following the rewatering treatment, plants were considered

survivors if they had green and healthy new leaves. All the experiments were repeated three times.

3.6. Determination of O_2^-, H_2O_2 and Physiological and Biochemical Indexes in Overexpressed Arabidopsis

The content of ROS in vivo was identified by 3,3′-diaminobenzidine (DAB) and p-nitroblue tetrazolium (NBT) histochemical staining, and the specific method was referred to as Fryer's method [27]. The content of H_2O_2 and O_2^- was calculated by using the acetone method, and the specific method was referred to as Wang's method [28]. The determination of water loss rates was referred to as Negi's method [29]. The content of malondialdehyde was detected by using the thiobarbituric acid method; fresh young leaves were soaked in 95% ethanol for 24 h under dark conditions. The absorbance of chlorophyll solution was measured at 649 nm and 665 nm, and the chlorophyll content was measured by Fitter's method [30]; the proline content was analyzed by the acid ninhydrin method [31]. The antioxidant enzyme activities of SOD, POD and CAT were measured according to Xiang's method [32].

3.7. Guard Cell Stomatal Aperture Observations

For the stomatal opening test, the leaf epidermis of 3-week-old *Arabidopsis* seedlings was incubated in a buffer (10 mM KCl, 200 μM $CaCl_2$ and 10 mM MES-KOH, pH 6.2) for 2 h. Then, the epidermis was immediately transferred to a dish containing 100 μM ABA in the stomatal opening solution for 2 h, and the ABA free epidermis was used as a control. Photographs were taken using a microscope and at least 30 stomata were observed for each treatment. The lengths and widths of stomata were measured and analyzed using the ImageJ software.

3.8. Statistical Analysis

Differences between data were determined by analyzing one-way variances using SPSS (SPSS Inc., Chicago, IL, USA). The test showed significant differences at $p < 0.05$ (*) or $p < 0.01$ (**).

4. Discussion

Glutathione S-transferase is an important enzyme and a superfamily of biotic and abiotic stress responses. Glutathione S-transferase plays a key role in the antioxidant enzyme system. The expression of GST genes plays an important role in response to plant oxidative stress, metal ions, drought, salinity and other abiotic stresses [33–36]. In this study, the differentially expressed *ZmGST26* under drought stress was screened by previous laboratory transcriptome sequencing data. On this basis, we performed drought stress on this gene in different tissues of maize. The results showed that *ZmGST26* was significantly sensitive to drought stress in roots. A previous study also found that *CsWRKY48* directly binds to the W-box element (TTGACC) in the *CsGSTU8* promoter and activates its expression by improving the clearance of excess ROS under drought conditions to indicate enhanced drought tolerances [37]. The promoter element analysis of 2000 bp upstream of *ZmGST26* showed that there were W-box response elements and a large number of elements involved in drought and oxidative responses. Therefore, we speculate that the W-box element (TTGACC) in the *ZmGST26* promoter may be regulated by WRKY transcription factors to cope with drought stress. Subsequently, the subcellular localization of *ZmGST26* was performed to verify its localization in the nucleus, and this conjecture will be further verified by experiments.

Many studies have determined the function of genes by phenotypic and physiological changes in transgenic *Arabidopsis* lines. It has been reported that the germination rate and root length of transgenic *Arabidopsis* overexpressing *TaSADR1* under drought stress were significantly lower than those of wild-type *Arabidopsis*, which confirmed that *TaSADR1* reduced the tolerance of plants relative to drought stress [38]. *PtGSTF1* increases biomass

production and salt tolerance by regulating xylem cell proliferation, ion homeostasis and ROS-scavenging in poplar [39]; the overexpression of *CsGSH2* reduces phytotoxicity in cucumbers by enhancing antioxidant and glutathione detoxification properties [40]; in this study, after drought stress, the root length, germination rate, chlorophyll content, proline content and survival rate of transgenic lines overexpressing *ZmGST26* were significantly lower than those of wild-type *Arabidopsis*, but the water loss rate and MDA content increased significantly. It showed that *ZmGST26* reduced the drought tolerance of transgenic *Arabidopsis*.

Drought stress leads to the production of ROS, and the excessive accumulation of ROS will cause damage to plant cells. Therefore, ROS is required to remove excessive ROS free radicals and O_2^- ions by activating the enzymatic antioxidant system in vivo. In previous studies, it was reported that *MruGSTU39* could detoxify ROS by upregulating the activities of antioxidant enzymes and GST to reduce its damage to membranes and thus enhance the drought tolerance of alfalfa [41]. *OsGSTU6* enhances rice abiotic stress tolerance by participating in intracellular ROS homeostasis [42]. *ThGSTZ1* transgenic *Arabidopsis* enhanced drought tolerances by scavenging excess H_2O_2 and other studies have shown that ROS can regulate plant drought tolerance [43]. In this study, NBT and DAB staining results, H_2O_2, O_2^- content and antioxidant enzyme activities were determined. The results showed that under drought conditions, the antioxidant enzyme activity of transgenic lines was significantly lower than that of wild-type plants, and the contents of ROS, H_2O_2 and O_2^- increased.

In recent years, many studies elaborated that stomatal regulation plays an important role in abiotic stress, and the overexpression of *AtPUB46* decreased the number of rosette leaves, stomatal density and water loss rate in *Arabidopsis*, thereby improving tolerance to drought and oxidative stress [44]; the overexpression of *GmPUB21* significantly increased stomatal density and water loss, thereby reducing plant resistance to drought and salt tolerance [45]. In this study, *Arabidopsis* overexpressing the *ZmGST26* transgenic line showed a significantly higher stomatal aperture and water loss rate than wild-type *Arabidopsis* under ABA stress conditions; thus, we speculated that overexpressing *ZmGST26* reduced plant tolerance to drought stress by regulating the stomatal opening and thus water loss. Subsequently, we performed qRT-PCR on drought-stress-related genes (*DREB2A*, *RD29A*, *RD29B* and *PP2CA*). The results showed that drought-stress-related genes were significantly downregulated. Therefore, we determined that ZmGST26 increased plant sensitivities to droughts by increasing the accumulation of reactive oxygen species, thereby reducing plant drought tolerances.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12122948/s1: Table S1. Cis-elements in the promoter region (~2 kb) of *ZmGST26*; Table S2. Primer information in this study.

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