

Article

Overexpression of *AgDREBA6b* Gene Significantly Increases Heat Tolerance in *Arabidopsis thaliana*

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Abstract

The APETALA2/ethylene response factor (AP2/ERF) is a class of plant-specific transcription factors, among which the dehydration-responsive element-binding protein (DREB) subfamily has been widely reported to enhance plant resistance to abiotic stresses. A high-temperature-related gene, *Apium graveolens* DREBA6b (*AgDREBA6b*; accession number: OR727346), was previously cloned from a heat-tolerant celery variety. In this study, we transformed this gene into *Arabidopsis thaliana* using an Agrobacterium rhizogenes-mediated method to explore its function. The results showed that overexpressing *AgDREBA6b* in *Arabidopsis thaliana* significantly improved plant growth under high-temperature stress (38 °C) compared to the *dreb* mutant and wild-type (WT) plants. The anatomical structure of the leaves revealed that the number and degree of stomatal openings in the overexpressed plants were significantly higher than those in the WT and *dreb* plants, suggesting that *AgDREBA6b* enhances stomatal opening. Additionally, the chlorophyll content, chlorophyll fluorescence properties, proline (Pro), malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) activities were higher in the transgenic plants, indicating better stress tolerance. qPCR analysis showed that four heat tolerance-related genes (*AtHSP98.7*, *AtHSP70-1*, *AtAPX1*, and *AtGOLS1*) were upregulated in the transgenic plants, with higher expression levels than in WT and mutant plants. This study provides valuable genetic resources for understanding the molecular mechanisms of celery's heat tolerance and offers insights for breeding heat-tolerant celery varieties.

Keywords: transcription factor; *AgDREBA6b*; heat tolerance; celery; antioxidant enzymes



Academic Editors: Umesh K. Reddy, Padma Nimmakayala and Georgia Ntatsi

Received: 6 May 2025

Revised: 10 June 2025

Accepted: 24 June 2025

Published: 27 June 2025

Citation: Xie, F.; Yang, S.; Peng, Z.; Li, Y.; Yang, Z.; Lv, R. Overexpression of *AgDREBA6b* Gene Significantly Increases Heat Tolerance in *Arabidopsis thaliana*. *Agronomy* **2025**, *15*, 1565. <https://doi.org/10.3390/agronomy15071565>

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1. Introduction

Plants are exposed to a variety of environmental threats in their natural environments, such as abiotic stresses like drought, salinity, and temperature extremes, which negatively affect plant adaptation to these stresses, biomass, and yield [1]. To adapt to these stresses, plants have evolved complex physiological and molecular responses, including changes in gene expression, metabolic pathways, and cellular structures [2]. Transcription factors (TFs) play a crucial role in regulating these responses by binding to specific DNA sequences and modulating the transcription of downstream genes [3]. TFs are a class of proteins that activate or repress the transcription of other downstream genes by recognizing and binding to DNA sequences of their promoters, such as dehydration-responsive element-binding

protein (DREB), basic leucine zipper (bZIP), myeloblastosis (MYB), no apical meristem (NAM), WRKY, and the NAM/ATAF/CUC (NAC). They control signal transduction and regulate gene expression under abiotic stress [4].

DREB (dehydration responsive element-binding protein), as a plant-specific transcription factor, belongs to a branch of the APETALA2/ethylene response factor (AP2/ERF) gene family and regulates the expression of downstream target genes by specifically identifying the dehydration responsive element/C-repeat sequence (DRE/CRT) cis-element, which, in turn, enhances plant resistance to abiotic stresses [5,6]. Currently, different numbers of *DREB* genes have been identified and functionally analyzed in an increasing number of species. Based on the similarity of AP2 structural domains, DREB family members can be classified into subfamilies A1 to A6, and DREB proteins show functional divergence in stress responses. For example, A1 members are mainly involved in the cold stress response [7,8], A2 members are mainly involved in drought and high-salt stress regulation [9], and A3-type DREBs play a role in regulating the response to abscisic acid (ABA) in seeds [10]. Among these, the A-1 (*DREB1*) and A-2 (*DREB2*) subgroups have been extensively investigated and are recognized as the primary categories of DREBs that play crucial roles in modulating plant responses to abiotic stresses [11]. Previous studies on DREB have focused on the A1/A2 subfamily, and *DREBA6* contains a large number of gene members, with marked differences in the function of the *DREB* (A-6) genes in different plants. There are few studies on the A6 subfamily, and the functions and mechanisms of the A-6-type genes are still unclear [12]. Therefore, there is a need to explore more genes from subgroup A-6 to better understand their functions and complex regulatory mechanisms in various plants.

Environmental factor stresses (drought, high temperature, high salt, and low temperature) often cause changes in plant physiological and biochemical indices, which are regulated by the protective system and related gene expression in the plant [13]. Among them, chlorophyll fluorescence parameters can be the first to respond to stress, which helps to clarify the site and extent of stress damage in the photosynthetic structure [14]. Plants can also overcome environmental stresses through the synergistic action of osmoprotectants and various antioxidants in the plant. For example, proline (Pro) is an important soluble osmotic pressure, which is an important indicator of changes in physiological parameters in response to plant adversity stress [15]. The antioxidant enzyme system in plants, including key enzymes such as peroxidase (POD), catalase (CAT), and superoxide dismutase (SOD), reduces the damage of adversity to cellular structures by rapid synthesis and activation of antioxidant defense mechanisms that lead to the accumulation of reactive oxygen species (ROS), which degrade the polyunsaturated lipids, leading to the formation of malondialdehyde (MDA). Overexpression of *Limonium bicolor* *Lb DREB* upregulates the expression of stress-related genes such as *Cu/ZnSOD* and *POD* [16]. After transfection of the *Eleusine coracana* *Ec DREB2A* gene in tobacco (*Nicotiana tabacum* L.), the transgenic tobacco could tolerate a high temperature of 42 °C but not salt stress and osmotic stress. Further studies found that the plants enhanced tolerance to heat stress mainly by increasing the activities of antioxidant enzymes (SOD, CAT, and POD), and increasing the content of MDA, and eight downstream related genes were also significantly upregulated [17]. Overexpression of *chrysanthemum* *CmDREB6* promoted the expression of *CmHsfA4*, *CmHSP90*, and the reactive oxygen scavenger genes *CmSOD* and *CmCAT* [18]. The above studies indicated that DREB transcription factors are important regulatory nodes for abiotic stress response in plants, and their overexpression enhances stress tolerance by activating antioxidant systems and thermo-protective mechanisms.

Celery is suitable for growing in cool and moist environments, and high temperature is the main environmental factor limiting the growth of celery [19]. In order to investigate the

molecular mechanism of high-temperature response in celery, the research group cloned an *AgDREBA6b* gene from celery, and found that under high-temperature stress, the expression of *AgDREBA6b* gene showed a sustained increased trend with the increase in time, suggesting that this gene may be actively involved in the response to high-temperature stress in celery [20]. On this basis, the *AgDREBA6b* gene was transformed into *Arabidopsis thaliana dreb* mutants in this study, and its role in regulating heat tolerance in plants was analyzed in the context of changes in cellular anatomy, phenotype, physiology, and related stress genes. This study expands the application prospects of DREB family transcription factors, which is conducive to a deeper understanding of the mechanism of high-temperature injury in celery, and lays the foundation for further exploration of its functions and the creation of new stress-resistant germplasm resources.

2. Materials and Methods

2.1. The *AgDREBA6b* Gene Overexpression Vector *Agrobacterium* Transformation

The *Arabidopsis dreba6* mutant seeds were purchased from the TAIR website (<https://www.arabidopsis.org/>) on 10 June 2023, and *pCAMBIA1301-AgDREBA6b* plasmids were preserved for our laboratory.

The recombinant vector *pCAMBIA1301-AgDREBA6b* was introduced into *Agrobacterium tumefaciens* GV3101 (Sangon, Shanghai, China) using the freeze–thaw method. Briefly, 2 µg of the vector was added to 100 µL of *A. tumefaciens* competent cells, mixed by tapping, and subjected to 5 min on ice, 5 min in liquid nitrogen, 5 min at 37 °C, and 5 min in an ice bath. Then, 700 µL of antibiotic-free LB medium was added, and the mixture was incubated at 28 °C for 2 h. After incubation, the cells were centrifuged at 6000 rpm for 1 min, resuspended in 100 µL LB, and spread onto YEB solid medium (pH 7.0) with 15 µL kanamycin and 15 µL rifampicin. The plates were incubated at 28 °C for 2 days. Positive clones were selected and verified by colony PCR with the following protocol: 95 °C for 3 min, followed by 35 cycles of 95 °C for 15 s, 55 °C for 15 s, 72 °C for 30 s, and a final extension at 72 °C for 5 min.

2.2. Cultivation of *Arabidopsis thaliana*

Several *Arabidopsis* seeds were loaded into sterile 1.5 mL centrifuge tubes as required for the experiment, and 1 mL of 75% ethanol was added. The results were mixed by inverting the tube up and down, discarding the supernatant, and repeating three times. Next, 1 mL of ultrapure water was added, the seeds were washed, the supernatant was discarded, and the process was repeated three times. In the ultra-clean bench with 1 mL pipette *Arabidopsis* seeds in the prepared MS plate spot seeding, the plate was sealed and inverted under dark conditions in 4 °C in the environment of the vernalization of 72 h; after the end of the vernalization, the plate was placed in a light incubator for vertical cultivation. The emergence of seedlings can be transplanted after seven days; with tweezers gently planted the seedlings into the soil of the small pots. The first plastic wrap was moisturized for 24 h and put in the plant growth chamber; seedlings were kept in the plant growth room for 24 h. The seedlings were cultured until the *Arabidopsis thaliana* grew (30 days); then, they could be used for transformation experiments.

2.3. Genetic Transformation of *Arabidopsis thaliana*

First, 10 µL of Rif and 20 µL of Kan (Sigma, St. Louis, MO, USA) were added into 20 mL of LB liquid medium and shaken well to inoculate the bacteria, and the bacteria were activated by shaking at 28 °C 220 rpm for 8–10 h to obtain the activation solution of *Agrobacterium*. Next, 100 µL of Rif and 200 µL of Kan were added into 200 mL of YEB liquid medium; then, 5–10 mL of the activated bacterial solution was added and

the culture shaken at 28 °C/220 rpm for 14–16 h. When the OD value reached 1.6–2.0, centrifugation was performed at 4500 rpm for 10 min, and the supernatant was discarded from the precipitated bacterial body, which was dried naturally. Next, 20 µL of SILWETL-77 (surfactant) solution and 100 mL of 5% sucrose was added to the precipitated bacterial body to re-suspend the bacteria, a pipette was used to blow evenly, and the bacteria were re-suspended repeatedly. The bacterial solution in the centrifugal bottle was added to the petri dish, and the inflorescence of *Arabidopsis thaliana* was closed, immersed in the petri dish, and gently shaken for 15 s. After the transformation, the bacterial solution was stirred and the plants were covered with a black bag; the plants were protected from light and kept in humidity for 24 h, and the transformation was repeated again a week later.

2.4. Screening of T₁ Generation Positive Plants

The *Arabidopsis* T₀ generation seeds were collected and planted. The T₀ generation seeds were sterilized, inoculated on MS screening medium containing 30 mg/L chaotropic acid (with 25 mg/L cephalothin added for bacterial suppression), light-cultured at 22 °C for 7–10 days, and screened to obtain the T₁ generation of positive plants (plants in which the seedlings and root systems grew normally). Then, the positive seedlings were transplanted to nutrient soil and covered with cling film for 2–3 days before the film was removed so they could grow normally. Genomic DNA was extracted from the leaves of the screened T₁-generation positive plants, the presence of the *AgDREBA6b* gene was confirmed by PCR, and the molecular verification of the target gene in the transgenic plants was carried out, which finally confirmed that the gene had been transferred into the T₁-generation positive plants.

2.5. T₂ Generation Positive Test of Transgenic Plants

T₁-generation positive plants were harvested individually to obtain T₁-generation seeds. These seeds were then subjected to thaumatin screening to identify T₂-generation positive plants, which were grown for 10 days. The plants were removed with forceps and stained using a GUS staining kit (Beijing Coolabo Technology Co., Ltd., Beijing, China) to verify the presence of the transgene in T₂-generation positive plants, *dre*b mutants, and WT. The confirmed positive plants were then transplanted and grown further. Genomic DNA was extracted from the leaves of these plants, and PCR molecular identification was performed to determine the T₂ generation positive plants.

2.6. Phenotype Observation of *Arabidopsis* Positive Plants

The *dre*b mutant seeds were sown in blistered nutrient soil at the same time as the T₁ generation seeds, purified at 4 °C for 72 h, and then moved into the plant growth room. When the leaves of *Arabidopsis* unfolded to 5 cm (20 d), high-temperature stress treatment was carried out, and *dre*b, WT, and 35S:*AgDREBA6b/dre*b plants were simultaneously placed in a light incubator at 38 °C to maintain the humidity at about 60% for 24 h.

2.7. Determination of Leaf Structural Properties of *Arabidopsis* Positive Plants

The *dre*b mutant, WT, and *AgDREBA6b* transgenic *Arabidopsis thaliana* plants were exposed to high temperature (38 °C) for 4 h. Leaf samples were collected with tweezers, fixed in FAA fixative for 24 h, dehydrated through an alcohol gradient, and cleared with anhydrous ethanol and xylene. The samples were embedded in paraffin, sectioned at 3 µm thickness, and stained with fenugreek-solid green. Sections were mounted on slides, sealed with neutral gum, and observed under a Nikon Eclipse E100 microscope. Each sample was processed in triplicate.

2.8. Measurement of Physiological and Biochemical Indices in Leaves of Arabidopsis Positive Plants

The *dreb* mutant plants, WT plants, and overexpressing *AgDREBA6b* plants in the mutant were treated at 38 °C for 24 h, and the relative chlorophyll content was determined using a portable chlorophyll meter and expressed as SPAD values. The chlorophyll fluorescence parameters of leaves were measured using a portable fluorometer after the whole Arabidopsis plants were treated in the dark for 30 min, including PSII light energy conversion efficiency (F_v/F_m) and photochemical quenching (q_N). They were measured in situ in the Arabidopsis culture environment three to five times, with three biological replicates for each strain, and the average values were taken. Arabidopsis leaves were collected for the determination of chlorophyll b, chlorophyll a, and total chlorophyll content using a 1:1 mixture of anhydrous acetone and anhydrous ethanol. The samples were frozen in liquid nitrogen and stored in a −80 °C freezer for the determination of Pro (colorimetric method), POD, and SOD activities, as well as CAT enzyme activity, with measurements performed according to the instructions of the kit provided by Nanjing Jiancheng Institute of Bioengineering Co., Ltd., Nanjing, China. Three plants were selected from each strain, three leaves were selected from each plant, and each leaf was measured three times. All the above experiments were repeated three times.

2.9. Validation of Quantitative Expression in Arabidopsis Positive Plants

The *dreb* mutant, WT, and 35S:*AgDREBA6b/dreb* transgenic plants were treated with high temperature at 38 °C for 0 h, 4 h, 12 h, and 24 h, respectively, and the leaves were quickly frozen in liquid nitrogen and then stored in an ultra-low temperature freezer at −80 °C. Total RNA was extracted from Arabidopsis leaves using Plant Total RNA Isolation Kit (Chengdu Fukuji Biotechnology Co., Ltd., Chengdu, China), and the total RNA extracted was reverse transcribed into cDNA using the Goldenstar™ RT6 cDNA Synthesis Mix Rnasin selected (Beijing DynaPro Biotech Co., Ltd., Beijing, China). For the qPCR analysis, *AtActin* served as the internal reference gene, and the 2×TSINGKE™ Master qPCR Mix kit was employed to assess the expression differences of the four heat-tolerance-related genes among the *dreb* mutant, the WT, and the transgenic plants. The PCR mixture (20 µL total volume) consisted of 2.0 µL template cDNA, 0.4 µL each of forward and reverse primers (10 mM each), 10 µL SYBR Green I master mix (Takara, Dalian, China), and 7.2 µL ddH₂O. The PCR conditions were as follows: 95 °C for 1 min for pre-denaturation, 40 cycles of 95 °C for 10 s for denaturation, and 58 °C for 15 s for annealing and extension. The relative expression levels were assessed using the $2^{-\Delta\Delta C_t}$ method [21], with $\Delta\Delta C_t$ calculated as $\Delta\Delta C_t = (C_t \text{ of the target gene} - C_t \text{ of } Actin) \text{ in the treatment group} - (C_t \text{ of the target gene} - C_t \text{ of } Actin) \text{ in the control group}$. The primer sequences are listed in Table 1.

Table 1. qPCR primers for four high-temperature-related genes.

Gene Name	Forward Primer (5' → 3')	Reverse Primers (5' → 3')
<i>AtHSP98.7</i>	F: TGCTTGGACGAGGTGAACTGAG	R: CGTTCGGTGATGTAGCGGTCTG
<i>AtHSP70-1</i>	F: TGCCTGAGATTGCTGAGGCTTA	R: CGGCTGTAGGCTCGTTGATGAT
<i>AtAPX1</i>	F: ACTACCCAACCGTGAGCGAAGA	R: TGCCATGCCAGTCGGACCAT
<i>AtGOLS1</i>	F: ATGGAGTCACACGCCGCAATAC	R: TGACGCCATAACATCGCAAGGA
<i>AtACT2</i>	F: GAAATCACAGCACTTGCACC	R: AAGCCTTTGATCTTGAGAGC

2.10. Data Analysis

Statistical analysis was performed using one-way analysis of variance (one-way ANOVA) with Excel 2021 and SPSS 19.0 software, with a significance threshold set at $p < 0.05$. All graphs were generated using Origin 2021.

3. Results

3.1. Identification of Transgenic *Arabidopsis thaliana*

In the early stage of the study, the *AgDREBA6b* gene (GenBank accession number: OR727346) was cloned and backfilled into the *dreb* mutant plants of *Arabidopsis thaliana*. Transgenic plants (*35S:AgDREBA6b/dreb*) were obtained from the T₂ generation. GUS staining analyses were performed on the T₂ generation of positive plants, the *dreb* mutant, and WT plants (Figure 1). The results showed that *35S:AgDREBA6b/dreb* plants turned blue, while the *dreb* mutant and WT plants did not. Additionally, PCR identification revealed the presence of the target band of *AgDREBA6b* in the transgenic *Arabidopsis thaliana* (Figure S1). These results indicate that *35S:AgDREBA6b/dreb* transgenic positive plants have been successfully obtained.

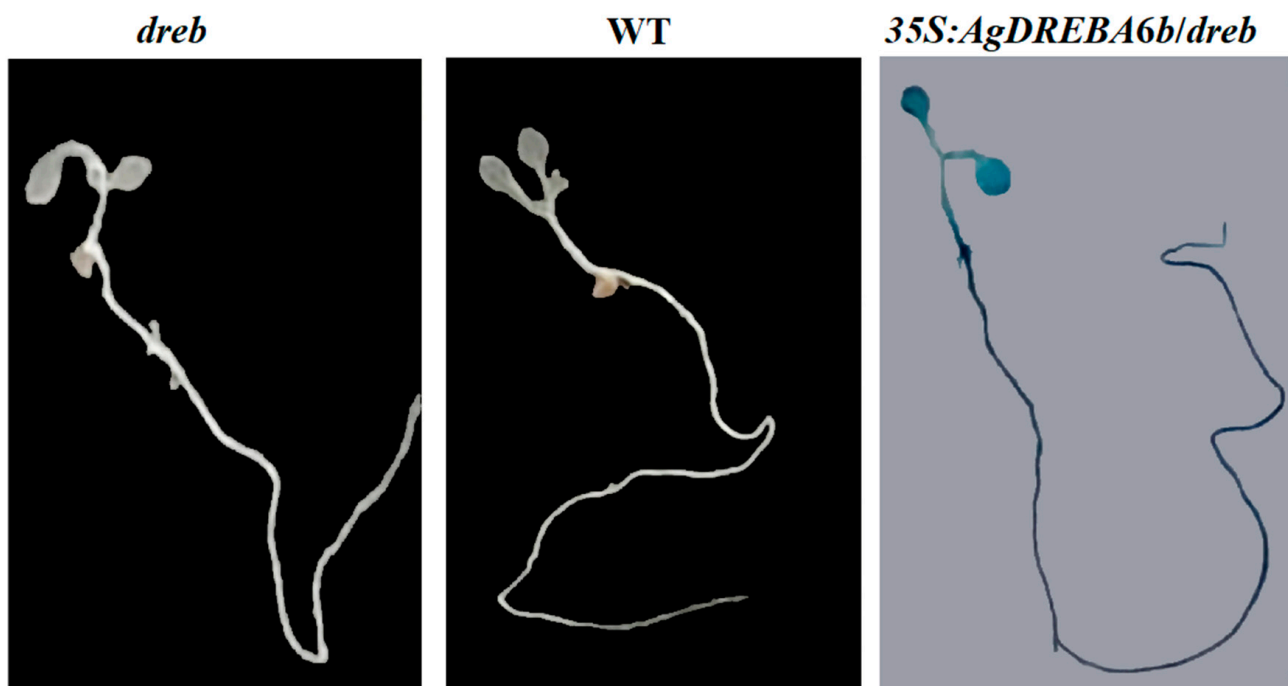


Figure 1. The GUS staining of transgenic T₂ generation plants of *Arabidopsis thaliana* with *AgDREBA6b* gene. From left to right: *dreb* mutant, WT plants, and *35S:AgDREBA6b/dreb* *Arabidopsis* plants.

3.2. The Phenotype and Leaf Anatomy of *Arabidopsis* Plants Under Heat Stress

As shown in Figure 2, the strains of *Arabidopsis thaliana* exhibited good growth and no significant differences before high-temperature treatment. After 24 h of high-temperature treatment, the *dreb* mutant plants showed the most severe leaf damage, followed by the WT plants. In contrast, the *35S:AgDREBA6b/dreb* plants exhibited the least leaf damage and the lowest degree of yellowing and wilting. Further leaf dissection revealed that the number and degree of stomatal openings in the *35S:AgDREBA6b/dreb* plants were significantly higher than those in the WT and *dreb* mutant plants (Figure 3). These results suggest that the *AgDREBA6b* gene may enhance heat stress tolerance through its regulatory mechanisms. Consequently, heat stress did not significantly damage the leaves of the transgenic plants, which retained well-preserved and undamaged stomata, unlike the damaged leaves of the WT plants.

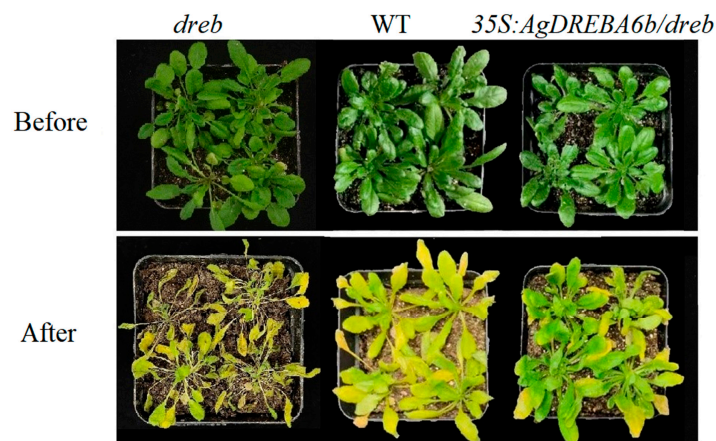


Figure 2. Growth of *dreb* mutant, WT, and *AgDREBA6b/dreb* under high-temperature treatment. From left to right: the Arabidopsis mutant plants with knockdown of *dreb*, WT plants of Arabidopsis, and *35S:AgDREBA6b/dreb* plants of Arabidopsis T2 generation, respectively.

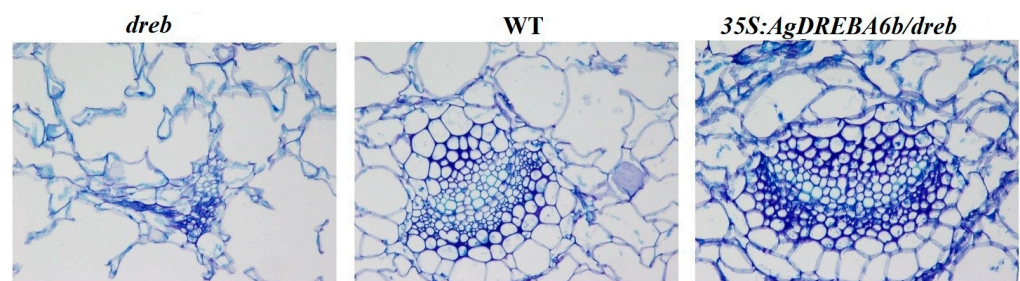


Figure 3. Stomatal anatomy of leaves of *dreb* mutant, WT, and *35S:AgDREBA6b/dreb* in Arabidopsis after 4 h of high-temperature treatment.

3.3. Effect of Heat Stress on Chlorophyll Content and Chlorophyll Fluorescence Properties of Leaves of Transgenic Plants

The content of chlorophyll, which serves as the material basis for energy conversion in photosynthesis, is an important indicator of crop senescence and photosynthetic capacity. To evaluate the photosynthetic performance of transgenic *Arabidopsis thaliana* plants, leaf chlorophyll content and chlorophyll fluorescence characteristics were determined in this study. As shown in Figure 4, the results indicated that the chlorophyll content at 0 h was higher in *35S:AgDREBA6b/dreb* plants than in the other two genotypes. Among the chlorophyll fluorescence characteristics, PSII did not differ significantly among the three strains, but dP was higher in *35S:AgDREBA6b/dreb* plants than in the other two. In contrast, the overexpression plants exhibited significantly lower non-photochemical quenching than WT plants. This may be because the overexpressing plants do not activate the NPQ pathway when unstressed. Under high-temperature stress, the intensity of photosynthesis in *35S:AgDREBA6b/dreb* transgenic Arabidopsis plants was significantly stronger than that in WT and *dreb* mutant plants. Specifically, while *dreb* mutant and WT plants showed no significant differences in chlorophyll a and chlorophyll b contents, the chlorophyll a, chlorophyll b, and total chlorophyll contents in *35S:AgDREBA6b/dreb* plants were increased by 3 mg/L, 2.3 mg/L, and 3 mg/L, respectively, compared with WT plants. In terms of PSII primary light energy conversion efficiency, chlorophyll non-photochemical burst coefficient, and chlorophyll photochemical burst coefficient, the values for the *35S:AgDREBA6b/dreb* transgenic Arabidopsis plants were 2-fold, 1.3-fold, and 1.5-fold higher than those for the *dreb* mutant plants, respectively. These results indicate that *35S:AgDREBA6b/dreb* Arabidopsis plants exhibited higher chlorophyll content and photosynthesis-related indices under high-temperature stress.

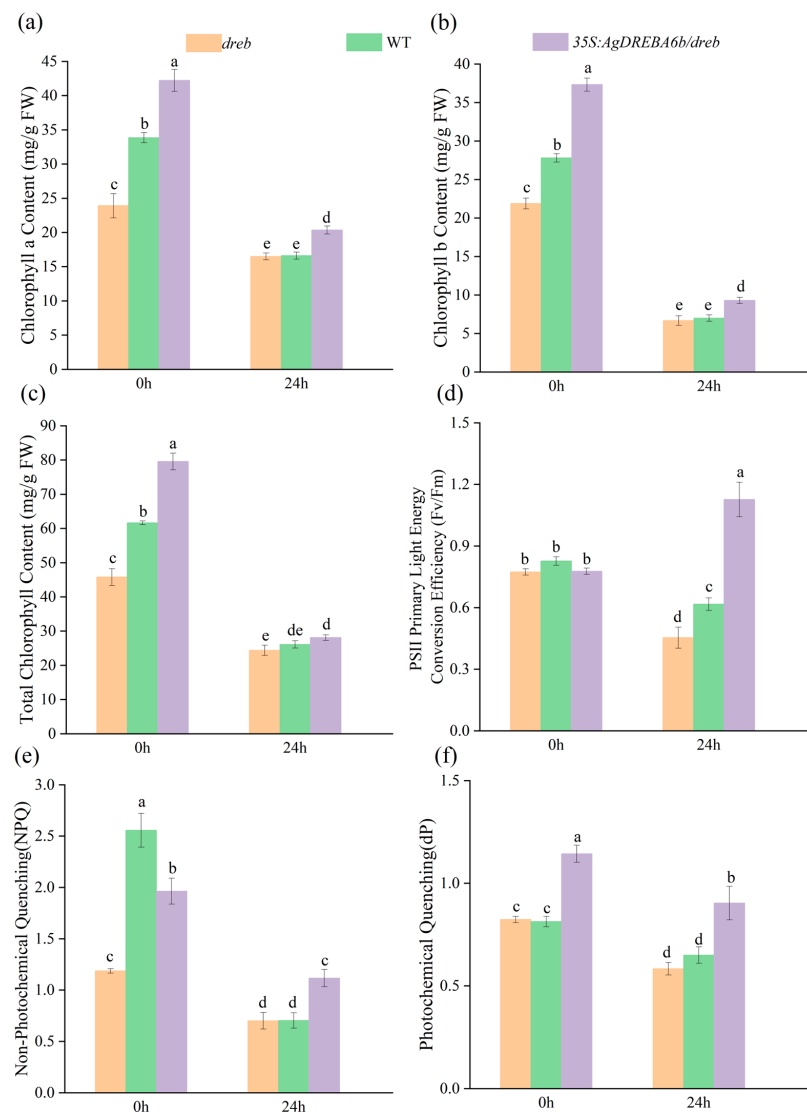


Figure 4. Chlorophyll a content and chlorophyll fluorescence characteristics of *dreb* mutant, WT, and 35S:AgDREBA6b/*dreb* transgenic Arabidopsis plants after 0 h and 24 h of high-temperature treatment. (a) Chlorophyll a content of the *dreb* mutant, WT, and 35S:AgDREBA6b/*dreb* transgenic Arabidopsis. (b) Chlorophyll b content of the *dreb* mutant, WT, and 35S:AgDREBA6b/*dreb* transgenic Arabidopsis. (c) Total chlorophyll content of the *dreb* mutant, WT, and 35S:AgDREBA6b/*dreb* transgenic Arabidopsis. (d) PSII primary light energy conversion efficiency in Arabidopsis *dreb* mutants, WT, and 35S:AgDREBA6b/*dreb* transgenic plants. (e) Non-photochemical quenching of the *dreb* mutant, WT, and 35S:AgDREBA6b/*dreb* transgenic Arabidopsis. (f) Photochemical quenching of the *dreb* mutant, WT, and 35S:AgDREBA6b/*dreb* transgenic Arabidopsis. Letters (a–e) on the vertical bars indicate significant differences at the 0.05 level. Each gene was tested with three biological replicates.

3.4. Effects of Heat Stress on Proline and Antioxidant Enzyme Activity in Transgenic Plants

To assess whether the overexpression of AgDREBA6b enhances heat stress tolerance in transgenic plants, we measured several stress tolerance indicators, including free Pro, MDA, CAT, SOD, and POD. As shown in Figure 5, no significant differences were observed in these indicators among *Arabidopsis thaliana* plants at 0 h. However, after 24 h of high-temperature treatment, the activities of Pro, MDA, CAT, SOD, and POD were significantly higher in the leaves of 35S:AgDREBA6b/*dreb* plants compared to the *dreb* mutant and WT plants. Notably, the Pro content in transgenic 35S:AgDREBA6b/*dreb* plants increased 7-fold and 3.5-fold compared to the *dreb* mutant and WT plants, respectively. This suggests that the transgenic plants can maintain intracellular osmotic pressure more effectively by

increasing Pro accumulation, thereby preventing excessive cell dehydration and structural damage. Additionally, the CAT content in transgenic plants was 4-fold and 2.8-fold higher than in the *dreb* mutant and WT plants, respectively, while POD enzyme activity was 4-fold and 1.9-fold higher. Although the SOD enzyme activity showed less variation, it was still 1.29 and 1.12 times higher in transgenic plants than in the *dreb* mutant and WT plants, respectively. The MDA content was also elevated, being 2 and 1.1 times higher in transgenic plants than in the *dreb* mutant and WT plants. These findings indicate that the introduction of the *AgDREBA6b* gene into the *dreb* Arabidopsis mutant enhances Pro content and antioxidant enzyme activity, reduces ROS accumulation, limits cell damage, and, ultimately, improves heat tolerance in transgenic Arabidopsis.

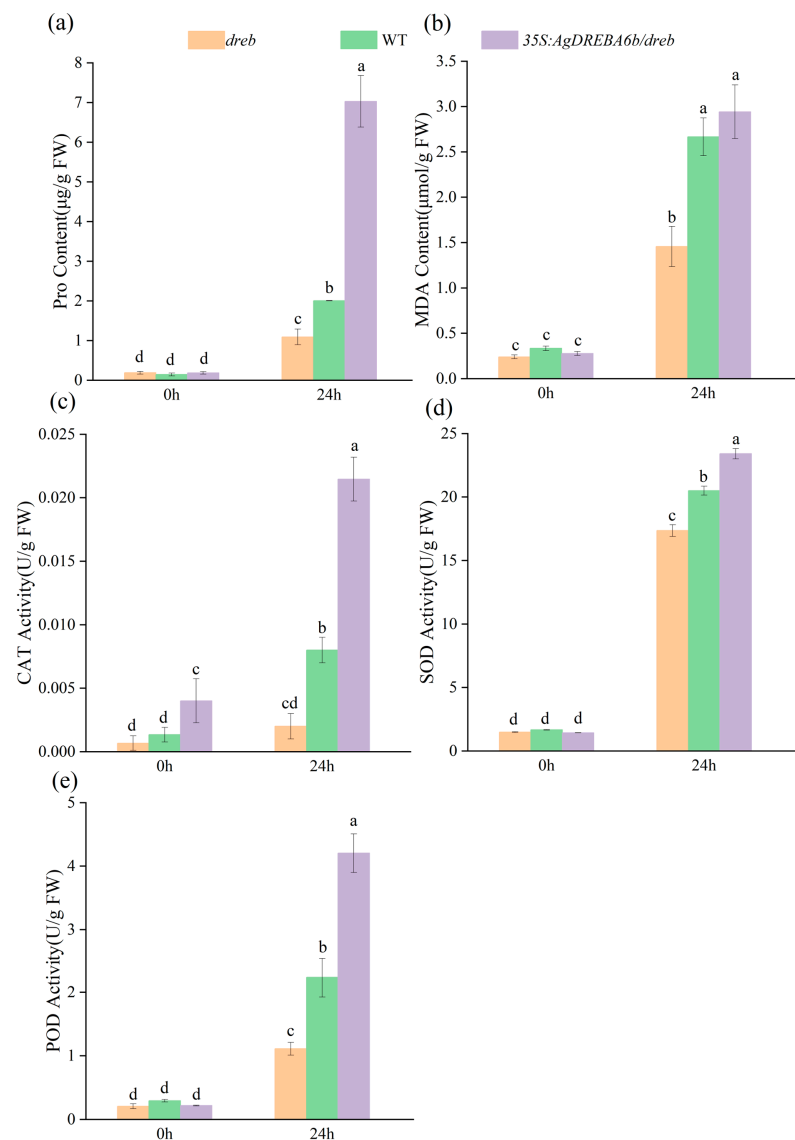


Figure 5. Determination of physiological indicators after 0 h and 24 h of high-temperature treatment in *dreb* mutant, WT, and 35S:AgDREBA6b/*dreb* Arabidopsis plants. (a) Pro content of the *dreb* mutant, WT, and 35S:AgDREBA6b/*dreb* transgenic Arabidopsis. (b) MDA content of the *dreb* mutant, WT, and 35S:AgDREBA6b/*dreb* transgenic Arabidopsis. (c) CAT activity in the *dreb* mutant, WT, and 35S:AgDREBA6b/*dreb* transgenic Arabidopsis plants. (d) SOD activity in the *dreb* mutant, WT, and 35S:AgDREBA6b/*dreb* transgenic Arabidopsis plants. (e) POD activity in the *dreb* mutant, WT, and 35S:AgDREBA6b/*dreb* transgenic Arabidopsis plants. Letters (a–d) on the vertical bars indicate significant differences at the 0.05 level. Each gene was tested with three biological replicates.

3.5. Expression Patterns of Four High-Temperature-Associated Genes Under Heat Stress

To determine the function of *AgDREBA6b* under heat stress induction, this study investigated the expression patterns of four high-temperature-responsive genes under different stress treatment times using qPCR. As shown in Figure 6, the results showed that the expression of the four related genes in *35S:AgDREBA6b/dreb* plants showed an increasing trend with the increase in treatment time, and the expression increased rapidly after 12 h and remained at a high level. Moreover, the expression of *AtHSP70-1*, *AtAPX1*, and *AtGOLS1* genes was higher than that of WT and *dreb* mutant plants, while the expression of *AtHSP98.7* gene was higher in overexpressed plants at 4 h and lower than that of WT plants thereafter. After 24 h of high-temperature treatment, *AtHSP70-1* gene expression was 2.3-fold and 1.3-fold higher than that of *dreb* and WT, respectively, *AtAPX1* gene expression was 3.3-fold and 1.9-fold higher than that of *dreb* and WT, respectively, and *AtGOLS1* gene expression was 3.8-fold and 1.7-fold higher, respectively. The results indicated that all four relevant genes in *35S:AgDREBA6b/dreb* transgenic plants responded to high-temperature stress, which might be related to their functions.

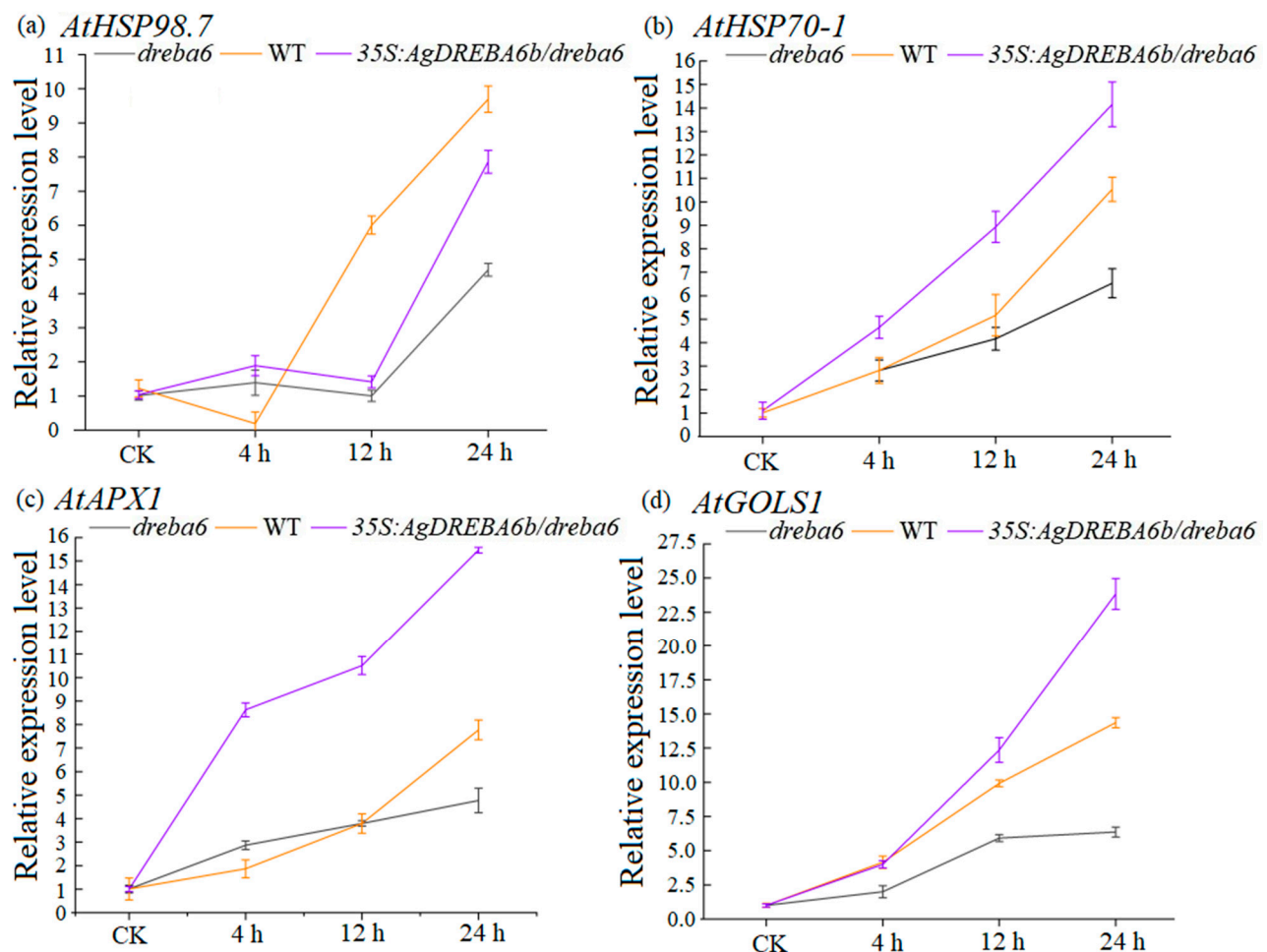


Figure 6. Relative expression of the four genes after high-temperature treatment. (a) Relative expression of the *AtHSP98.7* gene. (b) Relative expression of the *AtHSP70-1* gene. (c) Relative expression of the *AtAPX1* gene. (d) Relative expression of the *AtGOLS1* gene.

4. Discussion

High-temperature stress involves a variety of plant metabolism characteristics and complex processes of physiology and biochemistry, which is one of the main factors affecting plant growth and development, quality, and yield. Dehydration response element

binding protein (DREB) can play an important role in response to various abiotic stresses, especially in response to high-temperature stress, by regulating the expression of stress-related genes [22].

In this study, we successfully constructed Arabidopsis transgenic plants overexpressing the *AgDREBA6b* gene using genetic transformation technology and established a model of the *AgDREBA6b* regulatory network in response to heat stress (Figure 7). Under high-temperature stress, although all Arabidopsis plants showed heat damage symptoms such as leaf yellowing and wilting, the transgenic plants with *AgDREBA6b* were significantly less affected than the WT plants and the *dreb* mutant. This result indicated that the *dreb* mutant plants were more sensitive to heat stress, confirming that overexpression of the *AgDREBA6b* gene enhances heat tolerance in plants, which is consistent with the previous studies [23,24]. To validate our hypothesis, we further analyzed the leaf anatomical structures and found that stomatal opening of Arabidopsis plants overexpressing *AgDREBA6b* was significantly larger than that of WT and *dreb* mutant plants under high-temperature stress conditions, which suggested that *AgDREBA6b* may enhance the heat tolerance of the plants by regulating stomatal movement [25]. These results are consistent with the phenotype under heat stress and further demonstrate the positive role of *AgDREBA6b* in Arabidopsis plants in response to heat stress.

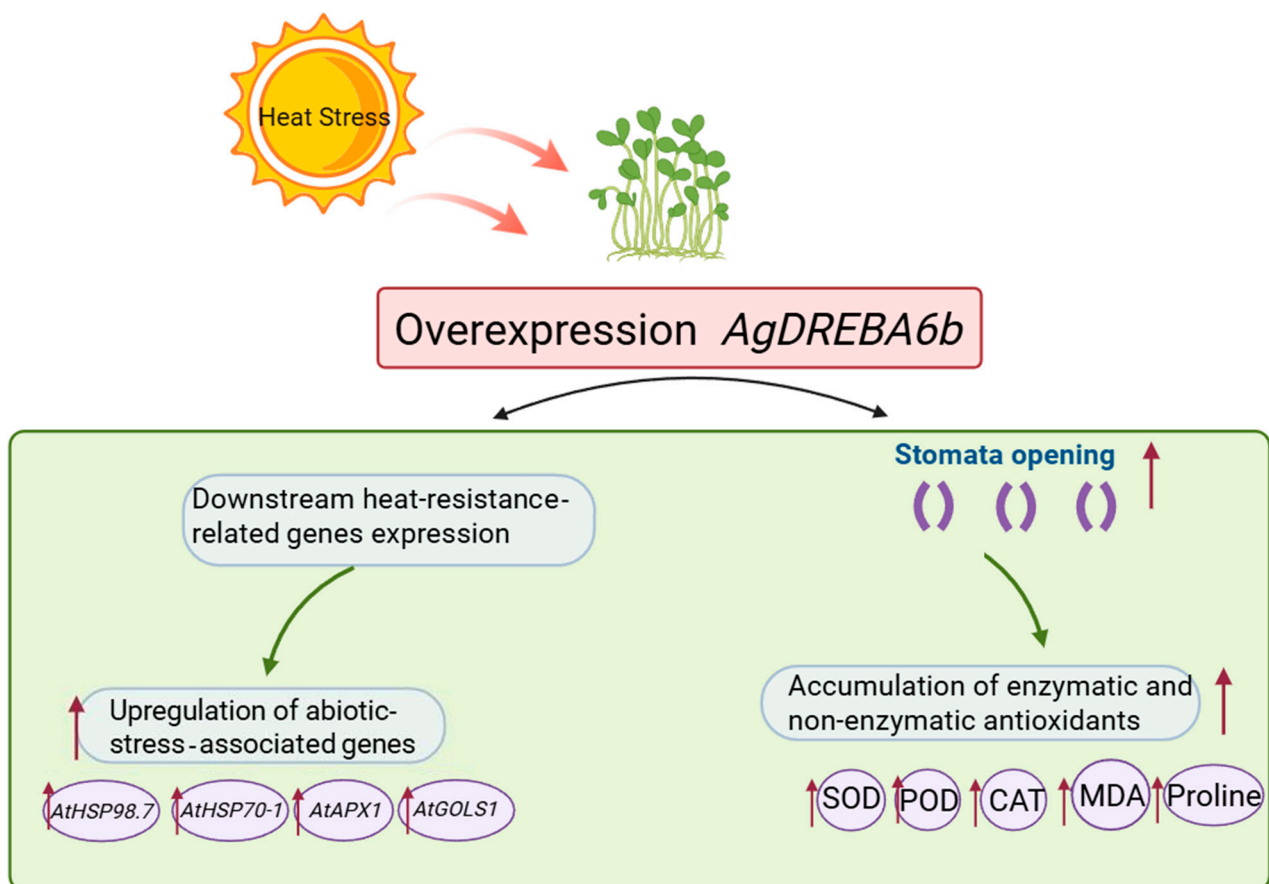


Figure 7. Module-centered pattern of the transcriptional regulatory network of *AgDREBA6b* in response to heat stress; purple arrows indicate increased.

Chlorophyll plays a direct role in numerous metabolic processes in plants, and its degradation directly impairs plant productivity and diminishes growth and stress tolerance [26]. In this study, we found that the chlorophyll a, chlorophyll b, and total chlorophyll contents in the transgenic plants were significantly higher than those of WT and mutants un-

der high-temperature stress, suggesting that the *AgDREBA6b* gene may be able to maintain the photosynthetic capacity by mitigating chloroplast damage. Chlorophyll fluorescence is an important index for evaluating the stability of the photosynthetic system and plant heat tolerance, which can reflect the efficiency of the absorption, transmission, distribution, and conversion of light energy in the plant photosystem, and it can respond to the stress first, which can help to clarify the site and degree of damage by adversity stress in the structure of photosynthesis [27]. PSII, as an important parameter reflecting the maximal potential photosynthetic capacity of the plant, had values that were significantly higher in the transgenic strain and significantly higher than those of the WT and the mutant, indicating that the transgenic plants were more efficient in converting light energy under heat stress, which could improve the heat tolerance of the plants by enhancing the photoprotective mechanism. Zha et al. [28] observed that the heterologous expression of *VvDREB2c* in *Arabidopsis thaliana* enhanced growth, drought tolerance, and heat tolerance. The *VvDREB2c* overexpressing lines dissipated excess light energy and converted it into heat, thereby reducing photodamage and enhancing photoprotection, ultimately improving tolerance to high temperatures. This is consistent with the results of the present study. In conclusion, overexpression of the *AgDREBA6b* gene not only reduces chlorophyll degradation and maintains PSII activity and light energy utilization efficiency, but also enhances the heat dissipation capacity, thus improving the heat tolerance and photosynthetic capacity of plants as a whole.

The plants will activate multiple regulatory mechanisms such as antioxidant enzyme systems, osmoregulation, and signaling pathways under high-temperature stress. Among these, the antioxidant enzyme system is crucial for scavenging reactive oxygen species (ROS) and protecting cells from oxidative damage. For example, the activities of SOD, POD, and CAT were enhanced by *AtDREB1C* and *SIDREBA4*, which, in turn, improved heat tolerance in transgenic *Arabidopsis thaliana* and tomato plants [29]. Overexpression of the *VuDREB2A-CA* gene in cowpea increased the antioxidant and osmoregulatory capacities of plants under heat stress [30]. In this study, we found that overexpression of *AgDREBA6b* *Arabidopsis thaliana* plants led to significantly higher SOD, CAT, and POD activities under high-temperature conditions. This result was consistent with the results of previous studies, indicating that DREB transcription factors generally function to regulate antioxidant enzyme systems. The enhanced activities of these enzymes effectively scavenged the high-temperature-induced generation of reactive oxygen species (ROS) and alleviated the cellular damage caused by oxidative stress. The contents of MDA were significantly higher in the transgenic plants than in the WT and the *dreB* mutant. MDA, a marker of membrane lipid peroxidation, was positively correlated with the degree of cell membrane damage [31]. Ren M et al. [32] reported that *CIRAP2.4*, a member of the DREB subfamily and the one most closely related to *AT1G22190*, was introduced into plants via *Agrobacterium*-mediated leaf disc transformation, yielding four overexpressing lines (OX-1, OX-2, OX-7, and OX-8). In these overexpressing lines, the activities of superoxide dismutase (SOD) and peroxidase (POD), as well as the content of proline, were higher than those in the wild type (WT), while the electrical conductivity and malondialdehyde (MDA) content were reduced. These findings indicate that plants overexpressing *CIRAP2.4* exhibit enhanced tolerance to cold stress. Previous studies have shown that plant stress increases Pro content, which has a number of important roles, including acting as an osmoprotectant, protecting the stability of cellular components and enzymes, scavenging ROS, and helping to maintain redox homeostasis in stressed plant cells [33]. Lotus showing overexpression of *LjDREB2B* lines had significantly higher Pro content than WT plants [34]. Transgenic *Arabidopsis thaliana* overexpressing the *MaDREB20* gene in banana showed higher survival, leaf relative water content, and Pro content for enhanced tolerance under high temperature,

drought, and the combined effects of drought and high temperature [35]. In the present study, compared to the mutant *dreba6* plants and WT plants, the *DREBA6b*-overexpression mutant plants produced higher concentrations of free Pro. This study was consistent with the results of previous studies. It indicated that the surge of Pro in transgenic plants provided a defense mechanism against adversity stress. In conclusion, the *AgDREBA6b* gene enhances heat tolerance in plants by decreasing ROS levels and alleviating membrane lipid peroxidation damage.

Transcription factors can regulate downstream high-temperature-responsive genes and related metabolic pathways and play important roles in the response to high-temperature stress. For instance, upregulation of the expression of heat, salt, and drought stress-responsive genes was observed in *DREB2A* transgenic Arabidopsis, resulting in higher abiotic stress tolerance [36]. *MsDREB2C* and *AtDREB2C* of Arabidopsis enhanced their heat tolerance by increasing the expression of *AtHsfA3* and *SIHSP90* in the transgenes [37]. In this study, the expression of *AtHSP70-1*, *AtAPX1*, and *AtGOLS1* high-temperature-responsive genes was significantly upregulated in *AgDREBA6b*-overexpressing Arabidopsis transgenic plants, with higher expression than that of WT and *dreba* mutant plants, whereas expression of the *AtHSP98.7* gene was higher in overexpressing plants at 4 h, and lower than that of WT plants thereafter. This may be because *AtHSP70-1*, *AtAPX1*, and *AtGOLS1* promoters contain activating DRE/CRT elements that are directly positively regulated by *AgDREBA6b*. Whereas *AtHSP98.7* may mainly depend on HSFs and non-DREB family regulation, the exact reason needs to be further verified. Previous studies have shown that *DREB* genes from other different species display heat tolerance, such as *EcDREB2A* in tobacco, *CmDREB6* in *chrysanthemum*, *LIDREB1G* in long lily [38], and *TaDREB3* in wheat [13], which is similar to the results of the present study, suggesting that *AgDREBA6b* may be an upstream transcription factor localized in the nucleus, which enhances heat tolerance by upregulating the expression of a series of stress-related genes to enhance heat tolerance in transgenic Arabidopsis, thereby mediating physiological processes associated with stress tolerance. Hence, transgenic Arabidopsis plants overexpressing *AgDREBA6b* exhibited enhanced heat tolerance, and the expression of physical metrics and stress-related genes further validated their function in coping with heat stress.

5. Conclusions

In this study, the *AgDREBA6b* gene from celery was transformed into *Arabidopsis thaliana* for overexpression, which initially revealed the role of *AgDREBA6b* gene in drought stress response. The leaves of transgenic *Arabidopsis thaliana* overexpressing *AgDREBA6b* showed less yellowing and wilting under high-temperature stress, and the degree of stomatal opening of leaf cells was significantly greater than that of WT plants and *dreba* mutant plants. The results of the physiological experiments showed that the transgenic *Arabidopsis thaliana* showed higher antioxidant enzymes and Pro and chlorophyll contents, and the relative expression levels of most high-temperature related genes were higher, indicating that *AgDREBA6b* overexpression lines could enhance the heat tolerance of plants by improving the activity of antioxidant enzymes and decreasing the degree of membrane lipid per-oxidation, which provided a basis for elucidating the molecular mechanism of the DREB gene family in response to high-temperature stress in plants. These results lay a foundation for clarifying the molecular mechanism of the DREB gene family in response to high-temperature stress in plants and provide theoretical support for exploring the cultivation of high-temperature-tolerant varieties of celery.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agronomy15071565/s1>, Figure S1: T2 generation of PCR assay for *Arabidopsis thaliana* transcribed with *pCAMBIA1301-AgDREBA6b* gene.

Author Contributions: Conceptualization, F.X. and Z.Y.; data curation, S.Y. and Y.L.; validation, F.X. and Z.P.; writing—original draft preparation, F.X. and S.Y.; writing—review and editing, F.X., R.L. and Z.Y.; funding acquisition, R.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Ruiheng Lv, through the Plan for Tackling Key Scientific and Technological Problems in Key Areas of the Xinjiang Production & Construction Corps (Grant No.: 2021AB022).

Data Availability Statement: The data supporting the reported results in this study are available within the article and its Supplementary Materials. Additionally, the genetic sequences related to the *AgDREBA6b* gene discussed in this study are available in the GenBank database under the accession number OR727346.

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

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