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Overexpression of *GhMPK3* from Cotton Enhances Cold, Drought, and Salt Stress in Arabidopsis

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Abstract: Cotton production is hampered by a variety of abiotic stresses that wreak havoc on the growth and development of plants, resulting in significant financial losses. According to reports, cotton production areas have declined around the world as a result of the ongoing stress. Therefore, plant breeding programs are concentrating on abiotic stress-tolerant cotton varieties. Mitogen-activated protein kinase (MAPK) cascades are involved in plant growth, stress responses, and the hormonal signaling pathway. In this research, three abiotic stresses (cold, drought, and salt) were analyzed on *GhMPK3* transformed Arabidopsis plants. The transgenic plant's gene expression and morphologic analysis were studied under cold, drought, and salt stress. Physiological parameters such as relative leaf water content, excised leaf water loss, chlorophyll content, and ion leakage showed that overexpressed plants possess more stable content under stress conditions compared with the WT plants. Furthermore, *GhMPK3* overexpressed plants had greater antioxidant activities and weaker oxidant activities. Silencing *GhMPK3* in cotton inhibited its tolerance to drought stress. Our research findings strongly suggest that *GhMPK3* can be regarded as an essential gene for abiotic stress tolerance in cotton plants.

Keywords: MAPK; cold; drought; salt; gene cloning; Arabidopsis transformation; virus-induced gene silencing

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1. Introduction

Plants are constantly subjected to different forms of biotic and abiotic stress during their life cycle, including infection, pests, water shortage, high salinity, and extreme temperatures. Unlike animals, which are sessile, plants have developed complex signaling systems that operate under different conditions and control various cellular functions. Stress signaling in plant cells is a complex network of interacting proteins organized into segmented cascades in which a molecule's function depends on the interaction and activation of another molecule. For an appropriate adaptive response, cell surface receptors sense the stimulus and transmit the stress signal through a specific pathway [1]. Drought stress is a significant limiting factor in the production of cotton, with more than half of the global cotton supply cultivated in areas with drought challenges. *Gossypium* (cotton) crops require improved yields and yield balance in both normal and moisture-stressed environments. Cotton plants have evolved a variety of complex signaling networks to respond to a variety of metabolic, physiological, and morphological changes throughout evolution [2]. High salinity is among the most significant environmental stress that plants experience. Roots are the first and most direct organs to detect a signal [3]. Salinity is

one of the most effective stresses affecting plant developments as well as agricultural practices around the world [4,5]. From germination to boll formation, salt stress affects cotton physiology, and the tolerance mechanism is well described. Temperature is a crucial environmental factor for plant development, metabolism, and productivity [6]. Plants can survive in a wide range of temperatures based on their species and growth rate. Plants suffer significant temperature damage if they are exposed to temperatures outside of these ranges. Germination, development, and metabolic activities can all be hindered by low temperatures. Cold stress also induces dehydration and disrupts membrane integrity by forming ice crystals [7].

Phosphorylation is a significant post-translational modification (PTM), and protein phosphorylation is the key signal transduction mechanism, allowing external signals to be transmitted and enhanced by changes in downstream gene expression and other biological processes. As a component of a key transduction module of signaling, the mitogen-activated protein kinase (MAPK) cascade plays important roles in responses to the abiotic stresses in plants [8]. There are three types of MAPK cascades Mitogen-activated protein kinase (MAPK), Mitogen-activated protein kinase kinase (MAPKK), and Mitogen-activated protein kinase kinase (MAPKKK) [9–12]. The MAPK gene goes through the following steps: MAPKKK triggers MAPKK by phosphorylating MAPKK's preserved S/T- (S/T stands for serine/threonine, and X stands for any amino acid) motif. Then, MAPKK activates MAPK by phosphorylating the TXY motif in MAPK (T stands for threonine, Y stands for tyrosine, and X stands for any amino acid). Then, MAPK stimulates downstream kinases, proteins, hormones, and other reaction variables, as well as transmitting extracellular environmental signals into cells [8].

MAPKs are essential genes that regulate a variety of cellular processes and stress responses, as per previous studies. The functions of MAPK genes have been reported in Arabidopsis, tomato, tobacco, wheat, rice, and soybean and cotton [5,10,13–17]. *Gossypium hirsutum* is a vital strategic fiber source that is extensively used in textile mills, producing 95% of the overall cotton crop globally and serving as a model for researching genome-scale emergence and polyploidization [18,19]. Drought, salinity, and cold are only a few of the environmental factors that restrict cotton yields [9,20]. Gene cloning and virus-induced gene silencing among others have resulted in the maximum transformation and production of high yield breed in many crop species. In this study, *GhMPK3,* a group A MAPK, was identified and characterized. The silencing of *GhMPK3* enhanced drought tolerance in *Gossypium hirsutum*. *GhMPK3* overexpression improves plant resistance to drought, cold, and salt stress.

2. Materials and Methods

2.1. Plant Materials

The tissue expression was assessed using quantitative real-time PCR on an Upland cotton (*Gossypium hirsutum L.* cv., "TM-1") grown in both the lab growth chamber at 25 °C with a 16 h light/8 h dark cycle and cotton field in Anyang (AY), Henan, China. Tissues such as cotyledon, young leaves, mature leaves, stems, and leaves, were collected from the growth chamber and flowers of the "TM-1" were collected from the field to analyze *GhMPK3 (Gh_D05G3876)* gene expression. The TM-1 cotton germplasm was developed at the Texas Agricultural Experiment Station and was mainly used for cotton genetic experiments [21].

H117 variety was used for virus-induced gene silencing, while *Gossypium hirsutum L.* cv. CCRI10 was used for *GhMPK3* gene cloning. Both H177 and CCRI10 (Zhong Mian Suo 10) were developed by the Chinese Academy of Agricultural Sciences, Institute of Cotton Research Anyang, China. H177 was used because it is extremely vulnerable to various environmental stressors, such as drought, while CCRI10 was used due to its great yielding and consistently improve fiber quality. Plants were germinated in a growth chamber room with a 16 h light/8 h dark cycle and a temperature of 25 °C. *Arabidopsis thaliana* (ecotype Col-0) was used to study overexpression. The plants were grown in a growth chamber with

a temperature of 22 °C, a photoperiod of 16 h of light/8 h of darkness, and relative humidity of 80%. T0–T3 seeds were screened, and T3 progeny were used for further research.

2.2. Gene Cloning, Sequence Analysis, Phylogenetic Analysis, and Cis-Regulatory Element Analysis

The gene cloning of the *GhMPK3* cDNA was done as previously described following Ma et al. (2020) [22]. The genomic sequences analysis was performed as described previously [19]. The phylogenetic tree was developed using protein sequences from *Arabidopsis thaliana* (AT), *Gossypium hirsutum* (Gh), *Oryza sativa* (*LOC*), and *Gossypium raimondii* (Gorai) with MEGA 7.0 (http://www.megasoftware.net), taking into consideration the 1000 replications [23]. For the regulatory region analysis of gene *GhMPK3* in cotton, a 2000 bp upstream sequence from the start codon was submitted to the Plant CARE program (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) [24].

2.3. Subcellular Localization

An online tool was used to predict GhMPK3 protein subcellular localization PSORT WOLF (http://www.genscript.com/wolf-psort.html). For confirmation, *GhMPK3* was also amplified using the transformed gene-specific primers by polymerase chain reaction (Supplementary Table S2). To obtain the pBI121-*GhMPK3*-GFP gene construct, the amplified gene was inserted into the plasmid, and the amplified gene was inserted upstream of the green fluorescent protein pBI121-GFP into the plasmid vector (GFP). The 35S:GFP construct was used as a control. Both the transformants and the control were infiltrated into 6-week-old leaves of *Nicotiana benthamiana* [22]. Fluorescence was observed 48 h after infiltration with a microscope (Leica DM2500, Solms, Germany).

2.4. Expression Analysis

The RNAprep Pure Plant Kit (TIANGEN, Beijing, China) was used to extract and purify RNA for gene expression analysis according to the manufacturer's instructions. RNA was transcribed into cDNA using a PrimeScriptTMRT reagent kit and a gDNA Eraser. As described, a real-time quantitative polymerase chain reaction was performed using gene specific primers (Supplementary Table S2) [25]. The cotton and Arabidopsis actin genes were used as housekeeping genes. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative expression levels [26]. Each experiment was repeated three times, and all experiments were done with three technical replicates.

2.5. Overexpressed of GhMPK3 in Arabidopsis Plants

2.5.1. Construction of Vector and Genetic Transformation of Overexpressed Plants

The vector construction and genetic transformation were performed as described previously [27]. Positively transformed plants were confirmed by DNA and PCR. The transgenic T3 lines and WT plants were used for further experiments.

2.5.2. Stress Treatment, Physiological Parameters Measurement, Oxidant and Antioxidant Determination in Overexpressed and Wild-Type Plants

Overexpressed lines L2, L3, L5, and WT plants were treated with 20% polyethylene glycol (PEG) for drought stress treatment, while 200 mM NaCl was used for salt stress treatment. For cold treatment, plants were transferred from normal conditions of 22 °C to 4 °C for 8 days, whereas control plants were placed in the dark at 22 °C for 8 days. After 8 days, samples from leaves were collected from both treatments (cold, drought, salt) and control. Samples collected were immediately frozen in liquid nitrogen and stored at −80 °C to be used for subsequent gene expression analysis.

Physiological parameters such as the relative leaf water content (RLWC), excised leaf water loss (ELWL), chlorophyll content, and membrane ion leakage were analyzed. RLWC determination was done as described previously [28]. ELWL determination was carried out as described in Mccaig and Romagosa (1989) [29]. Ion leakage from leaves was determined using the relative conductivity method to measure the electrolyte leakage [30]. Lastly,

chlorophyll was extracted by grinding the leaves with 80% acetone and kept at 4 °C in the dark for 48 h. After 3 min of shaking, the absorbance of 2 mL of each sample was measured at 663 and 645 nm [31]. To develop a greater understanding of the transgenic and WT plant's drought stress responsiveness, the concentrations of oxidant and antioxidant enzymes such as malondialdehyde (MDA), hydrogen peroxide (H_2O_2), catalase (CAT), and peroxidase (POD) were assessed. The updated $(NH_4)_2Fe(SO_4)$/xylenol orange procedure was used to determine the concentration of H_2O_2 [32]. For the malondialdehyde concentration assay, lipid peroxidation was determined as the volume of MDA measured by the thiobarbituric acid (TBA) reaction as described previously [6]. Catalase activity (CAT) was measured by determining the concentration level reduction of H_2O_2 [33], while peroxide (POD) was measured with the substrate 4-methyl catechol to test the POD behavior [34].

2.6. Expression Level of Stress-Responsive Genes in Transgenic and Wild-Type Plants under Cold, Drought, and Salt Stress

The level of expression of six stress-responsive genes was analyzed *GhMPK3* by PCR using gene-specific primers (Supplementary Table S3) to further explore the function of *GhMPK3* under cold, drought, and salt stress exposure in cotton plants. The six stress-responsive genes used include *AtABF4, AtCBL1, AtKIN1, AtERD15 AtRD29A,* and AtSOS1.

2.7. Virus-Induced Gene Silencing Analysis of GhMPK3

For VIGS analysis, 300-bp fragments were amplified from *GhMPK3* by PCR using gene-specific primers (Supplementary Table S2). A control vector (pCLCrVA) with pCLCrVA:GhMPK3 and pCLCrVA:PDS (Positive control), which have been combined with the helper vector (pCLCrVB) strain carrier in a 1:1 ratio, and OD_{600} = 1.5 were individually transferred into cotton cotyledons [35]. Gene expression analysis was performed after one month. Physiological parameters such as chlorophyll content, ion leakage, excised leaf water loss, and relative leaf water content were determined after 10 days of post-treatment. Oxidant and antioxidant concentrations for empty vector and silenced gene plants were also analyzed. The experiment was repeated three times, and each experiment was done with three technical replicates.

2.8. Transactivation Assay of GhMPK3

Autoactivation and toxicity were tested by fusing the cotton *GhMPK3* gene with the GAL4 DNA-binding domain into pGBKT7, pGBKT7-*GhMPK3*, pGBKT7, and pGADT7-largeT+pGBKT7-p53 were transformed into the AH109 yeast using the Clontech protocol. The strains were plated on SD/-TRP (synthetic dextrose medium without tryptophan) and SD/-TRP, HIS, ADE (synthetic dextrose medium lacking tryptophan, histidine, and adenine) respectively.

2.9. Statistical Analysis

GraphPad Prism version 8.4.3 was used to conduct statistical analyses. In this research, all the experiments were carried out in three replications. In each graph, error bars indicate the mean values ± standard error (SE) of three replicates. To test the statistical significance between measurements of different treatments, a multiple test selection with analysis of variance (ANOVA) was used. The significance level between the various time points has been placed ($p < 0.05$).

3. Results

3.1. Cloning and Characterization of GhMPK3

The true leaf of *G. hirsutum* was used to clone the *GhMPK3* gene (CCRI10). The CDS of *GhMPK3* is 1131bp long, with six exons and five introns. A 376-amino-acid protein with a molecular mass of 43.325, a charge of −5, and an isoelectric point of 5.872 are found in the genome The amino acid sequence of the gene with the homologous sequence of MAPK members as family in *Arabidopsis*, rice, and *Gossypium raimondii* were used to

develop the phylogenetic tree. Phylogenetic analysis between *GhMPK3* and MAPK in other plants revealed that *GhMPK3* can be found in group A (Figure 1A). This is in line with previous research findings reports in *G. raimondii* and *G. barbadense* [15,36]. In addition, sequence comparisons between *GhMPK3* and the sequence of MAPK from other organisms revealed that *GhMPK3* had six exons and five introns structures, similar to other group A MAPK members (Figure 1B). This result gives more confirmation that *GhMPK3* belongs to group A MAPK members. For cis-regulatory element analysis, 2kb upstream sequences from the gene transcription start were used. Based on the evaluation of the cis-regulatory elements of the coding sequence *GhMPK3* on the promoter regions, cis-regulatory elements responsible for the plant's stress tolerance were identified. Cis-regulatory elements present in the gene-promoting region indicate their role in stress control in a plant (Supplementary Table S1).

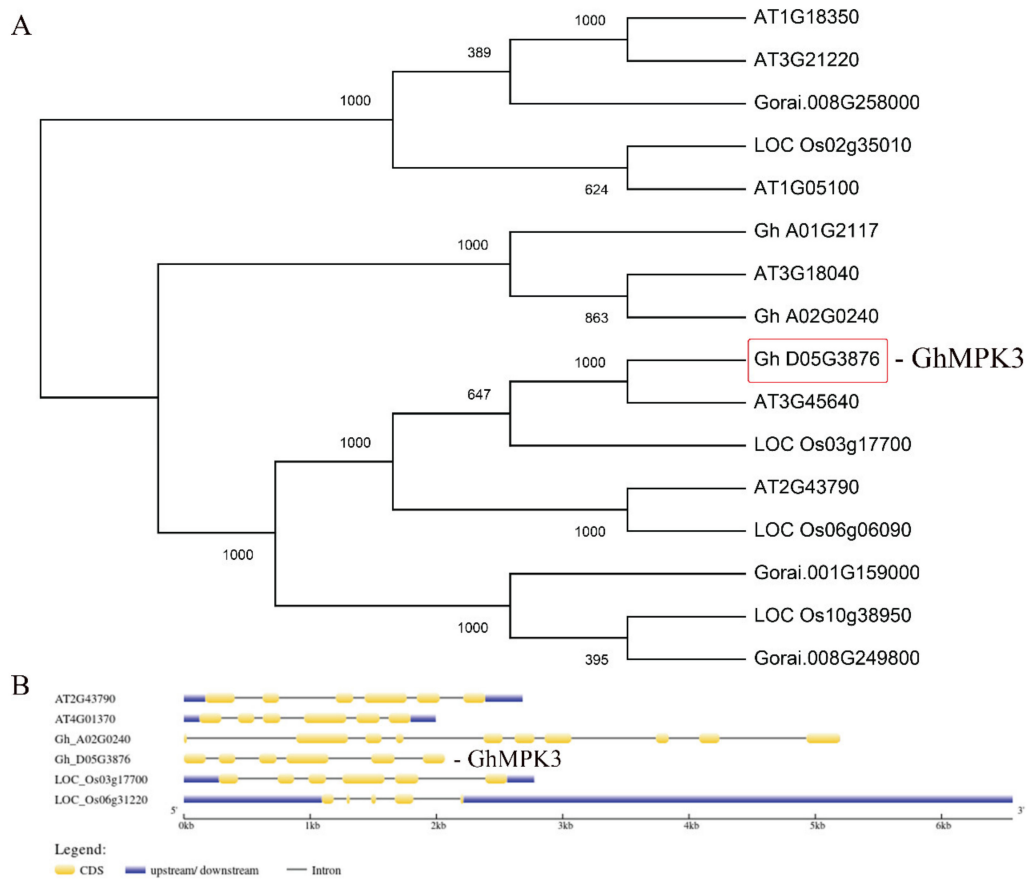

Figure 1. Comparison of the amino acid sequences of *GhMPK3* and closely related plant mitogen-activated protein kinase. (**A**) The phylogenetic relationships between *GhMPK3* and MAPK proteins in other plants. MEGA 7 was used for the development of the phylogenetic tree using the CLUSTALW by neighbor-joining (NJ) method. Bootstrap values of 1000 replicates are shown in each branch. Protein ID represents the gene names: *Arabidopsis thaliana –AT*, *Gossypium hirsutum*—Gh *Oryza sativa—LOC*, and *Gossypium raimondii—Gorai* (**B**) *GhMPK3* (*Gh_D03G1517)* genomic DNA structure comparison with other MAPK from other plants. The pattern of exons, introns, and untranslated regions is displayed in the legend (UTRs). The length of the sequence is indicated by the X-axis scale. The following are the abbreviations for the species names: At for *Arabidopsis thaliana*, Gh for *Gossypium hirsutum*, and Loc for Oryza sativa.

A protein's subcellular localization is closely related to its functions. *GhMPK3*-GFP and pBI121-GFP (control) were transformed into Agrobacterium tumefaciens to investigate its role. Figure 2A shows that fluorescence was detected in the cell membrane of pBI121-

GhMPK3-GFP, while in pBI121-GFP (control), fluorescence was distributed all over the cell. These findings indicate that the GhMPK3 protein is localized to the cytoplasm.

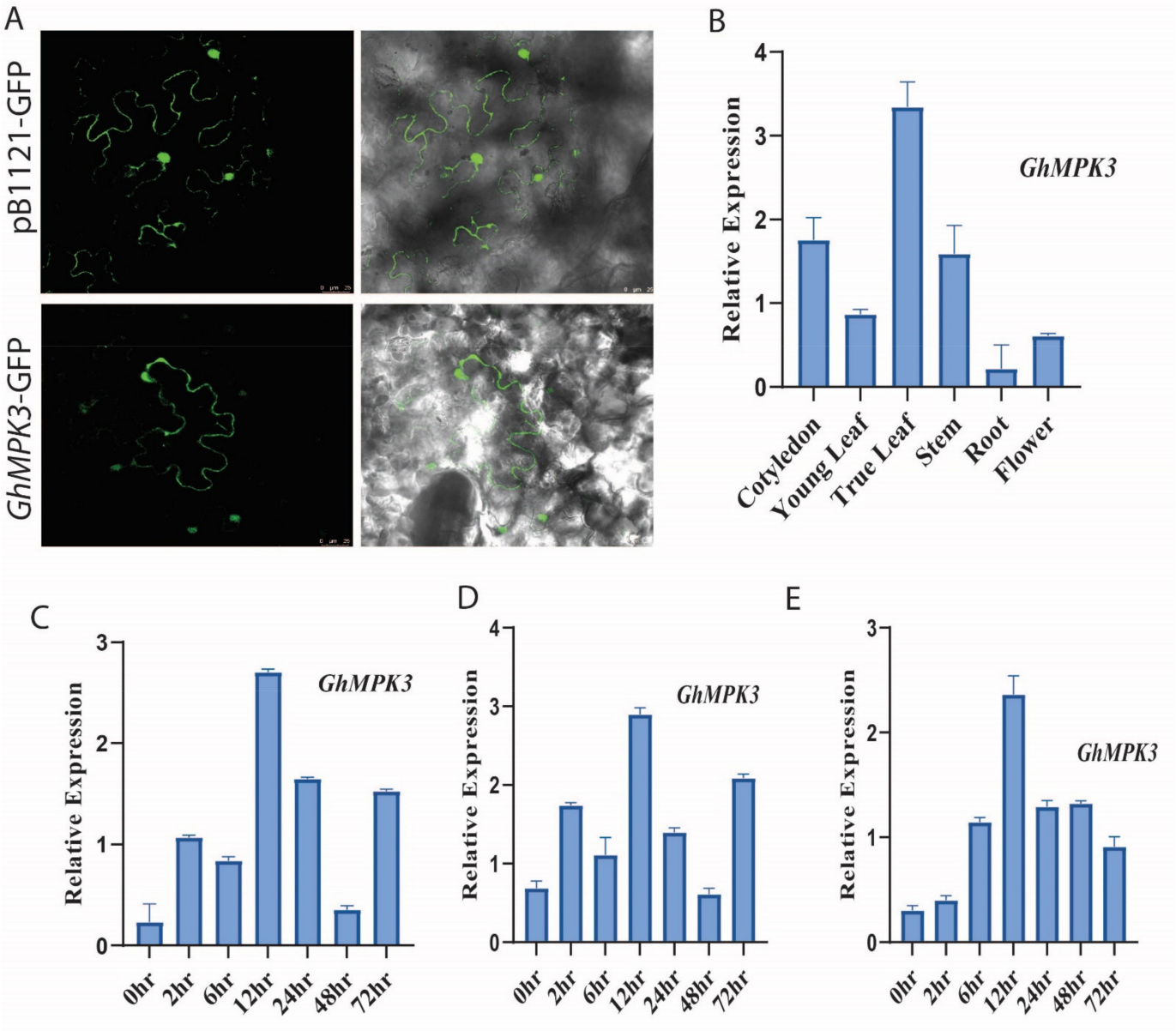

Figure 2. *GhMPK3*-GFP analysis in *Nicotiana benthamiana* and expression level in cotton. (**A**) *GhMPK3*-GFP analysis. (**B**) *GhMPK3* TM-1 tissue expression level. (**C**) *GhMPK3* expression pattern under cold stress. (**D**) *GhMPK3* expression pattern under drought stress. (**E**) *GhMPK3* expression pattern under salt stress. The standard deviation of three replications is reflected by error bars.

3.2. Profiling Expression of Cotton GhMPK3

To explore the biological function of *GhMPK3*, its expression pattern was analyzed in tissues and under stress treatments (cold, drought, and salt). Using designed primers (Supplementary Table S2), the expression level was analyzed by qRT-PCR. Tissue expression analysis results show that *GhMPK3* was expressed in all tissues with the highest expression in true leaves (Figure 2B).

For cold treatment expression, the genes show expression at all hours with the highest expression in 12 h of post-treatment (Figure 2C). After treatment with PEG (for drought treatment), expression levels were significantly increased at different stages, with maximum

expression at 12 h post-treatment; then, they showed a steady decrease (Figure 2D). For salt treatment, the gene was upregulated at various hours after treatment with the highest expression at 12 h post-treatment (Figure 2E). This shows that the *GhMPK3* gene has the highest level of expression after 12 h of exposure to stress.

3.3. Overexpression of Gh_D05G3876 Enhances Cold, Drought, and Salt Stress Tolerance

Lines L2, L3, and L5 are the overexpressed Arabidopsis lines with the highest gene expression among fifteen screened different lines (Supplementary Figure S1A) and were thus selected. Cold, drought, and salt stress conditions were chosen and used to carry out phenotypic and other functional evaluations. Overexpressed lines and WT plants were exposed to 4 °C for 8 days to induce cold stress, *GhMPK3* expression levels were highly expressed in transgenic plants but downregulation is observed in WT plants. After 8 days of drought treatment (20% PEG6000), transgenic lines expressed more *GhMPK3* than WT lines under the same condition. For salt treatment, the transgenic lines also show higher *GhMPK3* expression than the WT lines (Figure 3).

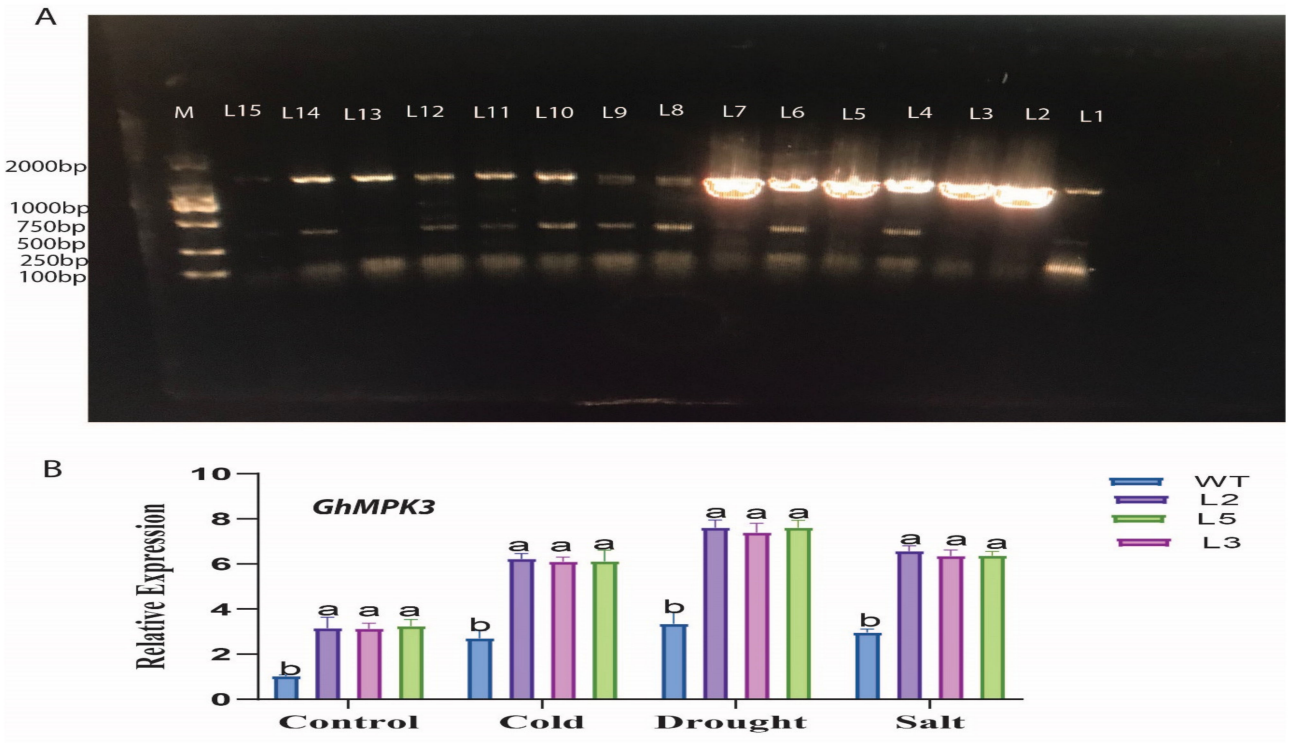

Figure 3. Gene expression analysis of *GhMPK3* (*Gh_D05G3876*). (**A**). PCR analysis result to check the 1200-bp CDS integration in transformed Arabidopsis plants, L 1–L14: *GhMPK3*-overexpressed lines, L15: WT. (**B**). *GhMPK3* gene expression level under abiotic stress (cold, drought, and salt) in transformed Arabidopsis and WT by qRT-PCR. Each experiment was performed three times. The bar represents a standard error (SE). Various letters above the columns indicate statistically significant differences (ANOVA, $p < 0.05$). WT: Wild type. L2, L3, L5: Overexpressed lines.

3.4. Physiological Characteristics and Enzymatic Activity of Evaluated under Cold, Drought, and Salt Stress Conditions

The physiological parameters of the transformed lines and WT plants were assessed at post stresses (cold, drought, and salt stress conditions) (Figure 4). Relative leaf water content (RLWC) was evaluated, and it was observed that the overexpressed lines have higher content compared with the WT under cold, drought, and salt stress conditions (Figure 5A). The excised leaf water loss (ELWL) showed a substantial decrease in overexpressed lines compared with the WT lines (Figure 5B). For chlorophyll content, the overexpressed lines had significantly higher chlorophyll content, while the WT leaves appeared chlorotic. In

both stress environments, the chlorophyll content of the WT plant decreased significantly, suggesting that WT plants were more vulnerable to oxidative damage than the transgenic line (Figure 5C). The ion leakage was evaluated as an indicator for cell membrane stability; in comparison to the transgenic lines, WT plants had higher levels of ion leakage due to exposure to stress (Figure 5D). All parameter measurements between the transgenic lines and the WT were similar under controlled conditions.

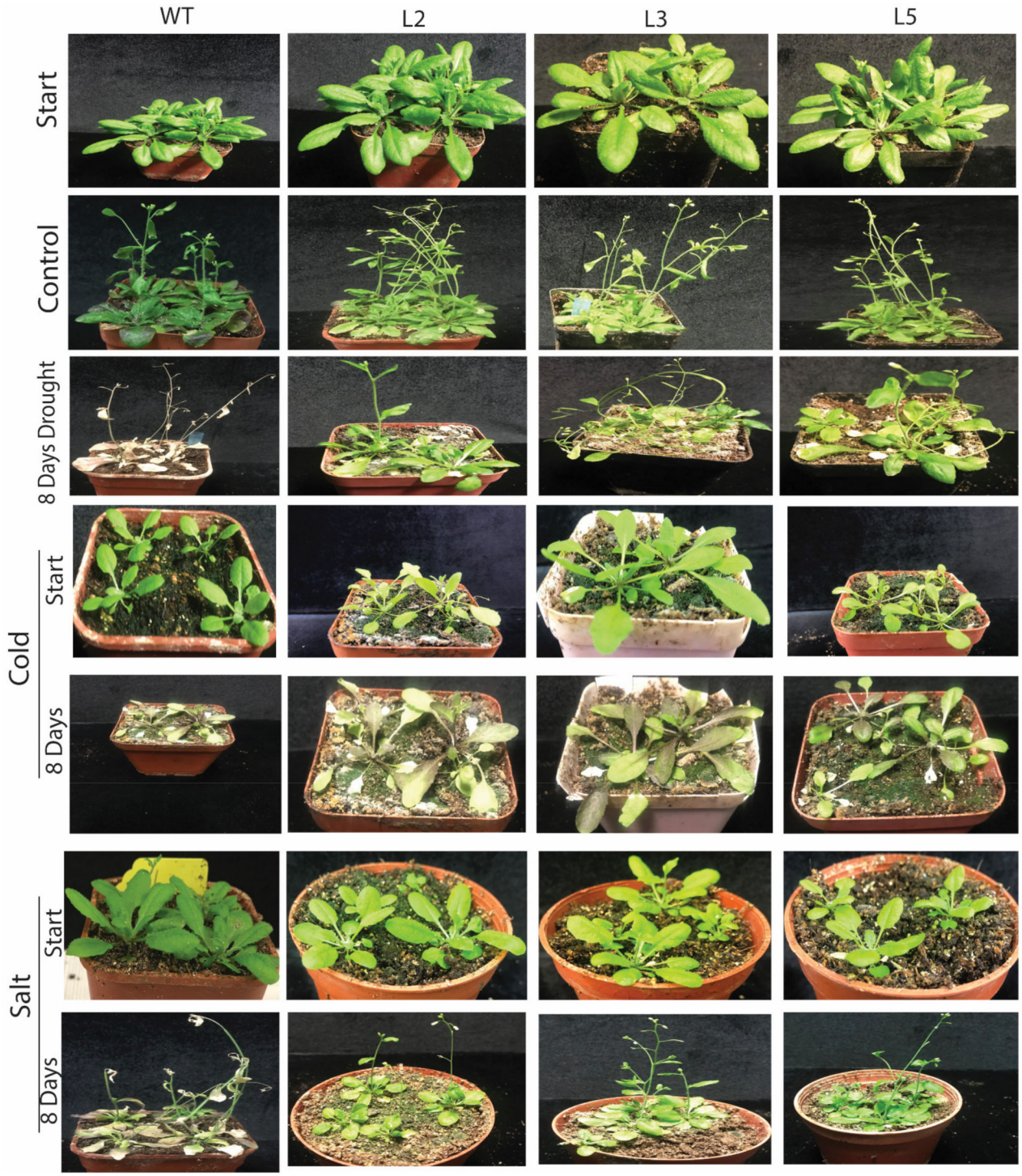

Figure 4. Physical features of *GhMPK3*-transformed lines and WT plants prior and post-cold, drought, and salt treatments.

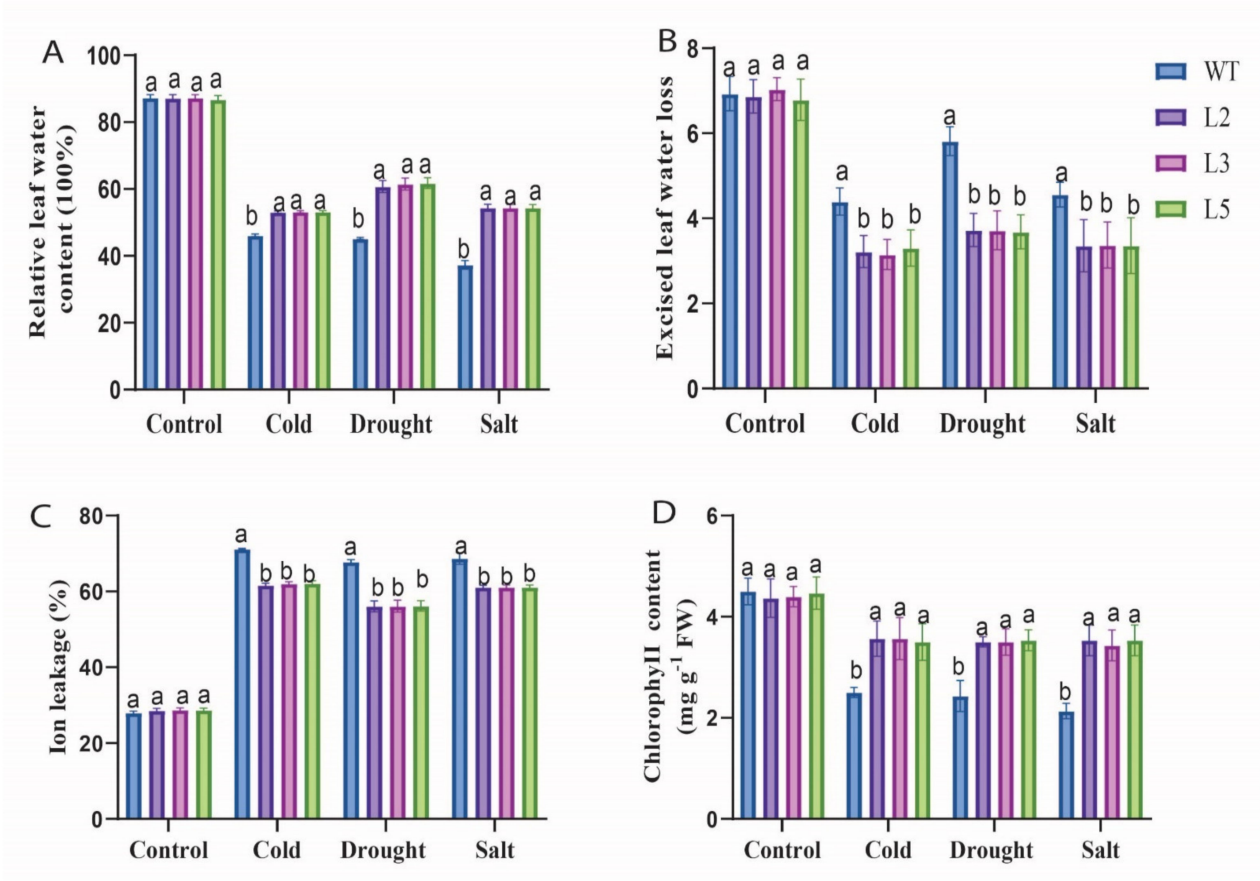

Figure 5. Assessment of physiological parameters in *GhMPK3* over-expressed lines and WT under drought and salt stress conditions. (**A**) Relative leaf water content (RLWC), (**B**) Excised leaf water loss (EWL), (**C**) Ion leakage, and (**D**) Chlorophyll content after 8 days of exposure to stress. Each experiment was performed three times. The bar represents a standard error (SE). Various letters above the columns indicate statistically significant differences (ANOVA, $p < 0.05$). WT: Wild type. L2, L3, L5: Overexpressed lines.

The enzyme activity in the transgenic lines and WT plants was analyzed using two antioxidants (CAT and POD) and two oxidants (MDA and H_2O_2), respectively. The antioxidant concentration in transformed lines was found to be higher than the WT (Figure 6C–D). Higher antioxidant levels (CAT and POD) in transgenic lines have shown that transgenic lines can greatly reduce ROS to normal levels during salt and drought stress [37]. The concentration of oxidants (MDA and H_2O_2) in the WT was found to be significantly higher and significantly lower in the transgenic line under both stress conditions (Figure 6A–B). The rates of enzyme activities in stressed plant's leaves indicate their ability to cope with the impact of the stress [38].

3.5. Expression of Stress-Responsive Genes in WT and GhMPK3-Overexpressed Arabidopsis under Cold, Drought, and Salt Treatments

To further explore the function of *GhMPK3* under abiotic stress, six stress-responsive gene expression levels were investigated in *GhMPK3* enhancing cold, drought, and salt stress in cotton plants. The stress-responsive genes investigated include *AtABF4*, *AtCBL1*, At*ERD15*, *AtKIN1*, *AtRD29A*, and AtSOS1. All the transgenic lines showed upregulated expression compared with the WT (Figure 7). This indicates that the overexpression of *GhMPK3* has a beneficial effect on the expression profile of the stress-responsive genes in these plants, implying that these gene regulations have a role in these plants' abiotic stress tolerance.

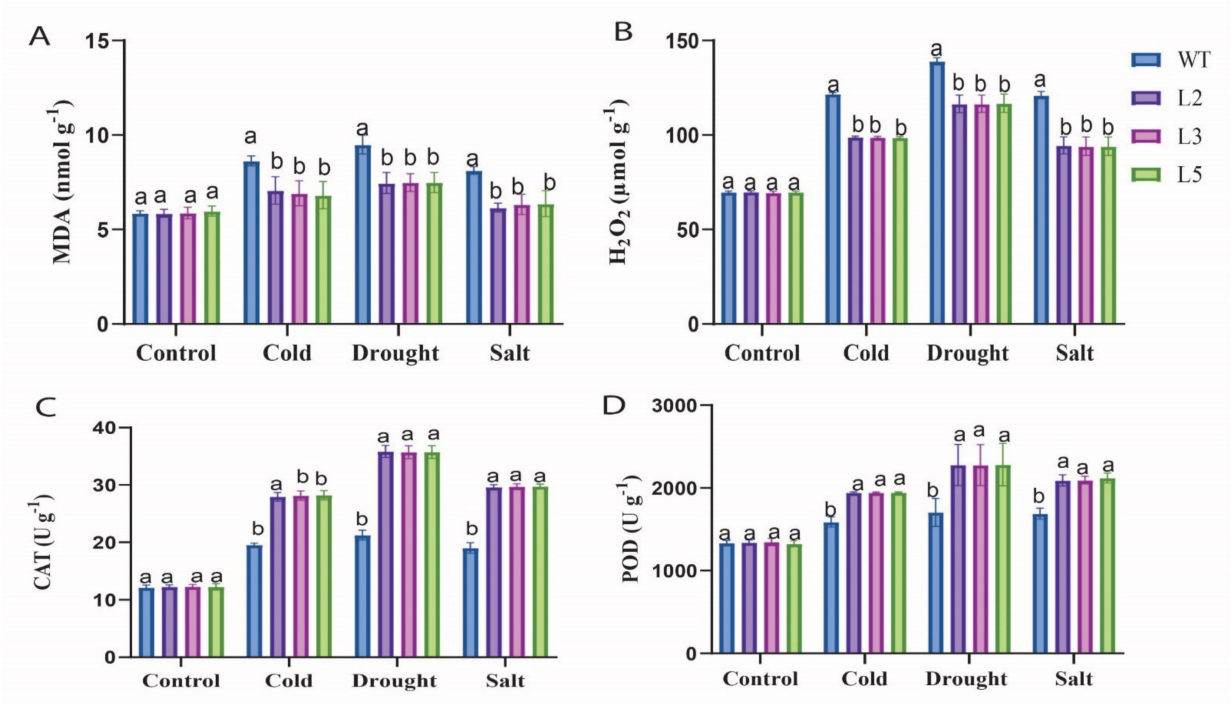

Figure 6. Determination of enzyme activity in *GhMPK3*-overexpressed and WT plants induced by stress treatments. (**A**) Malondialdehyde (MDA), (**B**) Hydrogen peroxide (H_2O_2), (**C**) Catalase (CAT), and (**D**) Peroxidase (POD) in post-treatment. Three replications were conducted in each experiment. The bar indicates a standard error (SE). "a" and "b" indicates major variations between the columns (ANOVA, $p < 0.05$). WT: Wild type. L2, L3, L5: Overexpressed lines.

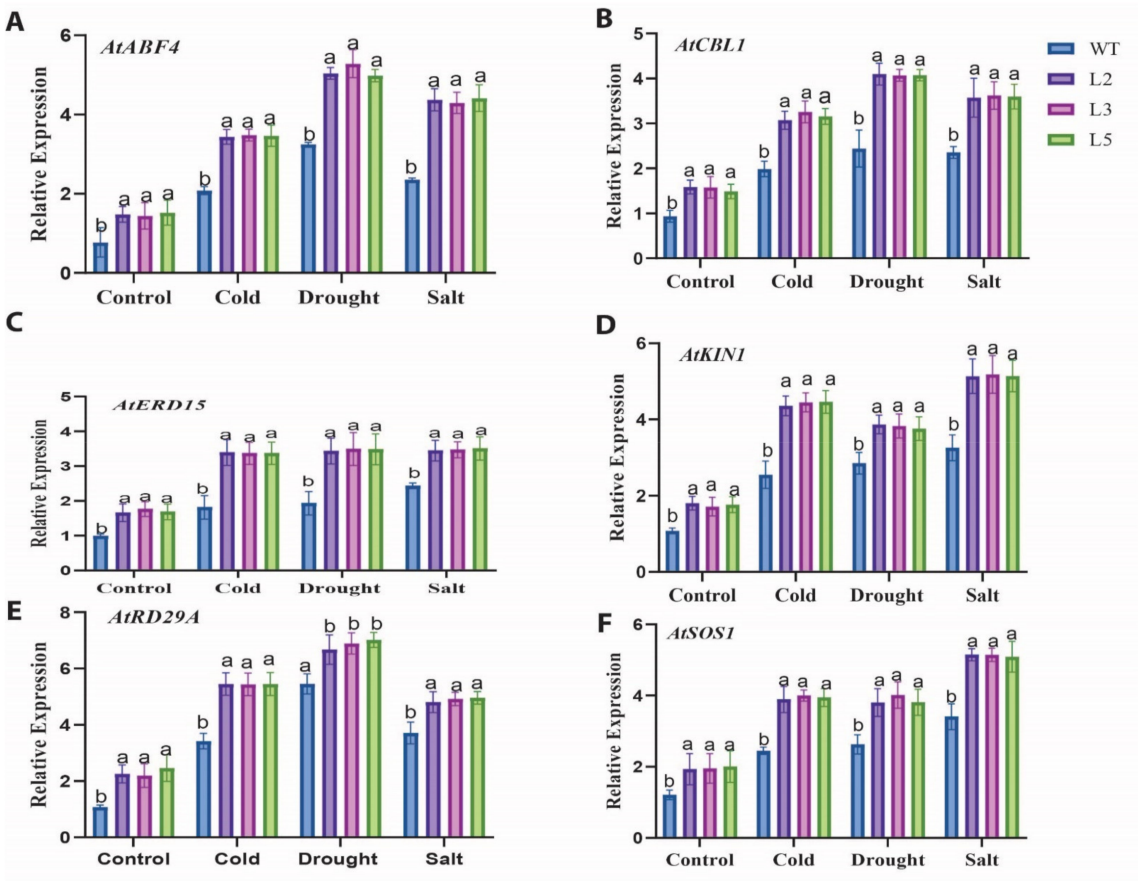

Figure 7. Expression profiles of abiotic stress-responsive genes. (**A**) *AtABF4*, (**B**) *AtCBL1*, (**C**) *AtERD15*, (**D**) *AtKIN1*, (**E**)

AtRD29A, and (**F**) *AtSOS1* in *GhMPK3*-transformed lines (L2, L3, and L5) and WT Arabidopsis under cold, drought, and salt stress. For the housekeeping gene, *AtACTIN2* was used. Three replications were conducted in the experiment. A standard error is indicated by the bar (SE). "a" and "b" indicate major variations between the columns (ANOVA, $p < 0.05$). WT: Wild type. L2, L6, L8: Overexpressed lines.

3.6. GhMPK3 Silenced Cotton Displays Sensitivity to Drought Tolerance

Virus-induced gene silencing (VIGS) technique serving as an effective method to analyze the role of *GhMPK3* under drought stress was carried out. Cotton plants infected with pCLCrVA:*GhMPK3*, plants infected with pCLCrVA:PDS (indicator), and plants infected pCLCrVA (control) were subjected to drought stress. Indicator (PDS) plants exhibited an albino phenotype after ten days. Phenotypic results for drought-subjected plants showed that pCLCrVA:*GhMPK3* leaves showed more shrinkage, whereas the leaves of the control plants appeared regular (Figure 8). The expression level of *GhMPK3* was analyzed by qRT-PCR, and the results show that infected plants (pCLCrVA:*GhMPK3*) have lower expression than the control plants (pCLCrVA).

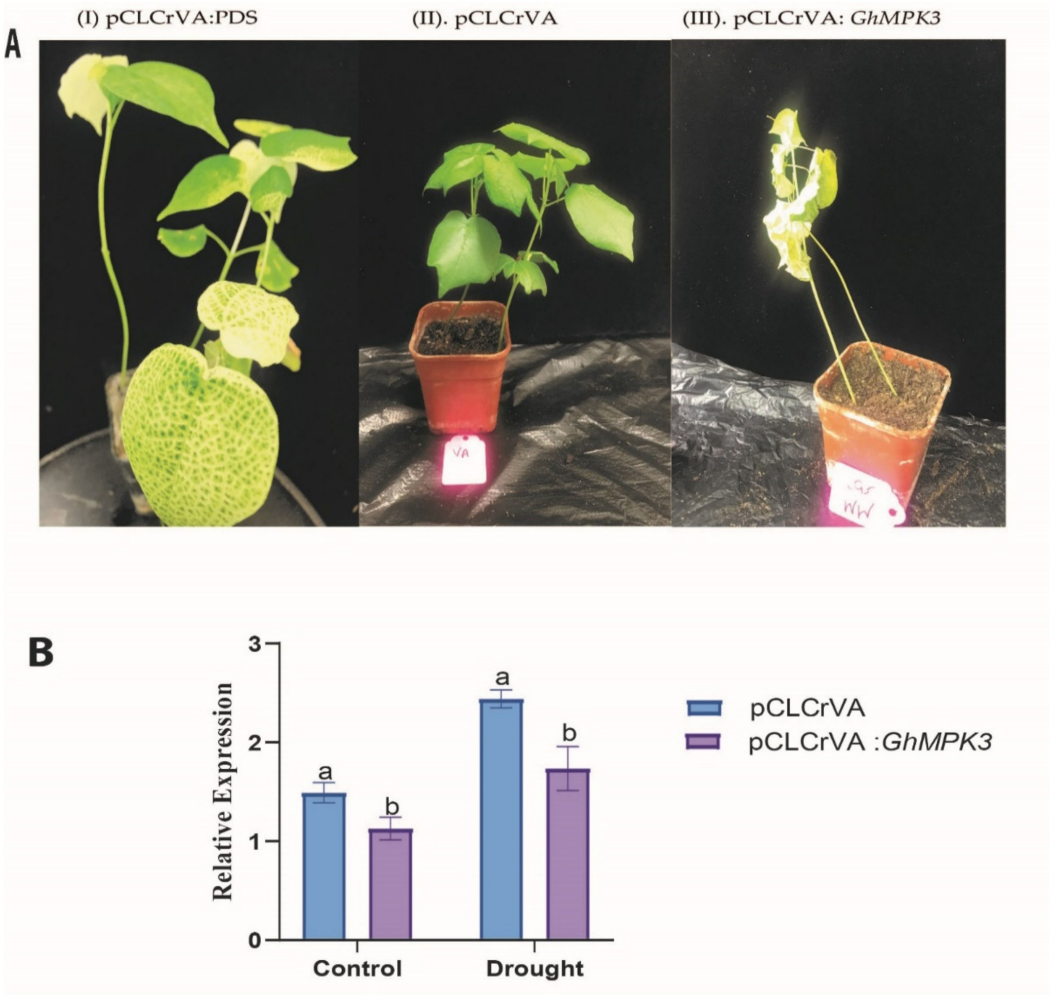

Figure 8. Phenotypic feature of *GhMPK3*-silenced plants (**A**) (I) pCLCrVA:PDS (II) pCLCrVA (III) pCLCrVA:*GhMPK3* (**B**). *GhMPK3's* expression level in empty control and *GhMPK3*-silenced plants. Each experiment was conducted three times. The error bar represents the three biological replicate's standard deviation. The significant differences between VA and *GhMPK3*-VIGS plants (ANOVA; $p < 0.05$) are indicated by different letters a/b.

Physiological parameters including relative leaf water content, excised leaf water loss, membrane ion leakage, and chlorophyll content revealed that the plants were less drought-tolerant than the control (Figure 9). Determination of the oxidant (MDA and H_2O_2) and antioxidant (CAT and POD) enzymes concentration in *GhMPK3*_silenced and VA plants under drought conditions. The results of the analysis showed that antioxidant concentrations were significantly reduced while the oxidant levels showed a sharp increase in the silenced plants compared with the control plants (Figure 10).

3.7. Transcriptional Activation Assay of GhMPK3

To investigate the activation activity of *GhMPK3,* the pGBKT7 and the construct of the pGBKT7-*GhMPK3* was inserted into a yeast strain AH109; these yeast cells were grown on the SD/-TRP, and SD/-TRP-HIS-ADE (selective medium). All transformants grew in SD/-TRP and SDO/X, but no growth is observed on SD/-TRP-ADE-HIS (Figure 11). This result suggests that the *GhMPK3* gene has no activation activity.

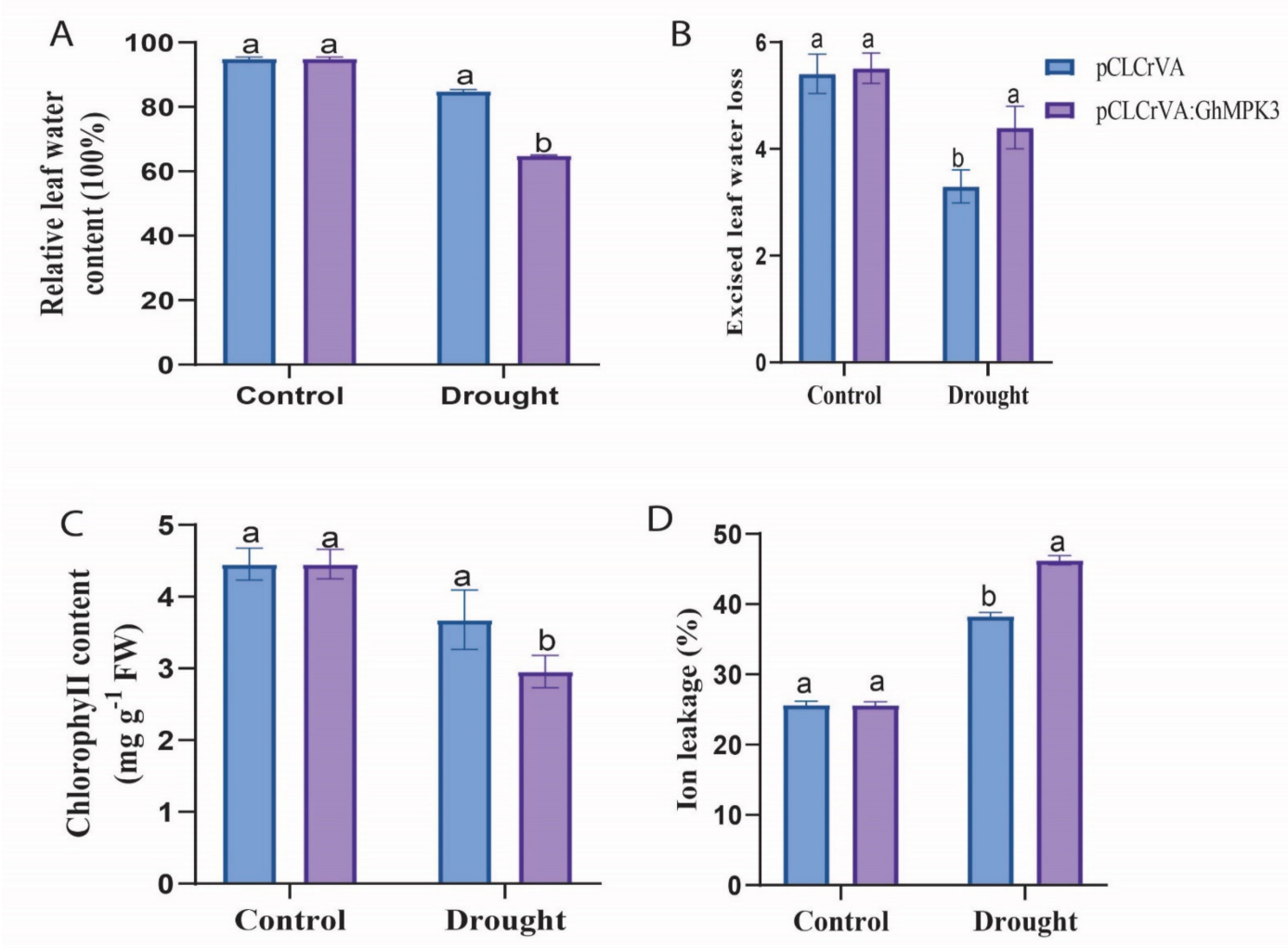

Figure 9. Evaluation of physiological parameters in *GhMPK3*-VIGS plants: (**A**) RLWC, (**B**) ELWL, (**C**) Chlorophyll, and (**D**) Ion leakage concentration in the leaves of empty vector and *GhMPK3*-VIGS, the assessment was performed after 10 days of drought exposure and control. Each experiment was conducted three times. The error bar represents the three biological replicate's standard deviation. The significant differences between VA and *GhMPK3*-VIGS plants (ANOVA; $p < 0.05$) are indicated by different letters a/b.

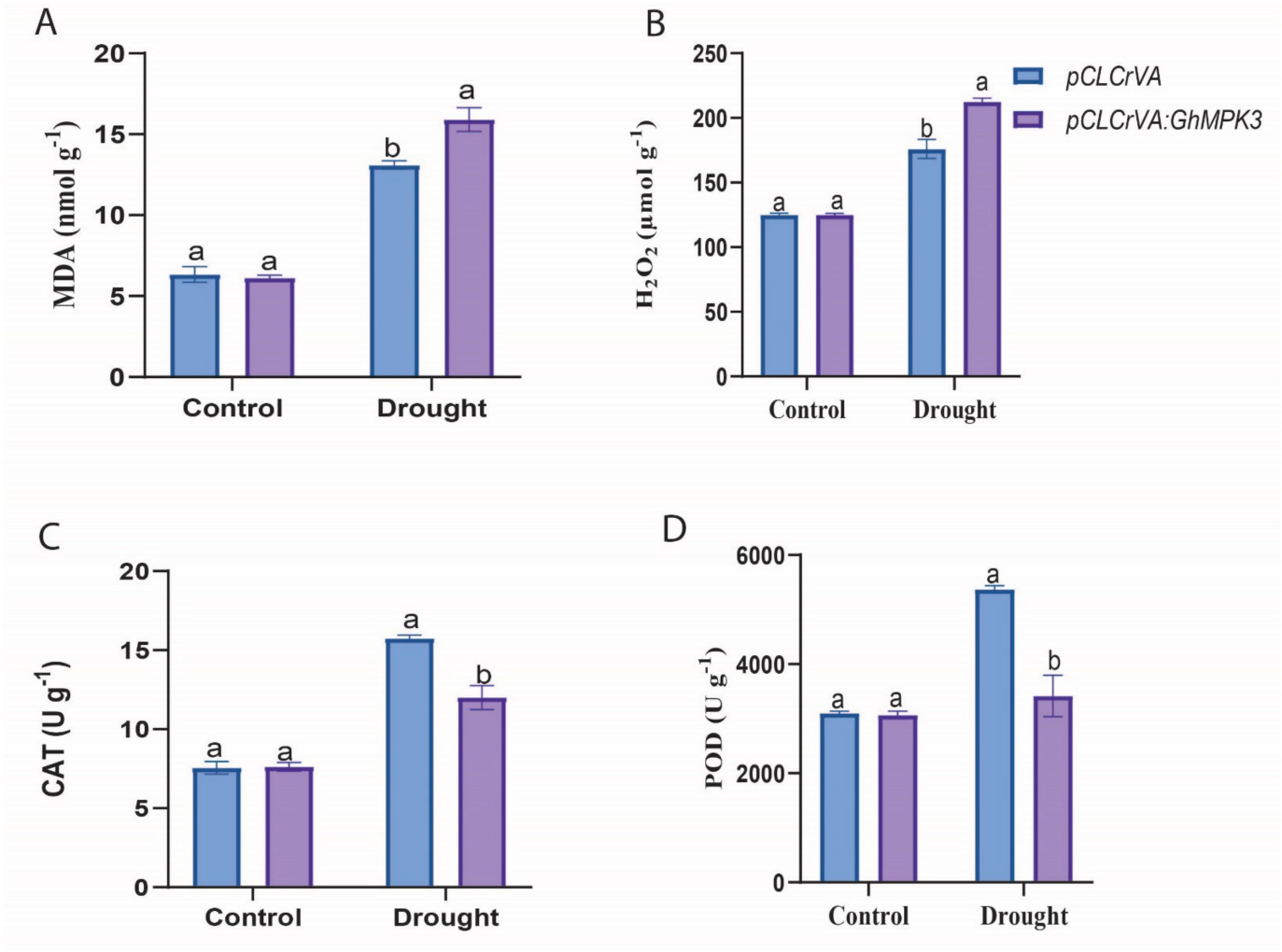

Figure 10. Enzyme activity analysis in *GhMPK3*-VIGS and VA cotton plant leaf under drought stress conditions. (**A**) MDA, (**B**) H_2O_2, (**C**) CAT, and (**D**) POD plants after 10 days of control and drought treatment. Each experiment was conducted three times. The error bar represents the three biological replicates' standard deviation. The significant differences between VA and *GhMPK3*-VIGS plants (ANOVA; $p < 0.05$) are indicated by different letters a/b.

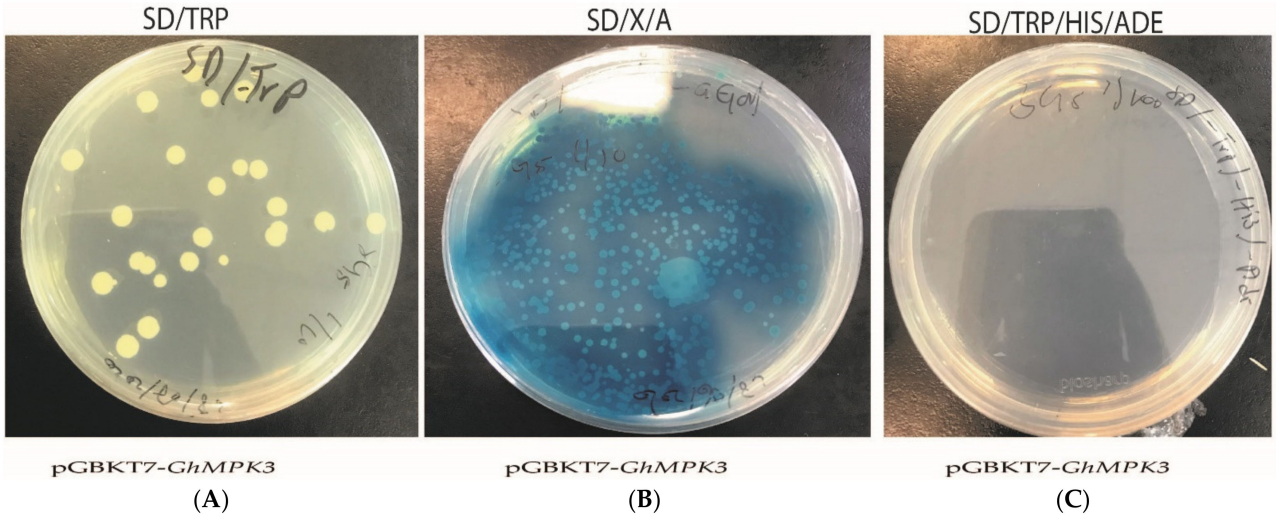

Figure 11. Determination of the auto-activation activity of *GhMPK3* gene. The constructs were transformed into Y2HGold yeast cells and plated on a *GhMPK3* gene on (**A**) SD/-TRP, (**B**) SDO/X/A, (**C**) SD/-TRP-HIS-ADE media and incubated for 3 days at 30 °C.

4. Discussion

Plant bodies are complicated, and their survival depends on well-coordinated internal activities due to ever-changing ecological parameters [39]. Abiotic stresses such as water shortage, salt stress, and severe temperatures have a major impact on crop growth and development [40]. Sessile plants have developed a variety of defensive strategies to cope with these harsh growing conditions. A few of these adaptations are the rapid activation of many genes that play important roles in stress tolerance [41]. Some recent studies have identified that MAPK genes perform an essential function in cotton by improving plant abiotic tolerance [5,15,28,36,42]. Thus far, further research is needed to know more about the complex role of other MAPK cascades in cotton plants. In this study, *GhMPK3*, a group A MAPK gene, was identified and characterized. The physicochemical properties of the Gh*MPK3* gene show that were hydrophobic, which is a property common among the membranous proteins [43]. Phylogenetic analyses and gene structure analysis revealed that the GhMPK3 gene and other group A MAPKs members have a similar protein structure The protein is characterized by the presence of TEY motif at the phosphorylation site between subdomains as seen in group A MAPKS of previous research findings [36]. These analyses confirmed that *GhMPK3* belongs to group A MAPKS. Subcellular localization analysis revealed that *GhMPK3* is localized in the cell membrane. Subcellular localization can reveal how a gene interacts with signaling pathways to function, provide important insights into its functions, and aid in their discovery to gain a deeper understanding of the complex pathways that govern biological processes at the cellular level [43]. MAPKs are found in both the cytoplasm and the nucleus, and their subcellular localization is highly associated with the cellular response [44]. The subcellular localization of MAPKs in the same family has yet to be determined. The fusion protein of GbMPK3, a group A MAPK from *Gossypium raimondii*, is found to be accumulated in the nucleus [36]. In addition, *AtMPK3* from *Arabidopsis thaliana* is located in the cytosol nucleus [45,46]. Subcellular localization analysis of MAPK in Tobacco with the use of CELLO v.2.5 revealed that *NtMPK7* was localized both in the nucleus and cytoplasm [47]. Our analysis of GhMPK3-GPF protein suggests that GhMPK3 was located in the cell cytoplasm, indicating that it may interfere with signaling pathways.

Abiotic stresses such as drought, salinity, and cold, among others, disrupt plant tissue functions, affecting both behavioral and environmental outcomes [34]. Plant responses are primarily regulated at the transcriptional level; transgenic species have developed a variety of stress-responsive genes that enable them to overcome the detrimental impacts of multiple stress factors. To determine if the gene had any effect on preserving the cell's normal biological function and electrolyte pressure, we measured RLWC, ELWL, ion leakage, and chlorophyll content in both transformed and WT under normal and stress conditions (drought, cold, salt). The findings revealed that the overexpressed lines showed stable physiological parameters such as relative water content, chlorophyll content, and lower water loss and excised leaves when compared to the WT plants. In addition, lower levels of ion leakage under drought, salt, and cold stress conditions were found in the transgenic plants compared to the WT plants. These results confirm that *GhMPK3* can maintain normal physiological functions in transformed plants. This follows prior research findings that MAK plays a role in sustaining cell physiological responses when plants are stressed by abiotic factors [48,49].

Plant's cellular activities and biochemical pathways change when they are exposed to stress conditions including cold, drought, and salinity. The excessive synthesis of ROS caused by cellular respiration resulted in oxidative stress, which results to plant death. Overexpressed plants can release antioxidant enzymes that control the amount of ROS within the cell. The *GhMPK3* overexpressed plants were found to have a higher level of antioxidants (CAT and POD) as compared with WT under each stress treatment. This indicates that the gene can control oxidative damage to the plants when they are exposed to cold, drought, and salt stress [50]. MAPKs genes were previously reported to be an essential factor for the production of antioxidant enzymes in plants [51,52]. The transgenic

plants had significantly lower oxidant (MDA and H_2O_2) concentrations than the WT. When the ROS level exceeds the cell's tolerance threshold, lipid peroxidation occurs, and this is among the most harmful process known to occur in plants exposed to stress. The level of oxidant concentration is used to determine the magnitude of lipid peroxidation [53]. Having a lower oxidants level implies that the transgenic lines had a greater ability to tolerate cold, drought, and salt stress, resulting in low or normal ROS production with no or low effects within the plant. The significance of MAPKs genes in ROS-related signal transduction has been previously identified. Arabidopsis MKK1 was thought to control ABA-induced CAT1 expression in response to H_2O_2 signaling [54]. Thus, the MKK1/MPK6 component is likely to be a key element of the ABA-dependent transcription factor that controls H_2O_2 development and stress responses [55]. In addition, *GhMPK17* overexpression in Arabidopsis improved plant resistance to salinity stresses, as well as changing H_2O_2 concentrations [44].

Six identified abiotic stress-responsive genes, *ABF4, CBL1, KIN1, ERD15, RD29A,* and *SOS1* were assessed and found to be significantly higher in all overexpressing lines compared to the wild type. This finding supports previous research findings that improved responsive gene products increased abiotic tolerance in transgenic Arabidopsis plants under abiotic stress [56]. These genes have been reported as stress tolerance genes in previous research [57–62]. The upregulation of all these abiotic stress-responsive genes in transgenic Arabidopsis plants revealed that the *GhMPK3* gene played a role in cold, drought, and salinity stress.

5. Conclusions

According to the results of this study, the *GhMPK3* performs a significant function in improving cold, drought, and salt stress tolerance. The overexpression of *GhMPK3* in Arabidopsis resulted in abiotic stress tolerance in transgenic plants, while silencing *GhMPK3* developed sensitivity to drought stress in cotton plants. Under cold, drought, and salt stress conditions, the transgenic lines had a stable cell membrane stability, as determined by the low level of ion leakage measured. Similarly, the transgenic lines had a low level of water loss but a high level of leaf water content. It has been discovered that overexpressed plants may integrate additional antioxidant enzymes to help facilitate the conversion of reactive oxygen species (ROS) to non-toxic compounds. In addition, *GhMPK3* overexpressed lines possess higher concentrations of antioxidant enzymes as compared to the WT plants. As indicated by increased levels of various oxidant enzymes measured, the VIGS plant's ability to withstand the drought stress was significantly reduced. Furthermore, all stress-responsive gene expression levels were upregulated in the overexpressed plants but were significantly downregulated in the WT under cold, drought, and salt stress. This research work lays the groundwork for more research into these genes to build a more robust cotton genotype that performs better under different environmental stress factors, such as cold, drought, and salt stress.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy11061049/s1, Table S1: Functions of Identified cis-regulatory element. Table S2: Gene primers used for qRT-PCR. Table S3: Stress Responsive Gene primers used for qRT-PCR.

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