

CbPLD γ gene from *Chorispora bungeana*: Gene cloning, characterization, expression, and expression analysis in drought

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Research

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Abstract

Background: *Chorispora bungeana* (*C. bungeana*) is a typical subnival alpine species, which shows high tolerance to multiple abiotic stresses. Phospholipase D (PLD) is a crucial enzyme participated in membrane phospholipid catabolism. In this study, to explore the function of *CbPLD γ* in drought stress, we cloned and characterized a *CbPLD γ* gene, which is a part of *CbPLD* gene family and from *C. bungeana*.

Methods: Use the gateway method for vector construction, using DNASTAR software, PCR machine, centrifuge, pipette, electrophoresis, gel imaging system, spectrophotometer, confocal microscope, etc. Spss, Origin software for statistical analysis.

Results: The *CbPLD γ* gene encodes 859 amino acids containing "FIYIENQYF" domain and two HKD domains. Bioinformatics analyses showed that the *CbPLD γ* is highly homologous with PLDs from other plant species. Real-time quantitative PCR (qRT-PCR) and Beta-glucuronidase (GUS) assay showed that *CbPLD γ* was accumulated dominantly in roots and stems. Compared with the control, the expression pattern of the *CbPLD γ* mRNA is in response to low temperature, salt, mannitol, and exogenous ABA have up-regulated. Subcellular localisation analysis showed that the *CbPLD γ* was localized in the cell membrane. Compared with wild-type *Arabidopsis thaliana*, *CbPLD γ* gene overexpression plants showed higher activities of antioxidant enzymes, and lower levels of malondialdehyde content and electrolyte leakage under drought stress.

Conclusions: In this study, novel *PLD γ* gene was amplified from *C. bungeana* and was called *CbPLD γ* . These confirmed that *CbPLD γ* involved in the response to drought stress, and has the potential to improve the drought tolerance of plants. This is the first report about cloning and characterizing the gene of *CbPLD γ* from *C. bungeana*. It laid a foundation for further research and improvement of the PLD gene family of *C. bungeana*.

Introduction

Phospholipids are critical in cell membranes and also play a pivotal role in cell development, biotic stress and signal transduction. The majority of phospholipase D (PLD) can hydrolyze of phospholipids to produce phosphatidic acid (PA) and soluble head group. All of the PLD gene superfamily involves two copies of conserved HxKxxxxD sequence, which is known as the HKD motif, and it is an active site. In addition, N-terminal domain contains the binding motif lipid and C-terminal domain contains the catalytic motif. Different PLDs seem to have different but somewhat overlapping functions in cellular processes (Lein W, et al. 2001). PLD gene has been cloned from many species such as *rice bran* (*Oryza sativa* L.), *Maize* (Ueki J, et al. 1995), *tobacco* (Lein W, et al. 2001), *cowpea* (*Vigna unguiculata* L. Walp) (Ali Y B, et al. 2007), *tomato* (Tiwari K, et al. 2011), *Arabidopsis thaliana* (Pappan K, et al. 1997) and other gene subfamily which have been reported in many species. PLD genes are classified into six subfamilies in the *Arabidopsis* genome: α (3), β (2), γ (3), δ , ϵ and ζ (2) (Wang X. 2005). The majority of PIP₂ and Ca²⁺ can influence activity of *PLD γ 1* and *PLD γ 2* (Qin W, et al. 1997; Qin C, et al. 2007), and previous studies have

showed that *PLD γ* are more tolerant to all stress in the *Arabidopsis* seedlings (Zhao J, et al. 2011). *PLD γ* is located in the nuclear, and this perhaps revealed that the *PLD γ* play a role of cell division and reproduction (Fan L, et al. 1999).

Chorispora bungeana (*C. bungeana*) is a cruciferae plant, which is a typical representative to subnival alpine plant. It can adapt to the environment of low oxygen partial pressure, cold and strong ultraviolet radiation. This is closely related to *Arabidopsis* (Zhao Z, et al. 2012). Long-term studies have shown that *C. bungeana* has no special morphological characteristics. The surface lint and wax layer can resist harsh environment, but it contains a lot of free fatty acid, neutral amino acid, soluble sugar, Mg^{2+} -ATPase activity, and unsaturated fatty acids (Song Y, et al. 2015).

Abiotic stresses, such as salt, drought, salicylic acid (SA), abscisic acid (ABA), can also induce the PLD expression (Hong Y, et al. 2016; Guo L, et al. 2016; Kalachova T, et al. 2013; Misugi Uraji, et al. 2012). For example, *PLD α 1* and *PLD δ* in ABA signaling have a synergistic effect in *Arabidopsis* guard cells (Kalachova T, et al. 2013). SA can active the PLD to induce of stomatal closure in *Arabidopsis* (Guo L, et al. 2016). In *Craterostigma plantagineum*, water deficiency triggers PLD activity (Frank W, et al. 2000). In addition, PLD is more tolerant to freeze stress in *C. bungeana* callus (Yang N, et al. 2013). Therefore, PLDs are thought to be involved in plant responses to abiotic stresses, and have multiple functions during plant growth and development (Pinhero R G, et al. 2003). Drought is a major stress factor that limits agricultural production worldwide. In order to adapt to survive under drought stress, plants will respond through changes in morphology and physiology, biochemistry and molecular response. When stress intensifies, the plant activates the mechanism of enzyme activity and membrane structure to avoid cell death, such as increased antioxidant activity, decreased membrane permeability, and control of reactive oxygen homeostasis (Hong Y, et al. 2008; Hong Y, et al. 2010.).

To the best of our knowledge, there is no detailed report was seen on the characterization of the *CbPLD γ* of the *C. bungeana*. Thus, in this study, we cloned and analyzed the full length of *CbPLD γ* cDNA sequence from *C. bungeana* and its promoter region, examined the expression pattern of *CbPLD γ* in different stress conditions, subcellular localization of *CbPLD γ* , organizational positioning and tolerance of overexpressing plants to drought stress. It provided a better insight into the molecular mechanisms *CbPLD γ* , and provides a theoretical basis for further research and improvement of the gene function of the PLD gene family of *C. bungeana*.

Material And Methods

Plant material: The *C. bungeana* of plantlet was obtained and had a little of modified by previously. *C. bungeana* seedlings were cultivated on Murashige and Skoog (MS) medium with 1 mg/l 6-benzyladenine (6-BA) and 3% (w/v) sucrose.

Arbidopsis and tobacco (*Nicotiana benthamiana*) seeds were disinfected and germinated on MS medium. After 5 days, the germinated seeds were transferred to the soil, and all the plants were grown in

a greenhouse under 25 °C light for 16 h and dark light for 8 h.

Stress treatment: The *C. bungeana* seedlings were grown in a growth chamber under 25 °C, photoperiods of 16 h light/8 h dark for 1 weeks. The seedlings were subjected to 150 mM NaCl, 0.3 M mannitol, 4 °C and 100 mM abscisic acid (ABA) induction, respectively, with three replicate. As controls, the plantlets were cultivated on MS. All the samples were immediately frozen in liquid nitrogen for RNA isolation and quantitative real-time PCR.

The overexpressing *Arabidopsis* seedlings were grown in a growth chamber under 25 °C, photoperiods of 16 h light/8 h dark for 2 weeks. The seedlings were growing without watering for 1 week, rehydrated for five days. This sample is used for electrolyte leakage, malondialdehyde (MDA) and antioxidant activity analysis.

Cloning of *CbPLD γ* gene: RNAiso Plus kit (Takara, Dalian) was used to extract total RNA from the plantlets of *C. bungeana* according to the manufacturer's instructions, The first strand of cDNA was synthesised using the PrimeScript™ II 1st Strand cDNA Synthesis Kit (Takara, Dalian) according to the manufacturer's instructions.

Based on other *PLD γ* homologous species, Two primers (CbPLD γ 1-F and CbPLD γ 1-R) (Table 1) corresponding to sequences of the conserved regions of PLD genes were used to amplify part of the *CbPLD γ* sequence using LA Taq DNA polymerase (Takara, Dalian). The amplified products were cloned as the pMD19-T vectors (Takara, Dalian) for the further sequencing. Design primers (CbPLD γ 2-F and CbPLD γ 2-R) (Table 1) at the 5' end of the first amplified fragment to amplify the middle fragment of *CbPLD γ* . The amplified products were cloned as the pMD19-T vectors (Takara, Dalian) for the further sequencing. The three sequences were combined to obtain the middle part of *CbPLD γ* .

The 5' and 3' rapid amplifications of cDNA ends (RACE) were performed using the SMARTer™ RACE cDNA Amplification (Invitrogen, USA). Based on the instruction manual, total RNA were isolated and produced templates for 5' and 3' race from the *C. bungeana*. The primers of 5'GSP, 5'NGSP, 3'GSP and 3'NGSP (Table 1) were designed to amplify of RACE products, and nest PCR products were cloned into pMD19-T vectors and sequences.

By comparing and aligning the sequences, the full length of *CbPLD γ* gene sequences were obtained, which were contained in middle region, the 5' and 3' RACE sequences. The full length of *CbPLD γ* gene sequences were verified by PCR using Primer Star HS DNA polymerase (Takara, Dalian) and sequencing.

Table 1. Primers list used for gene amplification and for RT-qPCR analysis

| Primers | Sequence(5'-3') |
|------------|----------------------------------|
| CbPLDy1-F | 5'-AAGAGACTCGCCGGTTTTTTCAA-3' |
| CbPLDy1-R | 5'-AAGCTTTAGCCAGCGTTCCT-3' |
| CbPLDy2-F | 5'-TGGATGCAGCAYTTYRATGT-3' |
| CbPLDy2-R | 5'-AGCTGTGACCACTTCCC-3' |
| 3'GSP | 5'-GGGGGAAGTGGTCACAGCTTTATA-3' |
| 3'NGSP | 5'-CCGAAGCATTCTCTATTTAGGACA-3' |
| 5'GSP | 5'-TCGAGATATACACTTGGAAGAGTA-3' |
| 5'NGSP | 5'-CAGTCATTACCAAAGCCAACACCC-3' |
| CDS-F | 5'-ATGGCGCATCATCCGGTTTATA-3' |
| CDS-R | 5'-GGTTGTTGACAGTACAAGGCAGG-3' |
| SP1-1 | 5'-CCATGTTGGGGAGATGTTTGGCT-3' |
| SP2-1 | 5'-TGACCCTCCTCCCATTGACATAGTCT-3' |
| SP3-1 | 5'-AAGATCGATCACTTGGTGGGCAATC-3' |
| SP1-2 | 5'-GCAGGCATGCAAGCTTGGCGTAATCA-3' |
| SP2-2 | 5'-GTGTAAAGCCTGGGGTGCCTAATGAG-3' |
| SP3-2 | 5'-GTGCCAGCTGCATTAATGAATCGG-3' |
| Promoter-F | 5'-ATCGTCTACCGGGTTGGACTCAAG-3' |
| Promoter-R | 5'-GGTGAAACGTTGACTTGAAGCGG-3' |
| qRT-F | 5'-TCGGAGGGCTAGACTTGTGTAACG-3' |
| qRT-R | 5'-TCTTGGTCCATCATCCGCAGTAGT-3' |
| Pro-CDS-F | 5'-ATCGTCTACCGGGTTGGACTCAAG-3' |
| Pro-CDS-R | 5'-GGTTGTTGACAGTACAAGGCAGG-3' |
| Pro-CDS-NR | 5'-TGCCACCGAGATCTGGGAAT-3' |
| SOD-L | 5'-GGATCTACCGATTCCTTTGCT-3' |
| SOD-R | 5'-CTGCAACTCCTTTCGCCATT-3' |
| POD-L | 5'-GAAAAGCATGGACCCGTACC-3' |
| POD-R | 5'-ACAACACTACATCTGATTGAGAGT-3' |
| CAT-L | 5'-CAATTTTCGACAGGGAACGGA-3' |

| | |
|-------|-----------------------------|
| CAT-R | 5'-AACCGGACAATGACAGGAGT-3' |
| Act-F | 5'-TGTGCCAATCTACGAGGGTTT-3' |
| Act-R | 5'-TTTCCCGCTCTGCTGTTGT-3' |

Cloning of the promoter of *CbPLD γ* . Genomic DNA was isolated from the *C. bungeana* using the TaKaRa MiniBEST Plant Genomic DNA Extraction Kit (Takara, Dalian) to clone the *CbPLD γ* promoter sequence. The promoter of *CbPLD γ* was cloned by high-efficiency thermal asymmetric interlaced PCR (hiTAIL-PCR) using Genome Walking Kit (Takara, Dalian). Adaptors were provided by the kit and SP primers (Table 1) were designed according to the sequence of *CbPLD γ* . The PCR products were purified using TIANGel Midi Purification Kit (TIANGEN, Beijing), cloned into pMD19-T vector and sequenced.

Bioinformatics analysis: The similarity of nucleotide and amino acid sequence were searched in BLAST of the NCBI. The opening reading frame (ORF) was selected using the Editseq in DNASTar software. The amino acid sequences of *CbPLD γ* were deduced in ExpASy website (<https://web.expasy.org/translate>). The Megalign was used in multiple alignments of the *CbPLD γ* amino acid and other species by DNASTar software. The phylogenetic trees were constructed with Neighbor-Joining method by MEGA-X.

Quantitative real-time PCR: Total RNA was isolated from *C. bungeana* were subjected to salt, mannitol, 4 °C, and abscisic acid (ABA) induction. The cDNAs were synthesized using the FastKing RT Kit (With gDNase) (TIANGEN, Beijing). Gene-specific RT primer pairs designed based on the *CbPLD γ* sequence (Table 1) were subjected to real-time quantitative PCR using TBGreen® Premix Ex Taq™ II (Tli RNaseH Plus) (Dalian Takala). The *Actin* genes were used as internal controls for normalizing gene expression levels. The results were displayed using the $2^{-\Delta\Delta Ct}$ method.

Histochemical location of *CbPLD γ* . Total RNA was isolated from *C. bungeana* roots, stems, and leaves using the Trizol reagent. The cDNAs were synthesized using the FastKing RT Kit, the Real-time quantitative PCR was performed. The *Actin* genes were used as internal controls for normalizing gene expression levels.

The promoter of *CbPLD γ* was cloned (Table 1) by Genomic DNA of *C. bungeana*, and the correct sequences were inserted into vectors. The final construct of pBIB-CbPLD γ -GUS was introduced into *Arabidopsis* Col-0 according the floral dip method (Yew C L, et al. 2017) by *Agrobacterium tumefaciens* GV3101. The homozygous seedlings were screened by herbicide basta and used to detect the histochemical location of GUS activity. Plant tissues were incubated for 16 h at 37 °C in 5-bromo-4-chloro-3-indolyl- β -D-glucuronic acid (X-Gluc) solution, and then were decolorized in destaining solution for approximately 10 h. The seedlings were washed and then observed under Olympus CX31 Microscope (Olympus, Japan).

Subcellular localization of *CbPLD γ* . The coding regions of *CbPLD γ* were amplified from *C. bungeana* by RT-PCR using primers (Table 1), and the PCR products were sequencing. The cloned gene fragment was recombined into the pMDC83 vector with recombinase. Competent DH5 α cells were transformed and

plasmids were verified by sequencing. The correct plasmids containing pMDC83-CbPLD γ -GFP vector was introduced into *Agrobacterium tumefaciens* GV3101. Sequentially, the recombinant plasmids were transformed into the tobacco with the epidermal cells for transient expression method (Sparkes I A, et al. 2006) and cultured in darkness at 25 °C for 48 h. The GFP fluorescence was captured by confocal microscope (Leica, German).

Transformation of *Arabidopsis*: The *CbPLD γ* promoter and coding regions was amplified by nested PCR using primers (Table 1). The cloned fragment was recombined into the pBIB vector with recombinase. Competent DH5 α cells were transformed and plasmids were verified by sequencing. The correct plasmids containing pro-pBIB-CbPLD γ vector was introduced into *Agrobacterium tumefaciens* GV3101. Sequentially, the recombinant plasmids were transformed into the *Arabidopsis* Col-0 according the floral dip method and cultured in darkness at 25 °C for 24 h. Transformants were selected on MS medium containing basta. The T₄ generation of transgenic *Arabidopsis* plants was used for further analyses.

Determination of electrolyte leakage, malondialdehyde content, and antioxidant enzyme activities:

Electrolyte leakage, malondialdehyde content, and antioxidant enzyme activities were analyzed to determine the mechanism by which overexpression of *CbPLD γ* conferred resistance to drought stress.

Electrolyte leakage was measured by the method of Walker et al. (Walker D J, et al. 2010). Take overexpression *Arabidopsis* plants with good growth vigor and similar shape and size, rinse three times in distilled water, wash away the surface grease and dust, and then use the filter paper to absorb the moisture on the surface of the leaves. Then incubated in 5 ml distilled deionized water at 25 °C for 2 h. The conductivity was determined E1 using a conductance bridge (DDS-11A, Yamei Electron Instrument Factory, Wuxi, China). Then, the samples were heated in boiling water for 30 min and cooled to room temperature, conductivity was read again E2.

The determination of MDA content is slightly modified according to the method of Lazzarino et al. (Lazzarino G, et al. 1995). Fresh leaf tissue was ground in 10% trichloroacetic acid (TCA). After sufficient grinding, the grinding liquid was centrifuged at 15000 \times g for 20 minutes at 4 °C. The supernatant was collected and mixed with 0.6% thiobarbituric acid (TBA) in 10% TCA. The mixture was heated in a water bath at 95 °C for 15 min, and then quickly placed on ice to cool. After the mixture was cooled, the sample was centrifuged at 10000 \times g for 10 min, and the absorbance of the solution at 532, 600, and 450 nm were recorded.

Antioxidant enzyme activity was measured by real-time fluorescent quantitative PCR. The qRT-PCR primers were designed based on SOD (At1g08830), CAT (At1g20630), POD (At1g05240) and Actin (AY825362) gene. The results were displayed using the $2^{-\Delta\Delta ct}$ method.

Statistical analyses: All experiments were repeated at least three times. Data were analyzed by one-way ANOVA using SPSS 19.0 for windows (SPSS Inc. Chicago, IL, USA).

Results

Cloning and sequence analysis of the *CbPLD γ* gene: Based on the *PLD γ* gene sequence in other homologous species, we have designed a pair of primers to amplify the middle of *CbPLD γ* gene sequence cDNA from *C. bungeana*. The single fragments about 480 bp were obtained. With the known cDNA sequences of the *CbPLD γ* genes, we obtained 712 bp after designed a pair of primers to clone the middle of *CbPLD γ* gene. 5'GSP and 5'NGSP were designed beside on the middle region sequence for 5'-RACE. We obtained a single fragment about 524 bp of two PCR amplified, which has a 160 bp 5' untranslated region (UTR) of the ATG codon upstream. We have designed 3'GSP and 3'NGSP for 3'-RACE beside on the middle region, the 1373 bp was cloned and contained 197 bp 3'UTR in the TAA codon downstream, including a poly A tail of 12 bp. By comparing and aligning the sequences, we have deduced the full-length cDNA from the known sequence of 2937 bp. Analysis by DNASTAR, the full-length cDNA contained a 2580 bp ORF, which encoded a protein of 859 amino acids with a calculated molecular weight of 96.3 kDa and with a pI of 7.88.

The *PLD ζ* gene in other plants was searched in NCBI, and sequence alignment was performed with DNASTAR. The *CbPLD γ* sequence is the most similar with other species *PLD γ* gene at the amino acid level. This protein contained two HKD motifs: HQKTVIVD is located in the 372-379 amino acids and HSKGMVVD is located in the 710-717 amino acids. In addition, *CbPLD γ* sequence was contained by the "FIYIENQFF", which was the conserved sequence in the entire plant PLD gene (Fig.1).

Using MEGA-X software, the *CbPLD ζ* gene was sequence aligned with other plant *PLD* genes and a phylogenetic tree was constructed. The phylogenetic tree was mainly illustrate that the *CbPLD γ* was classified to the *PLD γ* subfamily and more close to the *Arabidopsis PLD γ* gene compared with the *PLD* genes of other plant (Fig.2).

The expression of *CbPLD γ* is induced by abiotic stress: Under stress conditions, we have studied the expression changes of *CbPLD γ* , the gene specific primers were used for real-time quantitative PCR. We have studied the expression pattern of the *CbPLD γ* mRNA in response to low temperature, salt, mannitol and exogenous ABA respectively in different times and got the transcription level of *CbPLD γ* . After mannitol treatment, the relative expression of *CbPLD γ* gene increased significantly and was higher than that of the control group, with the treatment time, the gene expression level was also different, it reached the peak at 12 h and the transcription levels had a 2.97 fold change. Under NaCl treatment, the relative expression of the *CbPLD γ* gene increased significantly and was higher than that of the control group. At 12 h, the relative expression of the gene reached a peak and multiplied. Under the 4 °C treatment, the transcription level of *CbPLD γ* was up-regulated, and the transcription level had a fold change after 24 h of treatment. After exogenous ABA treatment of *C. bungeana* seedlings, the relative gene expression levels increased significantly (Fig.3). This result showed that the *CbPLD γ* is in responds to low temperature, salt, mannitol and ABA.

Histochemical location of *CbPLD γ* . In order to explore the functions of *CbPLD γ* , we selected roots, stems, leaves from *C. bungeana* within real-time quantitative PCR, the results indicated that *CbPLD γ* is expressed in all tissues examined, and has a significant enrichment in roots (Fig.4 A).

The GUS staining experiment is further confirming this expression pattern, the plasmid pBIB-CbPLD γ -GUS was introduced into *Arabidopsis* Col-0 according the floral dip method by *Agrobacterium* GV3101. The homozygous seedlings were screened by herbicide basta and used to detect the histochemical location of GUS activity, The GUS activity results further confirmed that *CbPLD γ* is expressed in all the tissues, but with a significant enrichment in roots (Fig.4 BC). The GUS assay and qRT-PCR have an identical result.

Subcellular localization of *CbPLD γ* . The subcellular localization of *CbPLD γ* is important for understanding its function. In silico subcellular localization analysis performed using WoLF PSORT and Cell-PLoc indicated that *CbPLD γ* is mainly localized in the cell membrane. In order to study the subcellular localization of *CbPLD γ* , the CDS was linked to pMDC83 vector with GFP tag, the construct was introduced into *Agrobacterium* GV3101. Sequencely, the recombinant plasmids was transformed into tobacco according the epidermal cells for transient expression method. The GFP fluorescence was captured by confocal microscope, and the results showed that the *CbPLD γ* was located in the membrane (Fig.5).

Determination of electrolyte leakage, malondialdehyde content, and antioxidant enzyme activities: The T₄ generation of transgenic *Arabidopsis* is adversely stimulated by the drought stress, its electrolyte leakage will increase significantly. In this experiment, we found that drought stress caused a significant increase in the conductivity of WT and OE(overexpressed plants), while the conductivity of WT plants was significantly higher than OE, indicating that under drought stress, the membrane damage of overexpressed plants is less than that of WT Plants (Fig.6).

MDA is a product of lipid peroxidation and is often used as an indicator of cell membrane free radical damage. Our measurement of MDA content showed that the MDA content of WT plants under drought treatment was always higher than that of transgenic lines (Fig.6). These physiological parameters indicated that transgenic lines were more resistant to drought than WT.

Under normal conditions, there was no significant difference in SOD, POD, and CAT relative expression between *CbPLD γ* transgenic lines and WT. However, the drought significantly increased the activities of SOD, POD, and CAT in the two plants, and the OE lines had higher antioxidant enzyme activities than the WT plants. This shows that compared with WT plants, transgenic lines are more resistant to drought stress (Fig.7).

Discussion

C. bungeana is a typical subnival alpine plant, its living environment is extremely harsh and the temperature changes greatly (Wu J M, et al. 2008.). In recent years, the research on *C. bungeana* has been reported from various levels, especially the anti-adversity response mechanism of *C. bungeana*. Phospholipase D (PLD) is involved in different plant processes, ranging from responses to abiotic and biotic stress to plant development (Distéfano Ayelen M, et al. 2015). In this study, the *CbPLD γ* (MF951104) gene was cloned on the basis of the cloned PLD gene family members of a *C. bungeana*, and its sequence was analyzed. The gene is 2937 bp in length and contains an open reading frame (ORF)

of 2580 bp, 5' and 3' untranslated regions are 160 bp and 197 bp, respectively, and also include a 12 bp poly A tail, encoding a protein of 859 amino acids. *CbPLD γ* includes two HKD domains, which are the active sites of PLD. In addition, *CbPLD γ* has the conserved motif "FIYIENQFF" in all plant PLDs, which may be related to the hydrophobic interaction between the methyl groups in the choline group, which is as important as the HKD domain (Yuan H, et al. 2005.) (Fig. 1). Phylogenetic analysis showed that *C. bungeana CbPLD γ* gene has high homology with other plant *PLD γ* genes. In addition, among the *PLD γ* subtypes, *C. bungeana* is closely related to *Arabidopsis* *PLD* gene (Fig. 2). This study explored the relative expression of *CbPLD γ* gene under salt, mannitol and 4 °C. It was found that 4 °C low temperature, salt and mannitol stress can induce the expression of *CbPLD γ* gene, indicating that *CbPLD γ* gene is widely involved in *C. bungeana* to many abiotic stress responses. Many plant growth regulators, such as ABA, participate in plant stress response and play an important role (Shaterian J, et al. 2005.). Exogenous ABA can induce the expression of *CbPLD γ* gene, and it is speculated that the biosynthesis of *CbPLD γ* may be through ABA dependent pathway under stress conditions (Fig. 3).

The different expression of PLD in various organs or different subcellular locations may be of great significance for further understanding the function and regulatory mechanism of each PLD. PLD exists in the plasma membrane, endoplasmic reticulum, and submicrosomes (Xu L, et al. 1996.). *PLD α* exists in soluble and membrane-related components, and the relative distribution between the two components depends on tissue and developmental stage (Dyer J H, et al, 1994.). Real-time fluorescence quantitative PCR confirmed that the average expression level of *PLD α 3* in buds, flowers, siliques, stems, old leaves, and roots was 1000 times lower than that of *PLD α 1*, indicating that the expression level of *PLD α 1* is generally much higher than *PLD α 3* (Hong Y, et al. 2008). Studies have shown that GmPLD γ -GFP fusion protein is expressed in transgenic *Arabidopsis* roots and in tobacco leaf mitochondria (Bai Y, et al. 2020). The expression of *AtPLD δ* gene in roots, flowers and stems is higher than that in leaves and pods, and the expression in old leaves, stems, flowers and roots is much higher than that of young leaves and pods. The subcellular localization of *AtPLD δ* gene in tobacco is also plasma membrane relate. *PLD γ* can be detected in the plasma membrane, intracellular membrane, nucleus and mitochondria, while *PLD δ* can only be detected on the plasma membrane (Fan L, et al. 1999). *PLD ϵ* was found in microsomes, but not in the soluble fraction. When *PLD ϵ* is transiently expressed in tobacco leaves, fluorescence is only detected on the plasma membrane (Hong Y, et al. 2009). *PLD α* , *PLD β* and *PLD γ* can exist in two states of soluble and membrane-bound state. The *CbPLD γ* gene is expressed in roots, stems, leaves, and is significantly enriched in roots and stems, and the expression level in leaves is low (Fig. 4). The difference in gene expression may be related to its function. The significant enrichment in roots may be because this gene is related to root growth and development. The results of subcellular localization indicate that the genes are significantly clustered on the cell membrane, which may imply that the *CbPLD γ* genes all perform their functions on the cell membrane (Fig. 5).

Drought is one of the most important limiting factors for plant growth and agricultural production (Sun X P, et al. 2013; Shahsavari N, et al. 2014). Current research further confirms the negative impact of drought on plant growth. When plants are stimulated by the external environment, they may resist the stimulation by activating the antioxidant defense system, but in the case of insufficient antioxidant defense, the

resistance of the plant can be increased by exogenous application of various hormone signaling molecules (Piotrowska A, et al. 2009; Kadioglu A, et al. 2011). As we all know, PLD plays an important role in the process of cytoskeletal assembly and plasma membrane reconstruction. The permeability and stability of plant cell membranes play an important role in plant growth and development, and PLD is involved in the degradation of plant cell membrane lipids. MDA is a product of lipid peroxidation, which can reduce the level of antioxidants, leading to membrane system damage and even cell death. MDA and electrolyte leakage are usually used to analyze the degree of membrane damage under environmental stress. In this study, our measurement of MDA and electrolyte leakage content showed that wild plants under drought treatment are always higher than that of transgenic lines. This indicates that under drought stress, the membrane damage of overexpressed plants is less than that of WT plants (Fig. 6).

ROS is a substance produced by plants under various stress conditions and an important medium for plants to respond to stress. Plants have an array of antioxidant enzymes that protect cells from oxidative damage. These enzymes include SOD, POD, CAT, which work together with other enzymes to scavenge ROS (Yue Y S, et al. 2011). In our study, drought significantly increased the activities of SOD, POD and CAT in the two plants, and the overexpressed lines had higher antioxidant enzyme activity than WT plants. This indicates that transgenic lines are more resistant to drought than WT plants (Fig. 7).

Conclusion

In this study, novel *PLD γ* gene was amplified from *C. bungeana* and was called *CbPLD γ* . The bioinformatics analysis was completed. The phylogenetic tree was revealed that *CbPLD γ* is classified to subfamily of *PLD γ* , and was more close to the *Arabidopsis PLD γ* gene compared with the other plants. Compared with the control, the expression pattern of the *CbPLD γ* mRNA is in response to low temperature, salt, mannitol, and exogenous ABA have up-regulated. Expression pattern of *CbPLD γ* is in roots, stems, leaves, but, it is mainly expressed in roots and stems compared with leaves. The *CbPLD γ* is located in cell membrane. Compared with WT *Arabidopsis*, *CbPLD γ* gene overexpression plants showed higher activities of antioxidant enzymes, and lower levels of malondialdehyde and electrolyte leakage under drought stress. These confirmed that *CbPLD γ* involved in the response to drought stress, and has the potential to improve the drought tolerance of plants. This is the first report about cloning and characterizing the gene of *CbPLD γ* from *C. bungeana*. It laid a foundation for further research and improvement of the PLD gene family of *C. bungeana*.

Declarations

Ethical Approval and Consent to participate

This article is to study the functional identification of plant genes and gene functions under drought. So ethical approval and consent to participate.

Consent for publication

All authors read and approved the final manuscript, and consent for publication.

Availability of supporting data

All data were obtained through repeated and parallel experiments, and the sequence was sequenced by Beijing Liuhe Huada gene technology company.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author' Contribution

Ning Yang experiments; Bo Liu carried out subcellular localization assay and qRT-PCR and histochemical GUS assays, genetically transformed tobacco, and study the resistance of transgenic plant and wrote manuscript; Peng-Jun Yang cloned the *CbPLD γ* gene, Hui Li, and Ya-Ping Zhou analyzed the data.

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Abbreviations

ABA - abscisic acid; *Chorispora bungeana* - *C. bungeana*; CAT – catalase; Col-0 - *Arabidopsis thaliana* Columbia ecotype; cDNA - complementary DNA; GFP - green fluorescent protein; GUS - β -glucuronidase; hiTAIL-PCR - high-efficiency thermal asymmetric interlaced PCR; MDA - malondialdehyde; MS - Murashige and Skoog; OE - overexpressed plants; PLD - phospholipase d; POD - peroxidase; PA - polymerase acidic protein; PCR - polymerase chain reaction; pI - isoelectric point; qRT-PCR - quantitative real time polymerase chain reaction; ROS - reactive oxygen species; RACE - rapid amplification of cDNA ends; SOD - superoxide dismutase; UTR - untranslated region; WT - wild-type; X-Gluc - 5-bromo-4-chloro-3-indolyl- β -D-glucuronic acid

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Figures

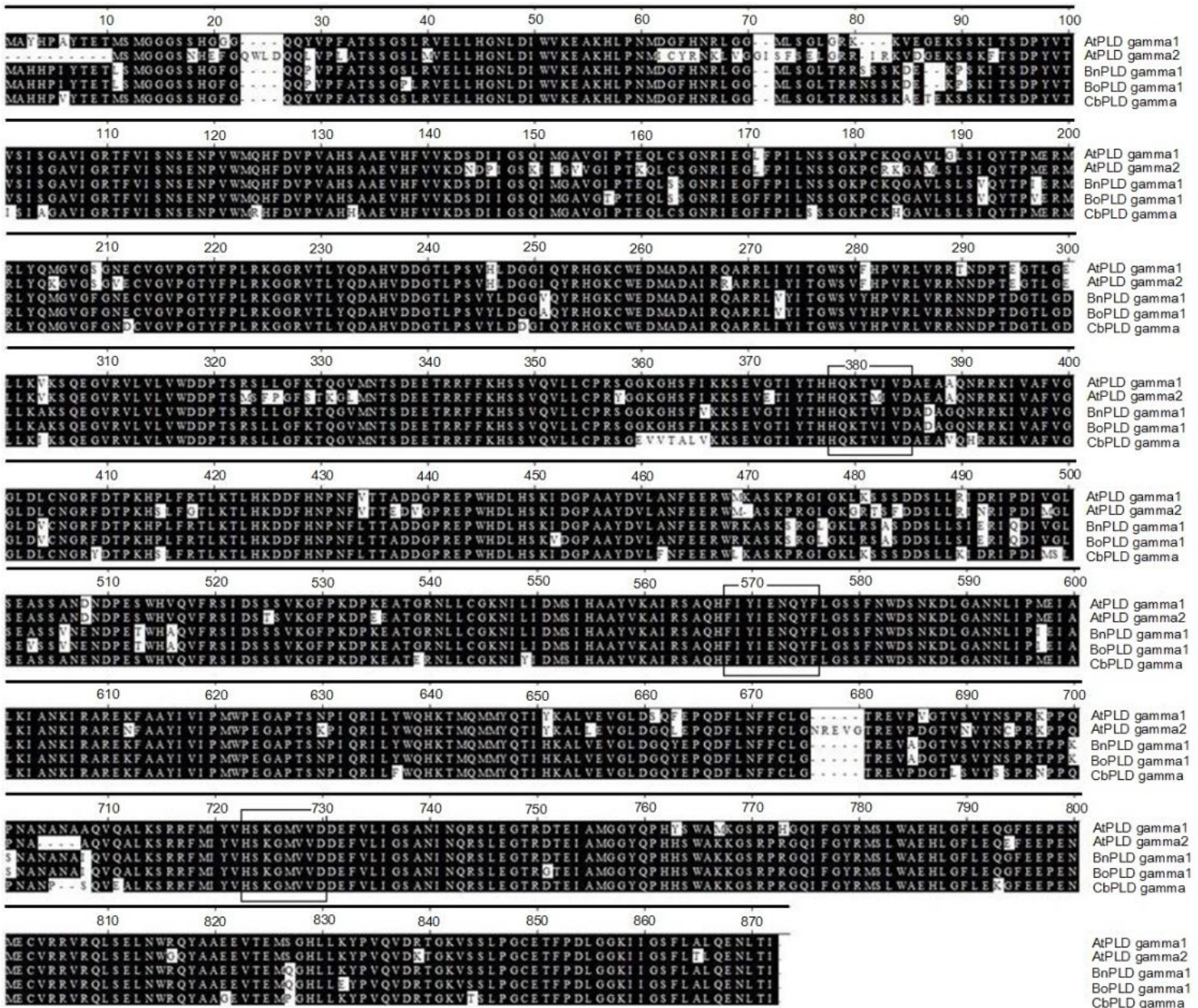


Figure 1

Alignment of the CbPLD gamma with PLDs from other plant species. The plants PLDs used for alignment are: Arabidopsis thaliana AtPLD gamma1 (GenBank Accession No. NM117255); AtPLD gamma2 (GenBank Accession No. NM117252); Brassica napus PLD gamma1 BnPLD gamma1 (GenBank Accession No. XM013806699); Brassica oleracea var. oleracea PLD gamma1 BoPLD gamma1 (GenBank Accession No. XM013756452). The conserved motifs HKD and FYIENQYF are underlined.

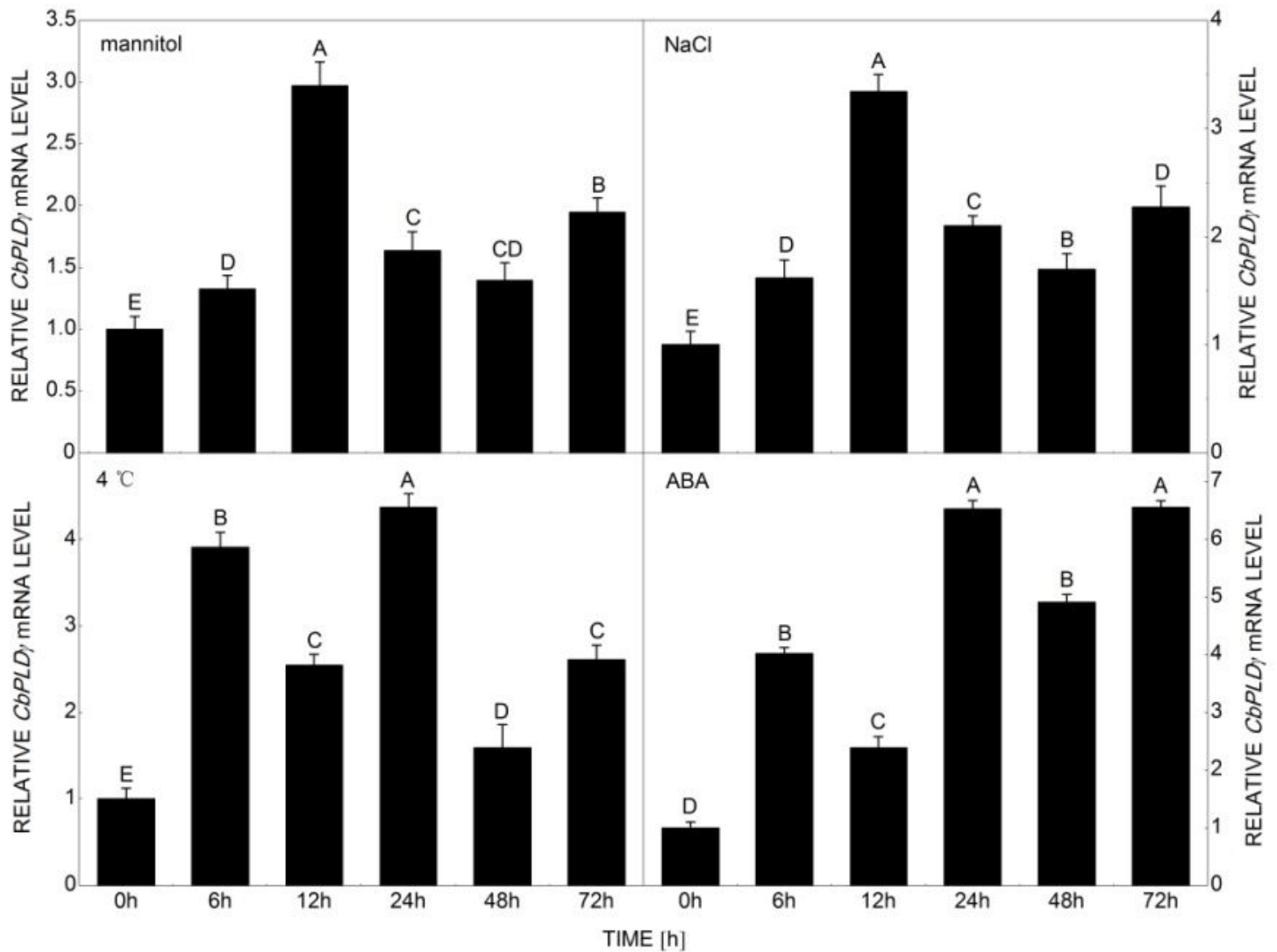


Figure 3

Effects of 4 °C, salt, mannitol, exogenous ABA on relative expression of *CbPLD γ* in *C. bungeana*. Values are the mean \pm SD of at least three independent experiments. Different lowercase letters in the figure indicate the significant differences between groups of same time at $P < 0.05$. Different capital letters in the figure indicate the significant differences at different times at $P < 0.05$.

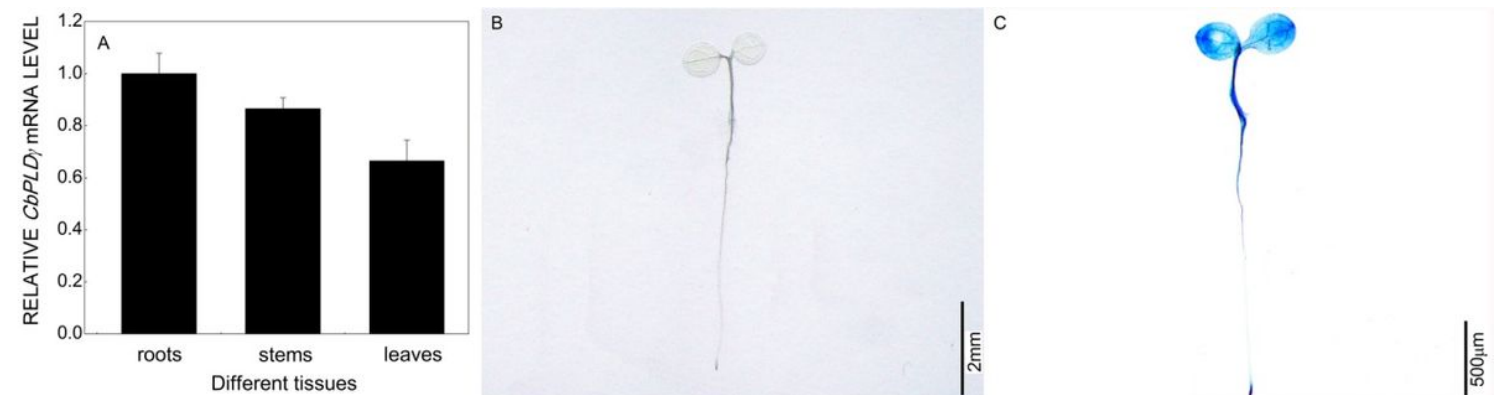


Figure 4

Expression pattern and relative mRNA levels of CbPLD γ in different tissues. (A) Total RNA were isolated from roots, stems and leaves of *C. bungeana* and subjected to qRT-PCR analysis. The relative mRNA level indicated a comparison between targeting gene CbPLD γ and internal control actin gene in each tissue. Values are the mean \pm SD of at least three independent experiments. (B-C) CbPLD γ -GUS activity of transgenic *Arabidopsis*. (B) Col-0 of *Arabidopsis* germinated after three days. (C) Transgenic seedling of *Arabidopsis* germinated after three days.

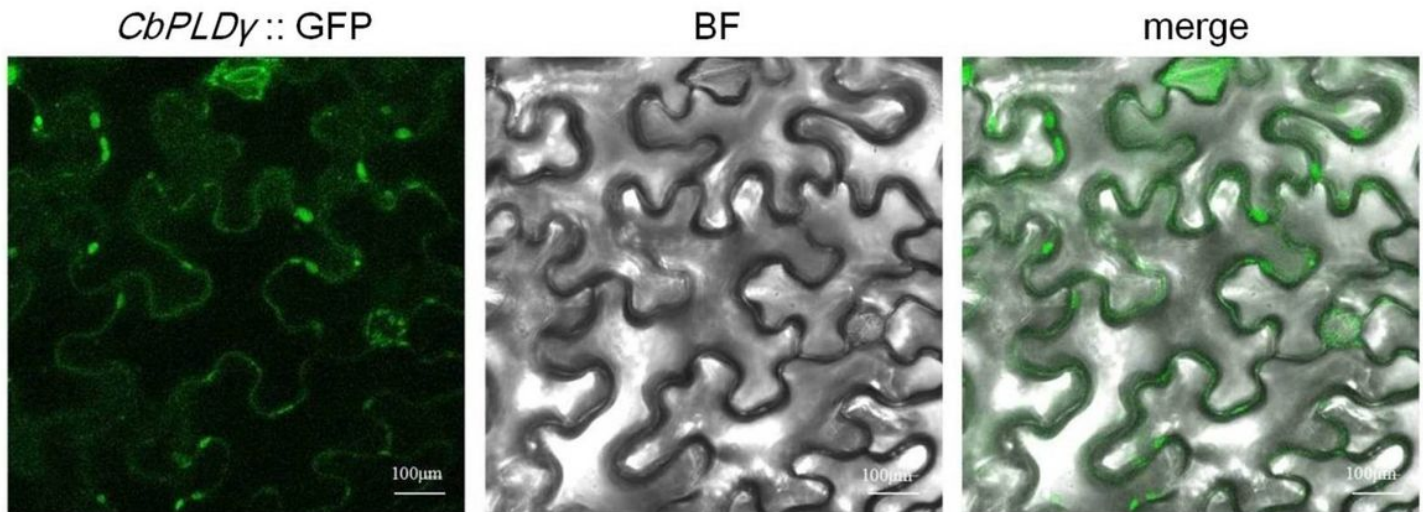


Figure 5

Subcellular localization of CbPLD γ . Construct pMDC83-CbPLD γ -GFP was transformed into tobacco epidermal cells. All images were observed with confocal microscope.

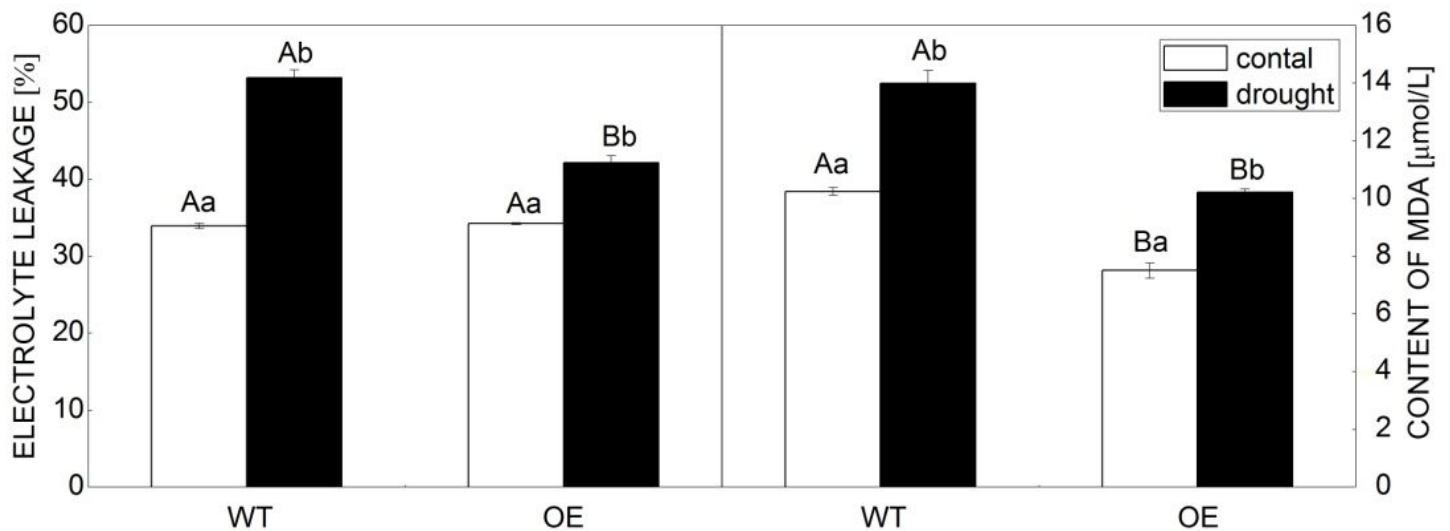


Figure 6

Effects of drought stress on electrolyte leakage and MDA content in CbPLD γ transgenic *Arabidopsis* and WT. Values are the mean \pm SD of at least three independent experiments. Different lowercase letters in the

figure indicate significant differences between the same group at $P < 0.05$. Different capital letters in the figure indicate significant differences at different times, $P < 0.05$.

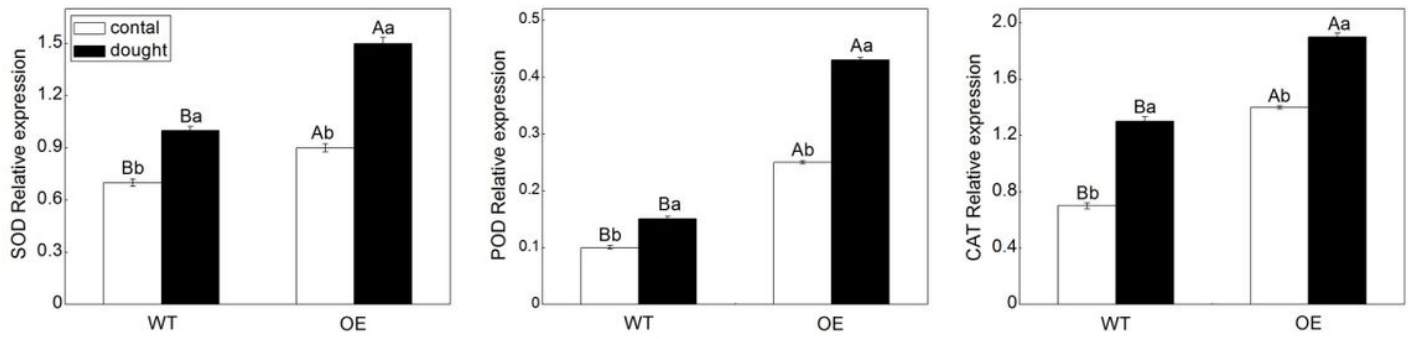


Figure 7

The effect of drought stress on the activity of antioxidant enzymes in CbPLD γ transgenic Arabidopsis and WT is the mean \pm SD of at least three independent experiments. Different letters in the figure indicate significant differences between the same group ($P < 0.05$).