horticulturae

MDPI

Article

BrDHC1, a Novel Putative DEAD-Box Helicase Gene, Confers Drought Tolerance in Transgenic *Brassica rapa*

Gangqiang Cao [1,†], Huihui Gu [1,†], Wenjing Jiang [1,2], Zhaoran Tian [1], Gongyao Shi [1], Weiwei Chen [1], Baoming Tian [1], Xiaochun Wei [2], Luyue Zhang [1], Fang Wei [1,*] and Zhengqing Xie [1,*]

[1] Henan International Joint Laboratory of Crop Gene Resources and Improvements, School of Agricultural Sciences, Zhengzhou University, Zhengzhou 450001, China
[2] Institute of Horticulture, Henan Academy of Agricultural Sciences, Graduate T and R Base of Zhengzhou University, Zhengzhou 450002, China
* Correspondence: fangwei@zzu.edu.cn (F.W.); zqxie@zzu.edu.cn (Z.X.)
† These authors contributed equally to this work.

Abstract: Drought can seriously hinder the growth of plants, resulting in reduced crop yield and quality. At present, the tolerance of DEAD-box helicases (*DHC*) to abiotic stresses, such as drought, high salinity, low temperature, and high temperature, has been confirmed in a variety of plants; therefore, using DEAD-box helicases to develop stress-resistant plants has great application prospects. In this study, *Brassica rapa* was used as a model to explore the response of the *BrDHC1* gene to drought stress by creating RNA interference and overexpressing lines in *B. rapa*. The mechanism of *BrDHC1* involved in drought resistance was revealed by the analysis of morphological characteristics, physiological indicators, and expression analysis of related stress response genes. The results showed that the overexpression of the *BrDHC1* gene was more conducive to enhancing the resilience of plants under drought stress in *B. rapa*. Taken together, these results confirmed *BrDHC1* as a newly identified DEAD-box helicase gene that could actively regulate plant growth and development under drought stress in *B. rapa*.

Keywords: *Brassica rapa*; DEAD-box helicase; *BrDHC1*; drought stress; chloroplast

Citation: Cao, G.; Gu, H.; Jiang, W.; Tian, Z.; Shi, G.; Chen, W.; Tian, B.; Wei, X.; Zhang, L.; Wei, F.; et al. *BrDHC1*, a Novel Putative DEAD-Box Helicase Gene, Confers Drought Tolerance in Transgenic *Brassica rapa*. *Horticulturae* **2022**, *8*, 707. https://doi.org/10.3390/horticulturae8080707

Academic Editor: Simone Landi

Received: 6 July 2022
Accepted: 2 August 2022
Published: 5 August 2022

1. Introduction

Plants are constantly exposed to various climatic disturbances, resulting in plants facing different types of external abiotic stress environments, such as drought, high salinity, high temperature, cold, etc., which will reduce the yield of crops [1–6]. As one of the most important abiotic stresses, drought limits plant growth, development, and productivity. In arid environments, the seeds will suffer from insufficient water supply, resulting in a decrease in the germination potential and germination rate of the seeds. In the early stage of water shortage, the water utilization rate of the root system is low, and the leaves cause water loss through transpiration, which in turn affects plant cells. The osmotic and ionic balance affects plant growth [7]. To cope with the arid environment, scientists have conducted extensive studies to reveal the mechanisms by which plants respond to drought, involving morphological characteristics, physiology, biochemistry, and molecular regulation [8]. The drought resistance methods of plants are divided into three types, drought-avoidant, drought-tolerant, and drought-resistant types. Plants generally absorb water efficiently from soil through changes in root structure and stomatal aperture to reduce water loss by transpiration, and cellular metabolism alteration to adapt to water loss [9]. The root system is the main organ for plants to detect water changes, and the length and surface area of the root system determine the absorption capacity of soil water [10]. Light energy can improve photosynthetic efficiency, thereby increasing plant yield with high chlorophyll content, which has also been considered as a physiological indicator that plants have certain drought resistance [11,12].

At the cellular level, drought stress could trigger the excessive accumulation of reactive oxygen species (ROS), affect cell homeostasis, lead to oxidative stress, and cause oxidative damage to plants, which are mainly manifested as a decrease in photosynthetic efficiency, cell damage caused by peroxidation, and cell membrane stability [13–16]. Enzymatic antioxidants could scavenge ROS, reduce the damage to plants, and enhance tolerance to stress. Superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), guaiacol peroxidase (GPX), and ascorbate peroxidase (APX) are common enzymatic antioxidants [17]. In addition, under drought stress, the accumulation of proline and soluble sugar could help reduce water loss in the body. To cope with water stress, drought-tolerant plants could maintain the osmotic balance of cells through osmotic regulation by increasing the content of soluble sugar, proline, betaine, and amino acids, thus reducing the osmotic potential and improving the water retention capacity of cells [18]. Therefore, the activity of antioxidant protective enzymes and the content of chlorophyll, proline, soluble sugar, etc., in plants, are related to alleviating drought damage, which can be used as physiological indicators of plant drought resistance.

DEAD-box helicase is the largest family of helicases, including RNA helicases and DNA helicases, but most are RNA helicases [19], which mainly utilize the energy obtained from the ATP hydrolysis destruction of hydrogen bonds between nucleic acid strands [20], and plays an important role in almost all biological processes, such as DNA and RNA replication, repair, recombination, transcription, and protein translation [21–23]. DEAD-box helicases have been widely found in prokaryotic and eukaryotic organisms, namely 58 in Arabidopsis [24], 50 in rice [24], 58 in longan [25], 29 in wild sweet potato [26], 80 in soybean [27], and 42 in tomato [28]. DEAD-box helicases usually act as pressure sensors, regulators, or effectors in diverse biological processes during plant growth and development, which also regulate plant responses to abiotic stresses, such as salt stress, osmotic stress, drought stress, cold stress, and responses to phytohormones, by participating in stress-induced pathways [29–31]. The research on plant DEAD-box helicases is mainly focused on the dicot- and monocotyledonous model plants, e.g., Arabidopsis and rice [32,33]. Over the years, studies on DEAD-box helicases have found that many DEAD-box helicases gyrase genes not only confer plants tolerance to a single stress, but also participate in multiple stress responses. Some genes promote plant growth and development through positive regulation to enhance plant tolerance to stress, whereas some negatively regulate plant growth and development upon stress. The directional regulation shows the sensitivity of plants to different stresses.

At present, DEAD-box helicase has been proved to confer stress resistance in a variety of plants, and the research on its connection to drought and salt stress is the most extensive [26,28,30,32,33]. Therefore, it is ideal to use genetic engineering to improve plant stress resistance and productivity, and the DEAD-box helicase genes could be used as candidates to develop plants with higher stress resistance. For this purpose, the functional analyses of a DEAD-box helicase gene were studied regarding different aspects.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

The self-pollination and seed setting *Brassica rapa* DH lines (cxl-45-05) and *Nicotiana benthamiana* were grown in a growth chamber under 16 h/22 °C in light, 8 h/16 °C in darkness, and a relative humidity of 70%.

2.2. Collinearity and Evolution Analysis

The GFF structure information files and the FASTA information files for *Arabidopsis thaliana* and *B. rapa* were obtained from the BRAD database (http://brassicadb.org/brad/index.php, accessed on 8 March 2022). The verified sequences of *AtRH7*, *AtRH8*, *AtRH22*, *AtRH38/LOS4*, *AtRH42*, *AtRH53*, *STRS1*, and *STRS2* of *A. thaliana* [24], *OsRH42*, *OsRH53*, and *OsABP* of *Oryza sativa* [33,34], *GmRH* of *Glycine max* [35], *BrRH22*, and *BrRH37* of *B. rapa* [36], and *PDH45* and *PDH47* of peas [37,38] were selected for the evo-

lutionary analysis. Then the DEAD-box helicase genes of different species were identified by nucleotide and amino acid similarities to construct the evolutionary tree. All protein sequences of the above genes were downloaded from the NCBI online website (https://www.ncbi.nlm.nih.gov/, accessed on 8 March 2022), and MEGA11 was employed for the maximum likelihood (ML) phylogenetic tree construction [39], as well as the sequence similarity analysis of the homologues of Bra040707 between *B. rapa* and *A. thaliana*, and *N. benthamiana*. The Bra040707 gene is named according to the homologous relationship of members of the DEAD-box family and the localization of their proteins in *A. thaliana*. Collinear relationships of different DEAD-box genes were visualized with Circos 2.0 [40].

2.3. Cis-Acting Regulatory Elements Analysis

The promoter sequences (2000 bp upstream of the ATG start codon) for the *BrDHC1* gene and *BrRH22* gene were downloaded from the Ensembl plants database (http://plants.ensembl.org/index.htmL, accessed on 8 March 2022), and then *cis*-acting regulatory elements analysis was carried out with online PlantCARE website (http://bioinformatics.psb.ugent.be/webtools/PlantCARE/htmL, accessed on 8 March 2022). Finally, the results were visualized by the Microsoft PowerPoint Presentation software.

2.4. Subcellular Location and Production of Transgenic Plants

The subcellular localization prediction of At3g02060 (homologous gene of Bra040707 in *A. thaliana*) was through the ePlant online website (http://bar.utoronto.ca/eplant, accessed on 30 July 2022). To further explore the subcellular location of *BrDHC1*, the recombinant gene expression vector was constructed for a transient expression system in *N. benthamiana*, and the transient transformation was performed as previously described elsewhere [41]. The full-length coding sequence (CDS) of *BrDHC1* was first cloned into the pEASY-T1 vector for amplification, and then subcloned into the pCAMBIA1300-EGFP vector using *Pst*I and *Kpn*I restriction sites. Subsequently, the recombinants were transformed into *Agrobacterium tumefaciens* (EH105), and then infiltrated with the 4-week-old *N. benthamiana* leaves using a 1 mL needleless syringe.

The preparation of tobacco protoplasts requires gentle manipulation of 20 mL of enzymatic solution, 0.2 mol/L $CaCl_2$ solution, and 20% sucrose solution. After successful protoplast preparation, we aspirated a small amount of tobacco protoplasts on a glass slide and observed the green fluorescence signal of EGFP (488 nm green excitation light, 40× objective) and the spontaneous red fluorescence signal of chloroplasts using Leica laser confocal microscopy (Leica, Weztlar, Germany). Figures shown in the manuscript are shown as the same result of at least three repetitive experiments.

The *BrDHC1* overexpressing plants (OE-4, OE-6, OE-8, and OE-9) were obtained by transforming the *Agrobacterium* strain GV3101 containing the pCAMBIAsuper1300-*BrDHC1*-EGFP vector into *Brassica rapa* (cxl-45-05) through the floral dip method [42]. For the production of RNA interference BrDHC1 transgenic plants (RNAi-7, Ri-7, Ri-9 and Ri-10), 356 bp fragment of the cDNA of *BrDHC1* gene were recombined into the pHELLSGATE12 vector through BP recombination, and the resulting *Agrobacterium* GV3101 containing the recombinant RNAi-*BrDHC1* vector was transformed into *Brassica rapa* (cxl-45-05) using the same transformation method. The expression levels confirmingOE-*BrDHC1* transgenic plants (OE-4, OE-6 and OE-8) and RNAi-*BrDHC1* transgenic plants (Ri-7, Ri-9 and Ri-10) were used for further analysis.

2.5. Drought Stress Treatment and Phenotype Observation

In this study, more than 20 seeds of wild-type *B. rapa*, OE-*BrDHC1* lines (OE-4, OE-6, and OE-8) and RNAi-*BrDHC1* (Ri-7, Ri-9, and Ri-10) transgenic plants were placed on the 1/2 MS medium or 1/2 MS + 200 mM mannitol medium, and the root growth status of each line (at least five plants for individual line, wild-type, OE-*BrDHC1*, and RNAi-*BrDHC1* transgenic lines, three times with a similar result) was observed after 8 days of growth. Subsequently, for the three types of the 3-week-old plants (at least ten plants for

one individual line, three times with a similar result), when watering was needed for the plants, we performed natural drought stress treatment (without watering for 10 days), and then the physiological and biochemical indices of each plant were determined with at least three replicates, three times with a similar result.

2.6. Measurement of Stomatal Opening Rate

The rapid imprinting technique was employed for stomatal aperture analysis [43]. The abaxial leaf surfaces were covered with transparent nail polish and air dried for 2 h at room temperature. The nail polish imprints were then placed on glass cover slips and photographed under an Olympus fluorescence microscope BX53 (Olympus, Tokoyo, Japan) with 40× magnifications. The stomatal opening rate was evaluated by the ratio of open stomata to total stomata.

2.7. Relative Water Content (RWC) Determination

To determine the relative water content (RWC), the leaves detached from the *B. rapa* plants were immediately weighed as the fresh weight (FW). After determining the FW, the leaves were kept in the distilled water for 12 h, and weighted as the turgid weight (TW). After drying the samples at 65 °C for 12 h, the dry weight (DW) was recorded. The (FW-DW)/(TW-DW) × 100% formula is used for the calculation of RWC [44]. Figures shown in the manuscript show the same result of at least three repetitive experiments, and more than three plants for each line.

2.8. Determination of Chlorophyll Content

The method mentioned by Guo et al., with slight modification, was employed to determine the content of chlorophyll a (Chla) and chlorophyll b (Chlb) in the leaves [45]. Three or more plants with the same growth stage of each line were selected for evaluation, and the leaves from the same area were taken and rinsed. Then, each 50 mg of fresh leaf sample was weighed and placed in a mortar, and thoroughly ground into a 2 mL centrifuge tube containing 1 mL solution of 80% acetone (ready until being used). The sample was labeled and subjected to 4 °C light-absorbing shock, and shaken for about 4 h until the leaf blade was completely decolored, and then submitted to a low temperature high-speed centrifuge at 4 °C at 9000 rpm for 2 min. We then took 500 µL of the sample supernatant and determined the absorbance of the sample at A_{663} and A_{647} using a UV spectrophotometer, took 80% acetone as a blank control, and used Chl a = $12.25 \times A_{663} - 2.79 \times A_{647}$; Chl b = $21.50 \times A_{647} - 5.10 \times A_{663}$ as the formula to calculate the content of Chl a and Chl b, respectively. The determination of antioxidant enzyme activity, osmotic regulator content, and MDA content was carried out in accordance with Griess reagent instructions.

2.9. Quantitative Real-Time PCR Analysis

The total RNA from *B. rapa* was extracted with the Plant Total RNA Isolation Kit Plus (Foregene, Chengtu, China), using 100 mg of fresh leaf tissue. The purified RNA was measured with an ultra-micro spectrophotometer, Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). The HiFiScript cDNA synthesis kit (CWBio, Beijing, China) was used for the cDNA synthesis. The Lunar® Universal qPCR Master Mix (NEB, Beijing, China) was used for qRT-PCR analysis on the Roche Light Cycler 480 Real-time Fluorescent Quantitative PCR system (Roche, Shanghai, China). The PCR conditions were 94 °C for 30 s, 40 cycles at 94 °C for 10 s, and 58 °C for 30 s, followed by a melting curve to determine the specificity of the amplification. The $2^{-\Delta\Delta Ct}$ method was employed for the calculation of relative expression levels of different genes [46], and the β-actin gene was used as the internal control, with three biological replicates. All the primers used are listed in Table A1 (Appendix A).

2.10. Statistical Analysis

More than ten replicates were grown for each type of plant, with a similar phenotype resulting at all times (more than three times). Each experiment of the parameters evaluated was carried out, with at least three independent biological replicates, three times with a similar result. All data graphs were analyzed using GraphPad Prism 5, and one-way analysis of variance (ANOVA) was employed to analyze the significance of differences between the experimental group and the control, and the Student's *t* test was applied. ** p value < 0.01 indicates very significant difference between the data, and * p value < 0.05 indicates significant difference.

3. Results

3.1. Evolutionary Analysis of Gene Collinear and DEAD-Box Helicase Members

The *Brassica* genus has undergone a unique whole-genome triplication (WGT) event after splitting from *A. thaliana*, so each single gene in *A. thaliana* should retained 3 copies in the *Brassica* plants. However, collinear analysis showed that the DEAD-box helicase member, *At3g02060*, retained only one copy in *B. rapa*, and is located in the scaffold sequence, which requires further refinement of genome assembly techniques (Figure 1A). TAIR database searching found that *Arabidopsis At3g02060* belongs to the DEAD-box protein family ATP-dependent DNA helicase, which has not been studied in Arabidopsis. Thus, it is speculated that *Bra040707* also belongs to the DEAD-box DNA helicase gene. It is named according to the homologous relationship of the DEAD-box family members and the localization of corresponding proteins. DEAD-box helicase genes are mainly involved in the responses to abiotic stress, so the DEAD-box helicases from Arabidopsis, rice, *B. rapa*, peanuts, and peas that have been identified as involved in plant abiotic stress were selected to construct a phylogenetic tree. The results showed that the BrDHC1 protein is closely related to the BrRH22 protein, suggesting that they may have similar functions (Figure 1B). A previous study showed that the BrRH22 protein is involved in salt, drought, and cold stress responses [36], indicating that BrDHC1 might also play a role in the abiotic stress response.

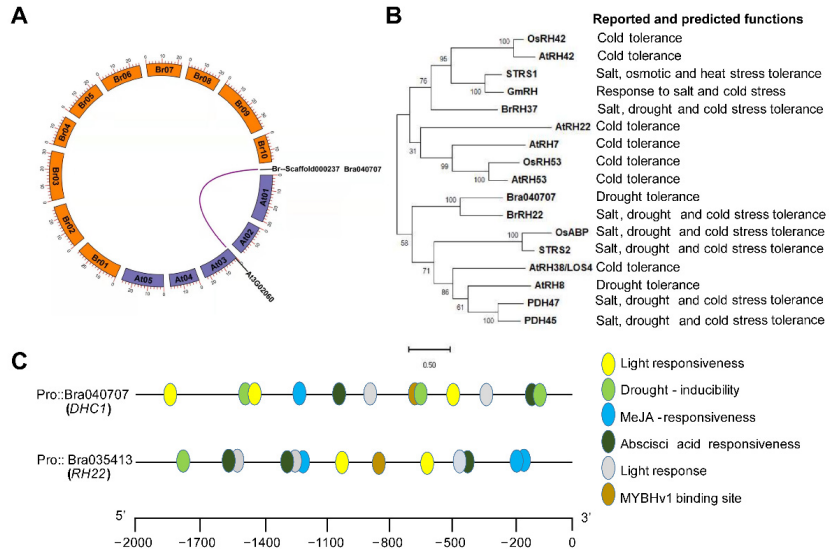

Figure 1. Collinear and phylogenetic analysis of *DHC1* genes, and *cis*-acting elements analysis of the promoters of the Dead-box helicases. (**A**) Collinearity of the *DHC1* gene between *Arabidopsis thaliana* and *Brassica rapa*. (**B**) Phylogenetic tree of the DEAD-box helicases from different species, constructed by MEGA 11. (**C**) The distribution of *cis*-acting regulatory elements on the promoters of *BrDHC1* and *BrRH22* genes.

For the sequence similarity analysis, we found that the percentage of similarity of the Bra040707 (BrDHC1) gene/protein with their homologous gene/protein (At3g02060) from

Arabidopsis thaliana was 90.17% (coding sequence), 95.33% (protein), and that from *Nicotiana tabacum* was 56.44% (coding sequence), 78.11% (protein), suggesting the high sequence (or function) conservation of DHC1 in Brassicaceae.

Promoter regulation can alter the expression of downstream genes, and the analysis of promoters plays an important role in studying gene expression and function [27]. In this study, the online tool PlantCARE was employed to analyze the *cis*-acting regulatory elements in the promoters of *BrDHC1* and *BrRH22* genes in *B. rapa* (Figure 1C). Three drought-responsive elements in the *BrDHC1* promoter and only one in the *BrRH22* were detected, suggesting that *BrDHC1* might play an important role in the drought stress response.

3.2. Expression Pattern Analysis and Subcellular Localization of BrDHC1 in B. rapa

The localization of gene expression products facilitates preliminary judgments about their function. Firstly, in this study, the total RNA of the roots, stems, young leaves (non-bolting stage), mature leaves (bolting stage), senescent leaves (senescence stage), immature buds, and mature buds of cabbage-type rapeseed (cxl-45-05) were extracted, respectively. The expression of *BrDHC1* in different sites was detected by qRT-PCR. The results showed that the highest expression amount of the *BrDHC1* gene was in the young leaves (unseen phase; Figure 2A), indicating its important role in plant leaf development.

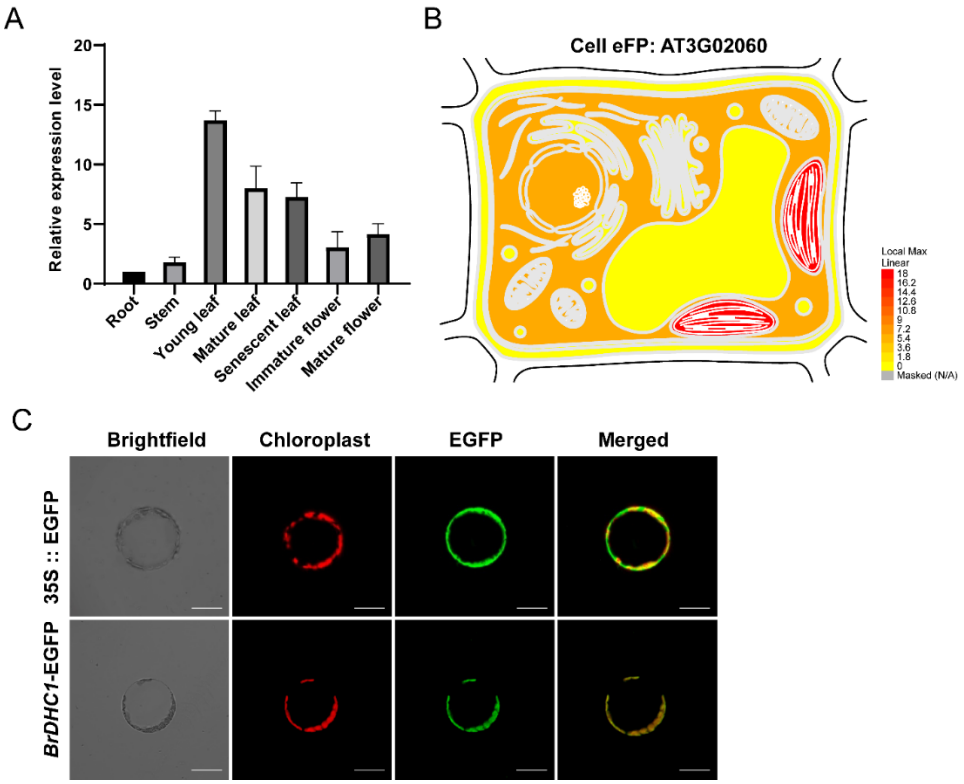

Figure 2. Expression and subcellular localization analysis of BrDHC1. (**A**) *BrDHC1* gene expression detected by real-time fluorescence quantitative PCR (data represent the means ± SD of 3 replicates). (**B**) Subcellular localization prediction of At3g02060 (homologous gene of Bra040707 in Arabidopsis) through ePlant. (**C**) Subcellular localization of BrDHC1 (fused *BrDHC1*-EGFP, pCAMBIAsuper1300-*BrDHC1*-EGFP) in *Nicotiana benthamiana* leaf protoplasts. The pCAMBIAsuper1300-EGFP construct is used as the control. Bars = 40 μm.

To explore the localization of BrDHC1 protein in cells, we then predicted the subcellular localization of At3g02060 (homologue of Bra040707 in Arabidopsis) using the ePlant online website (Figure 2B, new added figure), and predicted that the protein was localized in chloroplasts, suggesting that BrDHC1 protein might also be localized in the same or-

ganelle. To further explore the subcellular localization of the BrDHC1 protein in cells, the pCAMBIAsuper1300-*BrDHC1*-EGFP recombinant vector was successfully constructed. By transient transformation of tobacco leaves, pCAMBIAsuper1300-*BrDHC1*-EGFP recombinant plasmids and negative control pCAMBIAsuper1300-EGFP no-loaded *Agrobacterium* resuspension were individually inoculated into the epidermis of transfected recipient tobacco leaves using a needleless syringe, and 48 h later, tobacco protoplasts were prepared and placed on a laser confocal microscope for observation and photography. By observing the auto-red fluorescence of chloroplasts and the green fluorescence signal of EGFP (Figure 2B), we found that the green fluorescence signal of EGFP in the control (pCAMBIAsuper1300-EGFP) protoplasts not only localized in the chloroplasts (the auto-red fluorescence), but also scatter-distributed along the cytoplasm, while the green fluorescence signal of EGFP in the fused *BrDHC1*-EGFP (pCAMBIAsuper1300-*BrDHC1*-EGFP) protoplasts completely coincided with the auto-red fluorescence of chloroplasts, indicating the accurate chloroplast localization of BrDHC1. These results showed that *BrDHC1* were highly expressed in the chloroplasts of young leaves, which might play an important role in the growth and development of plant leaves.

3.3. BrDHC1 Regulates Root Development of Seedlings under Drought Stress

The growth and development of the root system strongly affects the absorption and utilization of water and nutrients in plants, and the growth state of the root system also reflects the sensitivity and adaptability of the plant to the external environment. Therefore, the root length of the plant and the fresh weight of the seedling can be used as important indicators for the preliminary study of plant drought resistance [47]. To explore the effect of *BrDHC1* on the root development of *Brassica rapa* under drought stress, we have constructed the OE-*BrDHC1* and RNAi-*BrDHC1* recombination vectors, and fortunately obtained the OE-*BrDHC1* and RNAi-*BrDHC1* transgenic *B. rapa* (cxl-45-05, Figure A1, Appendix B). The gene expression of *BrDHC1* in the OE-*BrDHC1* lines (OE-4, OE-6, OE-8, and OE-9) were upregulated (Figure A1A), while that of the RNAi-*BrDHC1* lines (Ri-7, Ri-9, and Ri-10) were downregulated (Figure A1B), suggesting the successful construction of transgenic plants. As the gene expression of OE-9 was not high, we just used the OE-*BrDHC1* lines (OE-4, OE-6, and OE-8) and the RNAi-*BrDHC1* lines (Ri-7, Ri-9, and Ri-10) for further drought stress treatment analysis.

Under normal and 200 mM mannitol conditions, the root growth status of wild-type, OE-*BrDHC1* transgenic plants and RNAi-*BrDHC1* transgenic plants after 8 days of growth were observed and analyzed (Figure 3A). The results showed that, whether under 1/2 MS medium or under 1/2 MS medium with 200 mM mannitol conditions, the roots of OE-4, OE-6, and OE-8 plants had a stronger elongation, and the growth of the primary roots and lateral roots were more developed than in the wild-type plants, which is more conducive to the growth and development of the plants. However, the root elongation of Ri-7, Ri-9, and Ri-10 plants was weak, and the root length was short, which is not helpful in the growth of plants in a water-scarce environment. In this study, the primary root length and the fresh weight of the seedlings were recorded for each plant (Figure 3B,C), and the seedlings of the overexpressing of *BrDHC1* lines were heaviest, followed by the wild type, and the seedlings of the RNA interference *BrDHC1* lines were lightest. These results showed that the OE-*BrDHC1* transgenic lines were more adaptable to the environment, with a stronger water absorption ability than the wild-type plants, whereas the RNA interference *BrDHC1* transgenic lines were more sensitive to the external environment, with weaker water absorption. Therefore, it can be inferred that the *BrDHC1* gene can promote root growth of *B. rapa* under drought stress.

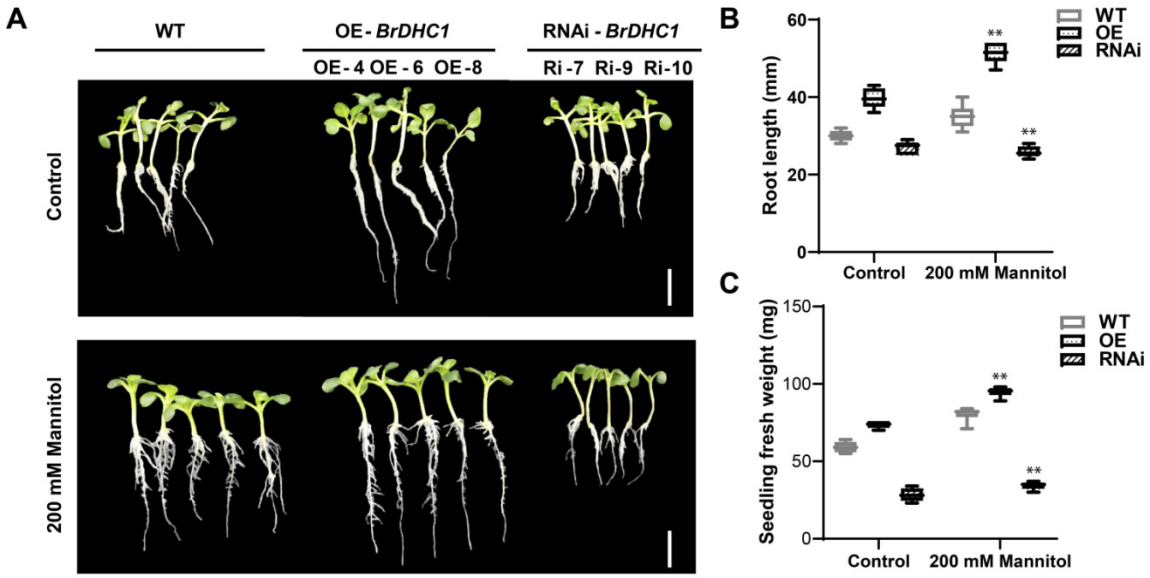

Figure 3. Root growth analysis of the wild-type and the transgenic *Brassica rapa* plants. (**A**) Root morphology of WT, OE-*BrDHC1*, and RNAi-*BrDHC1* plants under normal and 200 mM mannitol treatment (n = 10). (**B**) Main root length statistics of WT, OE-*BrDHC1*, and RNAi-*BrDHC1* plants under normal and 200 mM mannitol treatment (data represent the means ± SD of 5 replicates; Student's t test was performed to compare differences between the wild-type and the transgenic plants under 200 mM mannitol treatment, along with one-way analysis of variance (ANOVA), ** p value < 0.01). (**C**) Fresh weight statistics of the seedlings of WT, overexpressing *BrDHC1*, and RNAi-*BrDHC1* plants under normal and 200 mM mannitol treatment (data represent the means ± SD of 5 replicates; Student's t test was performed to compare differences between the wild-type and the transgenic plants under 200 mM mannitol treatment, along with one-way analysis of variance (ANOVA), ** p value < 0.01).

3.4. BrDHC1 Regulates Stomatal Aperture under Osmotic Stress

Under drought stress conditions, the plants could adapt to changes in the external environment by adjusting the opening of the stomata [9,43]. To assess the effects of *BrDHC1* gene expression on stomatal opening under drought (osmotic) stress treatment, the leaf stomata morphology of each line under normal conditions and with 20% PEG-6000 treatment was observed. The results showed that, under normal conditions (Figure 4A), there was no difference in the stomatal morphology of each plant of different lines; and under 20% PEG-6000 treatment, the stomatal closure degree of the OE-*BrDHC1* lines was very significantly (p value < 0.01) larger than that of the wild-type plant, while the stomatal closure degree of the RNAi-*BrDHC1* plant was the smallest (p value < 0.01). Then the stomatal aspect ratios of different lines were also evaluated (Figure 4B), and under normal conditions, there was no obvious difference in the stomata opening of different lines, but under 20% PEG-6000 treatment, the stomatal aspect ratio of the wild-type plants was about 58%, the stomatal aspect ratio of the OE-*BrDHC1* plants was about 35%, and the stomata aspect ratio of the RNAi-*BrDHC1* lines was about 66%. These results indicated that overexpression of *BrDHC1* conferred strong water retention ability (p value < 0.01) for *B. rapa* plants upon drought stress, while the water retention ability was weakened (p value < 0.01) in the RNAi-*BrDHC1* plants.

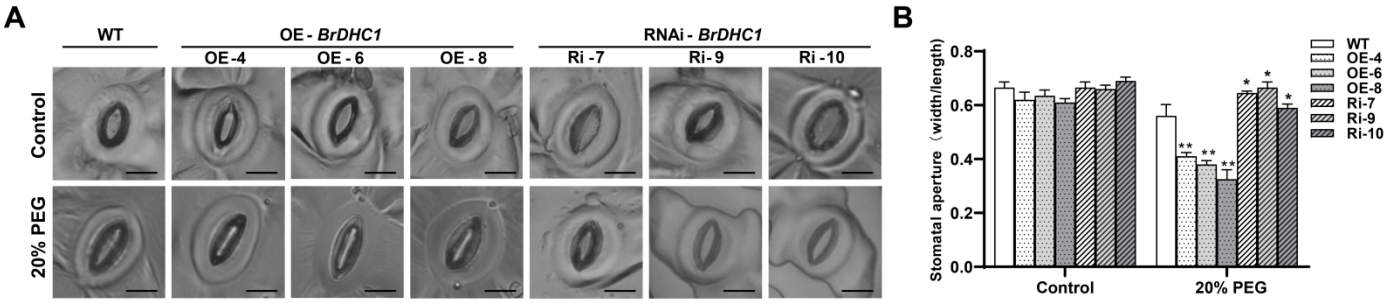

Figure 4. Stomata morphology analysis of different lines under drought treatment. (**A**) Leaf stomata morphology of WT, OE-*BrDHC1*, and RNAi-*BrDHC1* plants under normal and 20% PEG treatment (n = 10). (**B**) Statistical analysis of the width to length ratio of stomata from WT, OE-*BrDHC1*, and RNAi-*BrDHC1* transgenic plants under normal and drought stress treatment (data represent the means ± SD of 3 replicates; Student's t test was performed to compare differences between the wild-type and the transgenic plants under 20% PEG treatment, along with one-way analysis of variance (ANOVA), * p value < 0.05, ** p value < 0.01).

3.5. BrDHC1 Improves the Drought Resistance of B. rapa

The growth process of plants is a coordinated process between aboveground and underground parts, and studies of roots found that overexpression of *BrDHC1* plants improves the adaptability of roots under osmotic stress. To further explore the effect of *BrDHC1* gene expression on the growth of the aboveground parts of plants under drought stress, we performed a natural drought stress treatment for the 3-week-old seedlings of the three plant types (wild-type cxl-45-05, OE-*BrDHC1*, and RNAi-*BrDHC1* transgenic cxl-45-05, Figure 5A). The results showed that under normal growth conditions, there was no obvious difference in the growth state of each line: the leaves were normally unfolded, the leaf color was green, and the plants were robust. After the natural drought treatment (without watering for ten days), the plants showed different growth states: compared with the wild-type plants, some of the leaves of the OE-*BrDHC1* transgenic lines appeared light yellow due to the lack of water, while most of the leaves still showed a green phenotype; however, the leaves of the wild-type plants were partially curled with green and yellow leaves, but the leaves unfolded normally, whereas the leaves of the RNAi-*BrDHC1* transgenic lines were completely curled, with serious water loss folds, and they could not unfold normally.

The relative leaf moisture content and leaf water loss rate of each line was measured (Figure 5B,C), and the results showed that under drought stress, the average relative leaf moisture content of the OE-*BrDHC1* transgenic plants was 55%, while that of wild plants was 38%, and that of RNAi-*BrDHC1* transgenic plants decreased to 20%. RNAi-*BrDHC1* transgenic plants had the highest leaf water loss rate, followed by wild-type plants, and OE-*BrDHC1* transgenic plants had the lowest leaf water loss rate (p value < 0.01). In summary, it is clear that gene expression regulation of the *BrDHC1* gene positively improves the drought resistance of *B. rapa*, which has a positive effect on the drought resistance of the plants.

3.6. BrDHC1 Regulates Photosynthesis and Antioxidant Enzyme Activity of B. rapa under Drought Stress

The *BrDHC1* gene is located in the chloroplast, which is the focal place for photosynthesis. Photosynthesis provides plants with the necessary energy and organic matter for growth, and chlorophyll content directly affects the photosynthetic rate and the formation of photosynthetic products, ultimately influencing plant growth and development [48]. The determination of chlorophyll a and chlorophyll b contents in each line was shown in Figure 6: under normal growth conditions, the chlorophyll a and chlorophyll b contents of the OE-*BrDHC1* transgenic lines were higher than those of the wild-type plants, and those of the RNAi-*BrDHC1* transgenic lines were the lowest; there were no significant difference among plants of different types. Under drought treatment, the chlorophyll a content

and chlorophyll b content of OE-4, OE-6 and OE-8 transgenic plants were significantly higher than those of the wild-type plants, and those of Ri-7, Ri-9 and Ri-10 transgenic plants were significantly lower than those of the wild-type plants. The determination and analysis of chlorophyll a and chlorophyll b contents showed that upon drought stress, overexpression of *BrDHC1* transgenic plants improved the photoavailability of chlorophyll a and chlorophyll b, whereas the light energy utilization rate and the photosynthesis of RNAi-*BrDHC1* transgenic plants were reduced. These results indicate that the OE-*BrDHC1* transgenic lines possesses a stronger tolerance to drought stress than the wild-type line, while the RNAi-*BrDHC1* transgenic lines were more sensitive to drought stress than the wild-type plants.

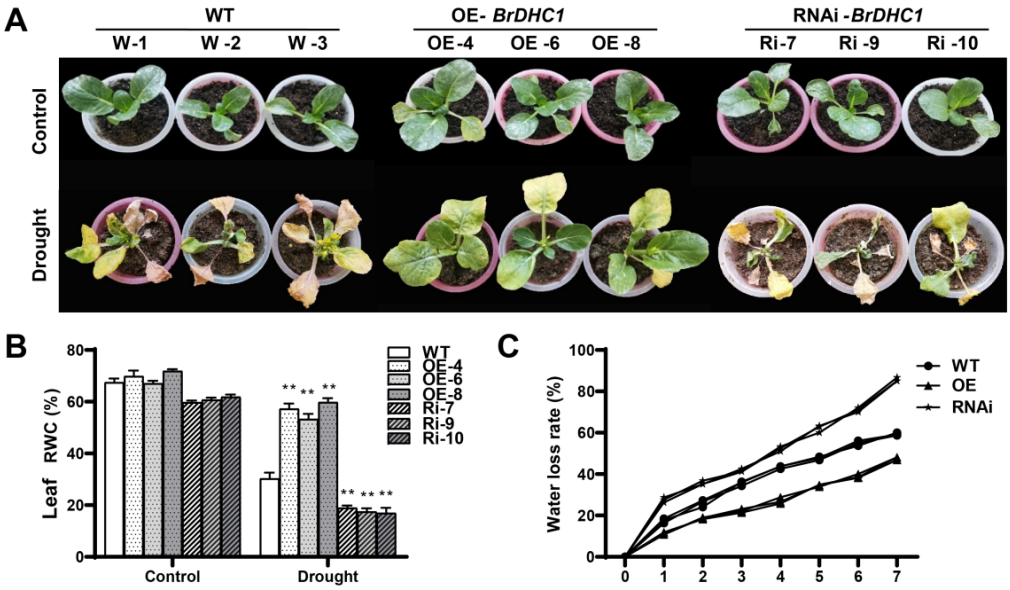

Figure 5. Phenotyping of the wild-type and transgenic plants under normal conditions and under natural drought stress. (**A**) Phenotypic analysis of WT, OE-*BrDHC1*, and RNAi-*BrDHC1* transgenic plants under 10 d natural drought (n = 6). (**B**) Analysis of leaf relative water content under natural drought of WT, OE-*BrDHC1*, and RNAi-*BrDHC1* transgenic plants for 10 d (data represent the means ± SD of 6 replicates; Student's t test was performed to compare differences between the wild-type and the transgenic plants under natural drought stress, along with one-way analysis of variance (ANOVA), ** p value < 0.01). (**C**) Leaf water loss rate analysis of WT, OE-*BrDHC1*, and RNAi-*BrDHC1* transgenic plants after 10 d of natural drought (data represent the means ± SD of 3 replicates).

When subjected to drought stress, the production and clearance of reactive oxygen species (ROS) within plant cells are out of balance, leading to the accumulation of reactive oxygen species and damage to cells in the plant. Plants can cope with drought stress through the regulation of antioxidant enzyme mechanisms, such as SOD, POD, CAT, etc. The resistant lines can effectively remove reactive oxygen species and prevent the excessive accumulation of reactive oxygen species, protecting plants from harmful abiotic stress [17]. Therefore, to test the drought resistance of different lines, the SOD, POD, and CAT activity of different lines were evaluated (Figure 6C): under natural drought stress, the SOD, POD, and CAT activities of the OE-*BrDHC1* transgenic lines OE-4, OE-6, and OE-8 were significantly higher than those of the wild-type plants, and the SOD, POD, and CAT activities of RNAi-*BrDHC1* transgenic lines Ri-7, Ri-9, and Ri-10 were all significantly lower than those of the wild-type plants. The antioxidant enzyme system is an important aspect for the study of plant physiological changes, and enhanced antioxidant enzymes activity can improve the adaptability of plants to arid environments. These results showed that the OE-*BrDHC1* transgenic plants showed strong adaptability and resistance to drought stress, whereas those of the RNAi-*BrDHC1* transgenic plants was weak.

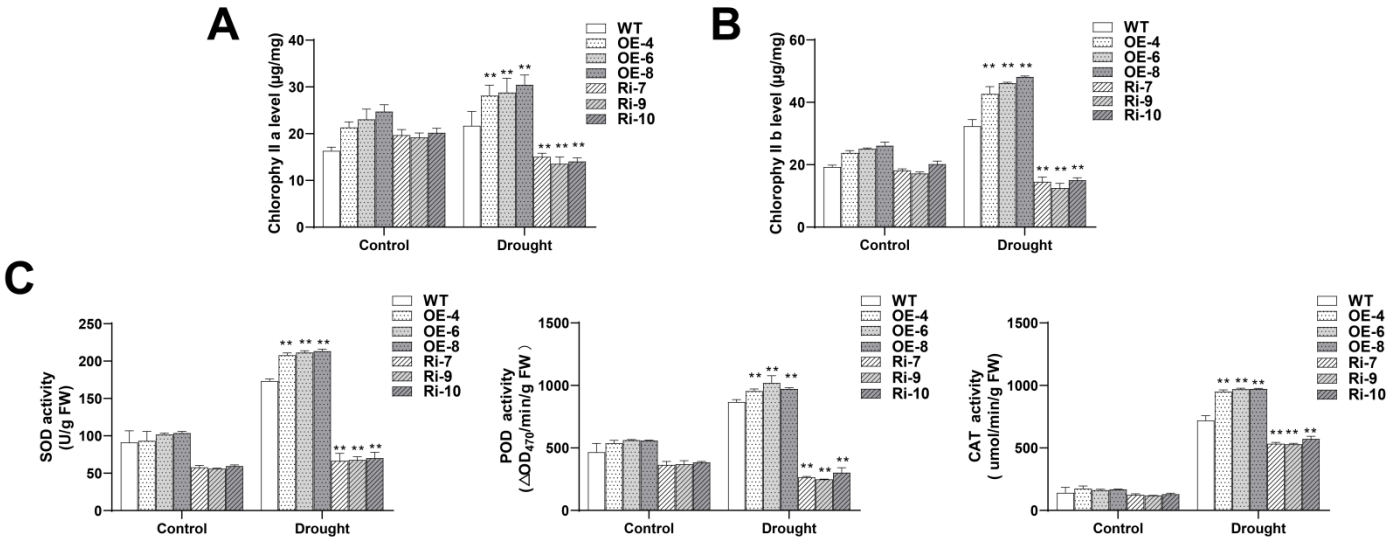

Figure 6. The determination of drought related physiological alternation under natural drought stress. (**A**) Analysis of chlorophyll a content of WT, OE-*BrDHC1*, and RNAi-*BrDHC1* transgenic plants (data represent the means ± SD of 6 replicates; Student's *t* test was performed to compare differences between the wild-type and the transgenic plants under natural drought stress, ** *p* value < 0.01). (**B**) Analysis of chlorophyll b content of WT, OE-*BrDHC1*, and RNAi-*BrDHC1* transgenic plants (data represent the means ± SD of 6 replicates; Student's *t* test was performed to compare differences between the wild-type and the transgenic plants under natural drought stress, along with one-way analysis of variance (ANOVA), ** *p* value < 0.01). (**C**) Analysis of antioxidant enzyme activities in wild-type, OE-*BrDHC1*, and RNAi-*BrDHC1* transgenic plants under drought stress (data represent the means ± SD of 6 replicates; Student's *t* test was performed to compare differences between the wild-type and the transgenic plants under natural drought stress, along with one-way analysis of variance (ANOVA), ** *p* value < 0.01).

3.7. BrDHC1 Regulates Osmotic Regulators and Malondialdehyde Contents of B. rapa under Drought Stress

An arid environment would lead to a decrease in osmotic pressure in plants, thus plants could not absorb water; however, the accumulation of osmotic regulators can maintain the water content and regulate the osmotic potential of cells, enhancing the adaptability of plants to arid environments [49]. The determination of the proline and soluble sugar content of WT, OE-4, OE-6, OE-8, Ri-7, Ri-9, and Ri-10 plants is shown in Figure 7A. Under normal conditions, there was no difference among different lines. However, under drought stress, the proline and soluble sugar contents of the OE-*BrDHC1* transgenic lines OE-4, OE-6, and OE-8 were significantly higher than those of the wild-type plants, while the proline and soluble sugar content of the RNAi-*BrDHC1* transgenic lines Ri-7, Ri-9, and Ri-10 were significantly lower than those of the wild-type plants. The results showed that overexpression of *BrDHC1* transgenic plants could regulate the permeability and maintain the moisture content of cells, which was conducive to plant growth under drought stress, whereas the RNA interference *BrDHC1* gene expression weakened the water absorption ability of plants, leading to drought-sensitive lines.

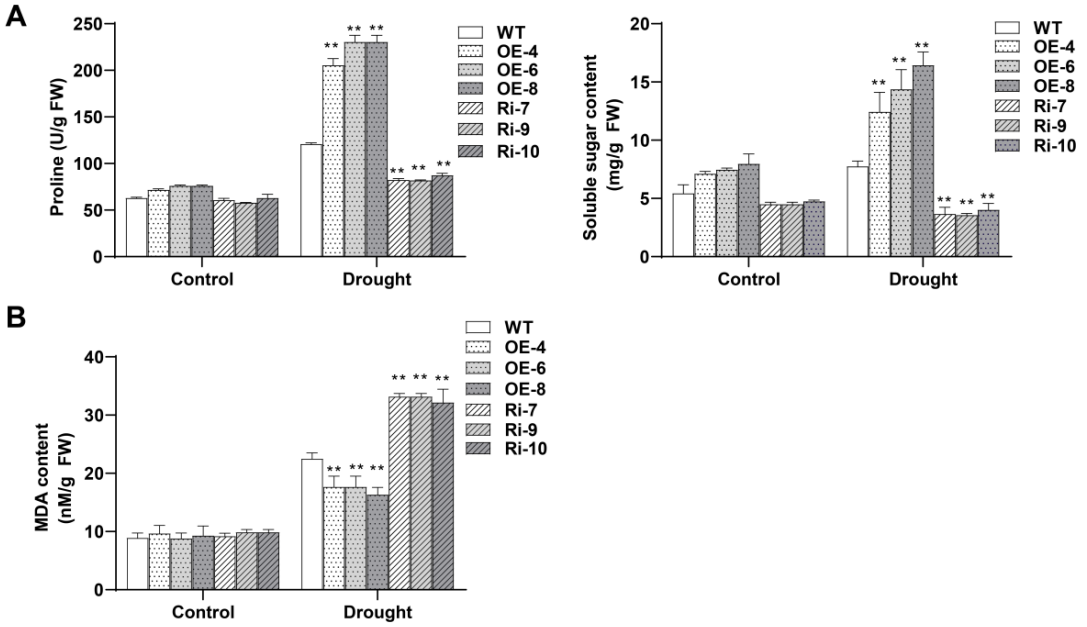

Figure 7. Evaluation of the osmotic regulators and malondialdehyde contents under drought stress. (**A**) Proline and soluble sugar contents in WT, OE-*BrDHC1*, and RNAi-*BrDHC1* plants (data represent the means ± SD of 3 replicates; Student's *t* test was performed to compare differences between the wild-type and the transgenic plants under natural drought stress, along with one-way analysis of variance (ANOVA), ** *p* value < 0.01). (**B**) Malondialdehyde content analysis of WT, OE-*BrDHC1*, and RNAi-*BrDHC1* plants (data represent the means ± SD of 3 replicates; Student's *t* test was performed to compare differences between the wild-type and the transgenic plants under natural drought stress, along with one-way analysis of variance (ANOVA), ** *p* value < 0.01).

Under drought stress, the active oxygen species accumulated in cells led to membrane lipid peroxidation, and the content of malondialdehyde (MDA) reflects the degree of cell membrane lipid peroxidation, as well as the degree of membrane lipid damage in the leaves [15]. In this study, the MDA contents of the leaf tissues from WT, OE-4, OE-6, OE-8, Ri-7, Ri-9, and Ri-10 plants were assessed (Figure 7B), and there was no difference among different lines under normal conditions. Compared with the control group, under drought stress, the MDA content of wide-type plants was increased, while the MDA contents of OE-*BrDHC1* transgenic lines were all significantly decreased; however, the MDA contents of the RNAi-*BrDHC1* transgenic lines were significantly increased over those of the wild-type plants, as well as the OE-*BrDHC1* transgenic lines. These results showed that under drought stress, the excessive expression of *BrDHC1* weakened the degree of membrane lipid peroxidation in plant cells and enhanced the stability of the cell membrane, whereas downregulation of *BrDHC1* aggravated the degree of membrane lipid peroxidation, the cells were seriously damaged, and the drought resistance of the plant was weak. The determination and analysis of proline, soluble sugar, and MDA contents showed that the OE-*BrDHC1* transgenic lines had stronger drought tolerance, enhancing the drought tolerance of *B. rapa*.

3.8. BrDHC1 Confers Drought Stress with the Synergy of Stress-Related Genes

Studies have shown that drought tolerance in plants is often the result of multi-gene control, not just a single gene, and when plants are subjected to drought stress, they can make adaptive adjustments at the molecular level of drought-related genes [6,14,15]. Therefore, the chlorophyll synthesis-related genes (*CAO* and *CHLG*), antioxidant enzyme-related genes (*POD*), proline metabolism-related genes (*P5CR*), and stress-related groups (*NAC2*, *MYB44*, and *ABRE2*) were quantitated to verify their gene expressions in different lines by qRT-PCR. The results are shown in Figure 8: compared with wild-type plants,

the gene expression levels of the *CAO*, *CHLG*, *POD*, and *P5CR* genes were significantly upregulated in the OE-*BrDHC1* transgenic lines, and those in the RNA interfering *BrDHC1* transgenic lines were significantly downregulated, whether under normal or drought stress treatment, which was consistent with the changes in chlorophyll content, *POD*, and proline content, as well as the gene expression alteration of *BrDHC1*. The expression of *NAC2*, *MYB44*, *ABRE2*, and other related stress genes also showed the same alteration pattern. These results showed that the *BrDHC1* gene responded to drought stress through the synergy of multiple genes, enhancing the drought resistance of plants.

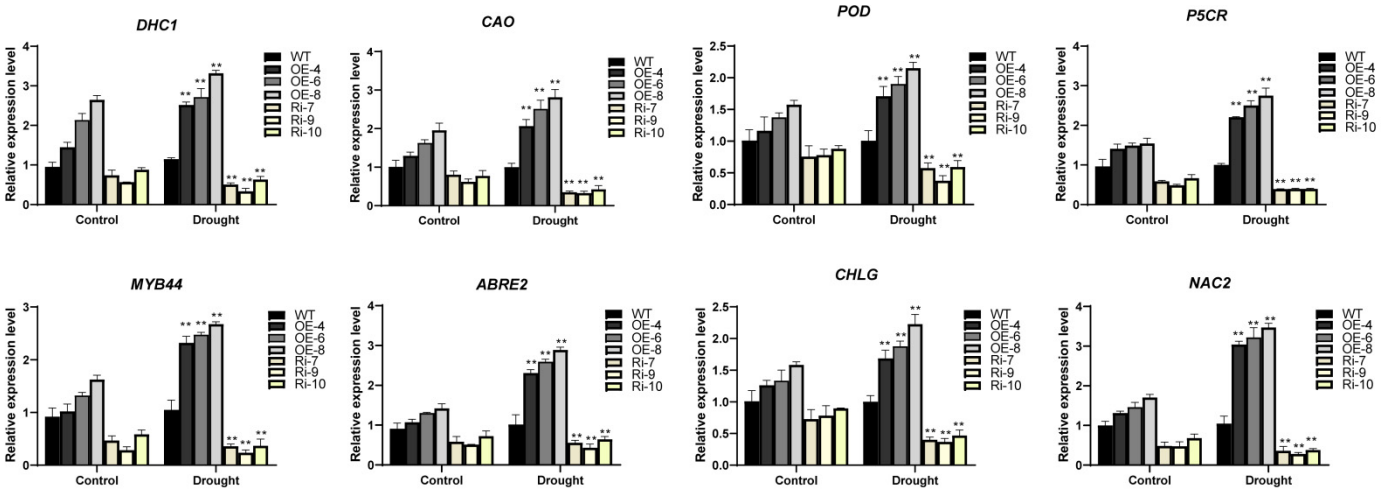

Figure 8. Quantitative analysis of the stress-related genes (*CAO*, *CHLG*, *POD*, *P5CR*, *NAC2*, *MYB44*, and *ABRE2*) in different lines (data represent the means ± SD of 3 replicates; Student's *t* test was performed to compare differences between the wild-type and the transgenic plants under natural drought stress, along with one-way analysis of variance (ANOVA), ** p value < 0.01).

4. Discussion

4.1. Expression Localization of BrDHC1 Aids Understanding Gene Function

The localization of gene expression products facilitates the preliminary judgments of gene function [50]. Tissue quantitative analysis showed that the *BrDHC1* gene could express in different tissues and was not tissue-specific (Figure 2A). It has the highest transcript level in the young leaves, suggesting that it may affect the photosynthesis of plant leaves. Subcellular localization analysis showed that the gene was localized in the chloroplasts, which are unique organelles of plant cells and are important places for plants to photosynthesize and produce various metabolic compounds, such as amino acids, vitamins, and secondary metabolites (Figure 2B) [51]. Thus we speculated that *BrDHC1* might affect the chloroplast photosynthesis of the leaves of the plant, consequently affecting plant growth and development.

Chloroplasts help maintain the cellular processes necessary for plants under external stress and normal growth condition [52,53]; chloroplasts also act as stress sensors for the external environments, and their photosynthesis could be greatly affected by the external environment [54]. To avoid the damage caused by abiotic stress, plants generally regulate cellular metabolic processes, such as photosynthesis, to increase plant productivity under external stress [55]. Reports show that the expression of chloroplast genes participates in the plant's response to the external abiotic stress, and the transcriptional regulation of these genes plays an important role in plant growth and development [51]. In cabbage-type rapeseed, DEAD-box RNA helicase *BrRH22*, localized in the chloroplasts, promotes the seed germination and plant growth of Arabidopsis under high salinity and drought stress [33,36]. Based on the bioinformatics analysis and subcellular localization analysis of *BrDHC1*, it is speculated that this gene may promote plant growth and development under abiotic stress.

4.2. BrDHC1 Positively Regulates Drought Stress

The study of plant drought resistance needs to be evaluated and analyzed in many aspects, and it must be verified at multiple levels, such as morphology, physiological biochemistry, and molecular mechanisms, which are interrelated and mutually restrictive. To explore the role of the *BrDHC1* gene under drought stress, OE-*BrDHC1* plants and RNAi-*BrDHC1* plants were created for functional analysis in this study. Under arid conditions, the RWC of the plant leaves is proportional to the drought resistance of the plants [56]. The results showed that under drought stress, compared with wild-type plants, the seed germination rate of OE-*BrDHC1 Arabidopsis* plants was significantly improved; these plants also showed longer primary root length, the closed leaf stomata, a reduced plant water loss and leaf water loss rate, leaves with a higher water content, and the improved water utilization rate of the leaves of the plant, indicating a strong adaptability to arid environments. However, the manifestation of these characteristics in the RNAi-*BrDHC1* plants was completely opposite.

After overexpression of the *PDH45* gene in rice, it was found that the salt stress tolerance was enhanced, and the antioxidant enzymes in transgenic plants, including SOD, APX, GPX, and glutathione reductase (GR) were significantly increased, indicating that the tolerance of salt stress in rice was achieved by the overexpression of *PDH45,* improving plant photosynthesis and antioxidant mechanisms [37]. Studies have shown that the stronger the antioxidant stress ability of plants, the stronger the drought tolerance [51,56]. Detailed analysis of the physiological and biochemical indices of each line found that under drought stress, the antioxidant enzyme activity and osmotic regulator content in the OE-*BrDHC1* transgenic plants were significantly enhanced and increased, while those of the RNAi-*BrDHC1* transgenic plants were significantly decreased under drought stress, suggesting that the drought tolerance of different types of plants was strongly related to the expression level of *BrDHC1*.

Under water deficiency or extreme environmental conditions, the peroxidation reaction of membrane lipids often occurs, resulting in the accumulation of MDA, the content of which implies the degree of membrane lipid peroxidation harm to the plant [15]. The accumulation speed of MDA content reflects plant's tolerance to adversity, and the increase in the MDA content in the strongly tolerant plant is less than that of the weakly tolerant plant. Under dehydration conditions, the MDA content of the OE-*BrDHC1* transgenic plants increased less than that of wild-type and the RNAi- *BrDHC1* transgenic plants, and the MDA content in the wild-type plants was less than that of the RNAi-*BrDHC1* transgenic plants.

To elucidate the molecular mechanism of drought resistance for *BrDHC1*, different photosynthesis-related and drought-related genes were quantitatively analyzed in this study. The results showed that under drought stress, the expression of photosynthesis-related genes (*CAO* and *CHLG*), drought stress-related genes (*NAC2*, *MYB44*, and *ABRE2*) of OE-*BrDHC1* transgenic plants was upregulated, while that of the RNAi-*BrDHC1* transgenic plants was downregulated, which was consistent with the change of chlorophyll content in each line. The results showed that under drought stress, *BrDHC1* responded to drought stress by co-responding with photosynthesis-related, stress-related, and probably other genes, and *BrDHC1* positively enhanced the drought tolerance of plants.

5. Conclusions

In this study, the effects of *BrDHC1* on the growth and development of cabbage-type rape under drought stress were investigated by using plant phenotype observation, physiological and biochemical indexes detection, and evaluation of molecular expression levels. The multi-level studies showed that the OE-*BrDHC1* transgenic plants are drought-tolerant and have a positive impact on plant growth and development. This is only a preliminary functional study of the *BrDHC1* gene, and further analysis is still needed for the evaluation of the molecular mechanisms of drought resistance. Based on the suggested pathways in which helicase may be involved, *BrDHC1* may, like pea *PDH47*, enhance DNA

helicase activity and ATPase activity through protein kinase C-mediated phosphorylation, thereby regulating gene expression at the transcriptional level [20].

Author Contributions: Conceptualization, G.C., F.W. and Z.X.; methodology, G.C. and H.G.; software, W.J. and Z.T.; validation, H.G. and W.J.; formal analysis, G.C. and W.J.; investigation, W.J.; resources, X.W.; data curation, H.G., W.C. and L.Z.; writing—review and editing, F.W. and Z.X.; visualization, W.J. and Z.X.; supervision, G.S. and B.T.; project administration, G.C. and F.W.; funding acquisition, F.W. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Program for Science and Technology Innovation Talents at the Universities of Henan Province (19HASTIT014), the Henan Provincial Natural Science Foundation of China (202300410366), and the Fostering Project for Basic Research of Zhengzhou University (JC21310015).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All the data are available in the manuscript file.

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

Appendix A

Table A1. Information of Primer sequences.

Primer Name	Primer Sequence (5′ to 3′)
BrDHC1-2469-F	AACTGCAGATGACATCCTTGCTCCTCCCAAATCC
BrDHC1-2469-R	GGGGTACCGTATTTGATAAGAGCAGGGAGTGATG
RNAi-*BrDHC1*-F	TTCCGGTGATTATGTGGTGC
RNAi-*BrDHC1*-R	CGAGACCCGTTTGGACTCAT
BrDHC1(RNAi)-BP-F	GGGGACAAGTTTGTACAAAAAAGCAGGCT TTCCGGTGATTATGTGGTGC
BrDHC1(RNAi)-BP-R	GGGGACAAGTTTGTACAAAAAAGCAGGCT CGAGACCCGTTTGGACTCAT
PDK(RNAi)-F	GACGAAGAAGATAAAAGTTGAGAGT
PDK(RNAi)-R	ACCTTGTTTATTCATGTTCGACTAA
EGFP-F	ATGGTGAGCAAGGGCGAG
EGFP-R	GCTCTTACTTGTACAGCTCGTC
β-actin-F	ATCAACTACCAGCCTCCAAC
β-actin-R	CTGCTGTGTTGTTGCTGATC
CAO-F	AATGCCCTTACCACGGATGG
CAO-R	AGGTCCATTACAAGCTCGGC
CHLG-F	GCTTTGGGAGGGTCCTTGTT
CHLG-R	ATCGCTATTCCCAACCCAGC
POD-F	GGTACGTGCTACACCTGGAC
POD-R	GCATCCACCCTGAAAGTCG
P5CR-F	AAGGCCATCACGGAAGTGAG
P5CR-R	CAACAAGTGCTCCGTCTT
NAC2-F	ACTGCATATCCCTTGGCGAG
NAC2-R	AGATTCCCACCAGGTTGCAC
MYB44-F	TCGGGAAAATCGTGTCGGTT
MYB44-R	CTTCAGCGTCGAGTTCCAGT
ABRE2-F	CTCGACCAGAAACCTTCCCC
ABRE2-R	GAAGATTGCGCTGCGTGTAG

Appendix B

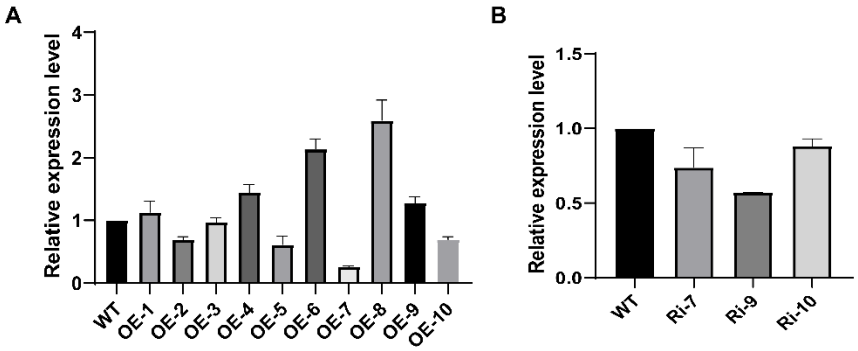

Figure A1. Relative expression level confirmation of the *BrDHC1* gene in OE-*BrDHC1* (**A**) and RNAi-*BrDHC1* (**B**) transgenic lines determined by qRT-PCR.

References

1. Xiong, L.; Schumaker, K.S.; Zhu, J.K. Cell signaling during cold, drought, and salt stress. *Plant Cell* **2002**, *14* (Suppl. 1), S165–S183. [CrossRef] [PubMed]
2. Seki, M.; Umezawa, T.; Urano, K.; Shinozaki, K. Regulatory metabolic networks in drought stress responses. *Curr. Opin. Plant Biol.* **2007**, *10*, 296–302. [CrossRef] [PubMed]
3. Shinozaki, K.; Yamaguchi-Shinozaki, K. Gene networks involved in drought stress response and tolerance. *J. Exp. Bot.* **2007**, *58*, 221–227. [CrossRef]
4. Umezawa, T.; Fujita, M.; Fujita, Y.; Yamaguchi-Shinozaki, K.; Shinozaki, K. Engineering drought tolerance in plants: Discovering and tailoring genes to unlock the future. *Curr. Opin. Biotechnol.* **2006**, *17*, 113–122. [CrossRef] [PubMed]
5. Yamaguchi-Shinozaki, K.; Shinozaki, K. Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. *Annu. Rev. Plant Biol.* **2006**, *57*, 781–803. [CrossRef] [PubMed]
6. Zhu, J.K. Abiotic stress signaling and responses in plants. *Cell* **2016**, *167*, 313–324. [CrossRef]
7. Kumar, A.; Gupta, A.; Azooz, M.M.; Sharma, S.; Ahmad Parvaiz Dames, J. Chapter 4—Genetic approaches to improve salinity tolerance in plants. In *Salt Stress in Plants: Signalling, Omics and Adaptations*; Springer: New York, NY, USA, 2013; pp. 63–78. [CrossRef]
8. Fang, Y.; Xiong, L. General mechanisms of drought response and their application in drought resistance improvement in plants. *Cell. Mol. Life Sci.* **2015**, *72*, 673–689. [CrossRef]
9. Daszkowska-Golec, A.; Szarejko, I. Open or close the gate-stomata action under the control of phytohormones in drought stress conditions. *Front. Plant Sci.* **2013**, *4*, 138. [CrossRef]
10. Fan, Y.; Wang, Z.; Liao, D.; Raza, M.A.; Wang, B.; Zhang, J.; Chen, J.; Feng, L.; Wu, X.; Liu, C.; et al. Uptake and utilization of nitrogen, phosphorus and potassium as related to yield advantage in maize-soybean intercropping under different row configurations. *Sci. Rep.* **2020**, *10*, 9504. [CrossRef]
11. Zhuang, J.; Wang, Y.; Chi, Y.; Zhou, L.; Chen, J.; Zhou, W.; Song, J.; Zhao, N.; Ding, J. Drought stress strengthens the link between chlorophyll fluorescence parameters and photosynthetic traits. *PeerJ* **2020**, *8*, e10046. [CrossRef]
12. Kalaji, H.M.; Bąba, W.; Gediga, K.; Goltsev, V.; Samborska, I.A.; Cetner, M.D.; Dimitrova, S.; Piszcz, U.; Bielecki, K.; Karmowska, K.; et al. Chlorophyll fluorescence as a tool for nutrient status identification in rapeseed plants. *Photosynth. Res.* **2018**, *136*, 329–343. [CrossRef] [PubMed]
13. Gebre, M.G.; Earl, H.J. Soil water deficit and fertilizer placement effects on root biomass distribution, soil water extraction, water use, yield, and yield components of Soybean [*Glycine max* (L.) Merrill.] grown in 1-m rooting Columns. *Front. Plant Sci.* **2021**, *12*, 581127. [CrossRef] [PubMed]
14. Choudhury, S.; Panda, P.; Sahoo, L.; Panda, S.K. Reactive oxygen species signaling in plants under abiotic stress. *Plant Signal. Behav.* **2013**, *8*, e23681. [CrossRef]
15. Choudhury, F.K.; Rivero, R.M.; Blumwald, E.; Mittler, R. Reactive oxygen species, abiotic stress and stress combination. *Plant J.* **2017**, *90*, 856–867. [CrossRef]
16. Hanin, M.; Brini, F.; Ebel, C.; Toda, Y.; Takeda, S.; Masmoudi, K. Plant dehydrins and stress tolerance: Versatile proteins for complex mechanisms. *Plant Signal. Behav.* **2011**, *6*, 1503–1509. [CrossRef]
17. Gill, S.S.; Tuteja, N. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiol. Biochem.* **2010**, *48*, 909–930. [CrossRef]
18. Mo, Y.; Wang, Y.; Yang, R.; Zheng, J.; Liu, C.; Li, H.; Ma, J.; Zhang, Y.; Wei, C.; Zhang, X. Regulation of plant growth, photosynthesis, antioxidation and osmosis by an Arbuscular Mycorrhizal fungus in watermelon seedlings under well-watered and drought conditions. *Front. Plant Sci.* **2016**, *7*, 644. [CrossRef] [PubMed]
19. Linder, P. DEAD-box proteins: A family affair—Active and passive players in RNP-remodeling. *Nucleic Acids Res.* **2006**, *34*, 4168–4180. [CrossRef]

20. Vashisht, A.A.; Tuteja, N. Stress responsive DEAD-box helicases: A new pathway to engineer plant stress tolerance. *J. Photochem. Photobiol. B Biol.* **2006**, *84*, 150–160. [CrossRef]
21. Mcglynn, P. Helicases that underpin replication of protein-bound DNA in *Escherichia coli*. *Biochem. Soc. Trans.* **2011**, *39*, 606–610. [CrossRef]
22. Tuteja, N.; Tuteja, R. Unraveling DNA helicases, motif, structure, mechanism and function. *Eur. J. Biochem.* **2004**, *271*, 1849–1863. [CrossRef] [PubMed]
23. Jankowsky, E. RNA helicases at work: Binding and rearranging. *Trends Biochem. Sci.* **2011**, *36*, 19–29. [CrossRef] [PubMed]
24. Umate, P.; Tuteja, R.; Tuteja, N. Genome-wide analysis of helicase gene family from rice and Arabidopsis: A comparison with yeast and human. *Plant Mol. Biol.* **2010**, *73*, 449–465. [CrossRef] [PubMed]
25. Xu, X.; Chen, X.; Shen, X.; Chen, R.; Zhu, C.; Zhang, Z.; Chen, Y.; Lin, W.; Xu, X.; Lin, Y.; et al. Genome-wide identification and characterization of DEAD-box helicase family associated with early somatic embryogenesis in *Dimocarpus longan* Lour. *J. Plant Physiol.* **2021**, *258–259*, 153364. [CrossRef]
26. Wan, R.; Liu, J.; Yang, Z.; Zhu, P.; Cao, Q.; Xu, T. Genome-wide identification, characterisation and expression profile analysis of DEAD-box family genes in sweet potato wild ancestor *Ipomoea trifida* under abiotic stresses. *Genes Genom.* **2020**, *42*, 325–335. [CrossRef]
27. Wang, Y.; Liao, J.; Wu, J.; Huang, H.; Yuan, Z.; Yang, W.; Wu, X.; Li, X. Genome-wide identification and characterization of the soybean DEAD-Box gene family and expression response to Rhizobia. *Int. J. Mol. Sci.* **2022**, *23*, 1120. [CrossRef]
28. Xu, R.; Zhang, S.; Lu, L.; Cao, H.; Zheng, C. A genome-wide analysis of the RNA helicase gene family in *Solanum lycopersicum*. *Gene* **2013**, *513*, 128–140. [CrossRef]
29. Jiang, Y.; Zhu, Y.; Liu, Z.J.; Ouyang, S. The emerging roles of the DDX41 protein in immunity and diseases. *Protein Cell* **2017**, *8*, 83–89. [CrossRef]
30. Owttrim, G.W. RNA helicases: Diverse roles in prokaryotic response to abiotic stress. *RNA Biol.* **2013**, *10*, 96–110. [CrossRef]
31. Tuteja, N.; Tuteja, R. Prokaryotic and eukaryotic DNA helicases, essential molecular motor proteins for cellular machinery. *Eur. J. Biochem.* **2004**, *271*, 1835–1848. [CrossRef]
32. Wang, D.; Qin, B.; Li, X.; Tang, D.; Zhang, Y.; Cheng, Z.; Xue, Y. Nucleolar DEAD-box RNA hlicase TOGR1 regulates thermotolerant growth as a pre-rRNA chaperone in rice. *PLoS Genet.* **2016**, *12*, e1005844. [CrossRef] [PubMed]
33. Nawaz, G.; Kang, H. Chloroplast or mitochondria-targeted DEAD-box RNA helicases play essential roles in organellar RNA metabolism and abiotic stress responses. *Front. Plant Sci.* **2017**, *8*, 871. [CrossRef] [PubMed]
34. Macovei, A.; Vaid, N.; Tula, S.; Tuteja, N. A new DEAD-box helicase ATP-binding protein (OsABP) from rice is responsive to abiotic stress. *Plant Signal. Behav.* **2012**, *7*, 1138–1143. [CrossRef] [PubMed]
35. Chung, E.; Cho, C.W.; Yun, B.H.; Choi, H.K.; So, H.A.; Lee, S.W.; Lee, J.H. Molecular cloning and characterization of the soybean DEAD-box RNA helicase gene induced by low temperature and high salinity stress. *Gene* **2009**, *443*, 91–99. [CrossRef]
36. Nawaz, G.; Lee, K.; Park, S.J.; Kim, Y.O.; Kang, H. A chloroplast-targeted cabbage DEAD-box RNA helicase BrRH22 confers abiotic stress tolerance to transgenic Arabidopsis plants by affecting translation of chloroplast transcripts. *Plant Physiol. Biochem.* **2018**, *127*, 336–342. [CrossRef]
37. Shivakumara, T.N.; Sreevathsa, R.; Dash, P.K.; Sheshshayee, M.S.; Papolu, P.K.; Rao, U.; Tuteja, N.; UdayaKumar, M. Overexpression of Pea DNA Helicase 45 (*PDH45*) imparts tolerance to multiple abiotic stresses in chili (*Capsicum annuum* L.). *Sci. Rep.* **2017**, *7*, 2760. [CrossRef]
38. Vashisht, A.A.; Pradhan, A.; Tuteja, R.; Tuteja, N. Cold- and salinity stress-induced bipolar pea DNA helicase 47 is involved in protein synthesis and stimulated by phosphorylation with protein kinase C. *Plant J.* **2005**, *44*, 76–87. [CrossRef]
39. Tamura, K.; Stecher, G.; Kumar, S. MEGA11: Molecular evolutionary genetics analysis version 11. *Mol. Biol. Evol.* **2021**, *38*, 3022–3027. [CrossRef]
40. Krzywinski, M.; Schein, J.; Birol, I.; Connors, J.; Gascoyne, R.; Horsman, D.; Jones, S.J.; Marra, M.A. Circos: An information aesthetic for comparative genomics. *Genome Res.* **2009**, *19*, 1639–1645. [CrossRef]
41. Sparkes, I.A.; Runions, J.; Kearns, A.; Hawes, C. Rapid, transient expression of fluorescent fusion proteins in tobacco plants and generation of stably transformed plants. *Nat. Protoc.* **2006**, *1*, 2019–2025. [CrossRef]
42. Zhang, X.; Henriques, R.; Lin, S.S.; Niu, Q.W.; Chua, N.H. Agrobacterium-mediated transformation of *Arabidopsis thaliana* using the floral dip method. *Nat. Protoc.* **2006**, *1*, 641–646. [CrossRef] [PubMed]
43. Zhao, Z.; Zhang, W.; Stanley, B.A.; Assmann, S.M. Functional proteomics of *Arabidopsis thaliana* guard cells uncovers new stomatal signaling pathways. *Plant Cell* **2008**, *20*, 3210–3226. [CrossRef] [PubMed]
44. Browne, M.; Yardimci, N.T.; Scoffoni, C.; Jarrahi, M.; Sack, L. Prediction of leaf water potential and relative water content using terahertz radiation spectroscopy. *Plant Direct* **2020**, *4*, e00197. [CrossRef] [PubMed]
45. Guo, Y.; Yin, G.; Sun, H.; Wang, H.; Chen, S.; Senthilnath, J.; Wang, J.; Fu, Y. Scaling effects on chlorophyll content estimations with RGB camera mounted on a UAV platform using machine-learning methods. *Sensors* **2020**, *20*, 5130. [CrossRef]
46. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}{}_{T}$ method. *Methods* **2001**, *25*, 402–408. [CrossRef]
47. Wu, L.M.; Fang, Y.; Yang, H.N.; Bai, L.Y. Effects of drought-stress on seed germination and growth physiology of quinclorac-resistant echinochloa crusgalli. *PLoS ONE* **2019**, *14*, e0214480. [CrossRef]

48. Liu, X.; Li, L.; Li, M.; Su, L.; Lian, S.; Zhang, B.; Li, X.; Ge, K.; Li, L. AhGLK1 affects chlorophyll biosynthesis and photosynthesis in peanut leaves during recovery from drought. *Sci. Rep.* **2018**, *8*, 2250. [CrossRef]
49. Hatzig, S.; Zaharia, L.I.; Abrams, S.; Hohmann, M.; Legoahec, L.; Bouchereau, A.; Nesi, N.; Snowdon, R.J. Early osmotic adjustment responses in drought-resistant and drought-sensitive oilseed rape. *J. Integr. Plant Biol.* **2014**, *56*, 797–809. [CrossRef]
50. Herath, V.; Verchot, J. Insight into the bZIP gene family in solanum tuberosum: Genome and transcriptome analysis to understand the roles of gene diversification in spatiotemporal gene expression and function. *Int. J. Mol. Sci.* **2020**, *22*, 253. [CrossRef]
51. Zhang, Y.; Zhang, A.; Li, X.; Lu, C. The role of chloroplast gene expression in plant responses to environmental stress. *Int. J. Mol. Sci.* **2020**, *21*, 6082. [CrossRef]
52. Nishiyama, R.; Le, D.T.; Watanabe, Y.; Matsui, A.; Tanaka, M.; Seki, M.; Yamaguchi-Shinozaki, K.; Shinozaki, K.; Tran, L.S. Transcriptome analyses of a salt-tolerant cytokinin-deficient mutant reveal differential regulation of salt stress response by cytokinin deficiency. *PLoS ONE* **2012**, *7*, e32124. [CrossRef] [PubMed]
53. Muhammad, I.; Shalmani, A.; Ali, M.; Yang, Q.H.; Ahmad, H.; Li, F.B. Mechanisms regulating the dynamics of photosynthesis under abiotic stresses. *Front. Plant Sci.* **2020**, *11*, 615942. [CrossRef] [PubMed]
54. Nowicka, B.; Ciura, J.; Szymanska, R.; Kruk, J. Improving photosynthesis, plant productivity and abiotic stress tolerance-current trends and future perspectives. *J. Plant Physiol.* **2018**, *231*, 415–433. [CrossRef] [PubMed]
55. Gururani, M.A.; Venkatesh, J.; Tran, L.S. Regulation of photosynthesis during abiotic stress-induced photoinhibition. *Mol. Plant* **2015**, *8*, 1304–1320. [CrossRef]
56. Meher Shivakrishna, P.; Ashok, R.K.; Manohar, R.D. Effect of PEG-6000 imposed drought stress on RNA content, relative water content (RWC), and chlorophyll content in peanut leaves and roots. *Saudi J. Biol. Sci.* **2018**, *25*, 285–289. [CrossRef]