

Overexpression of *Chorispora bungeana* CbPLD γ enhances drought tolerance in *Arabidopsis*

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Research Article

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Abstract

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, a *CbPLD γ* gene, which is a part of *CbPLD* gene family and from *Chorispora bungeana* (*C. bungeana*) was cloned and encoded a protein of 859 amino acids with a calculated molecular weight of 96.3 kDa and with a PI(Isoionic Point) of 7.88. Real-time quantitative PCR (RT-qPCR) and Beta-glucuronidase (GUS) assay showed that *CbPLD γ* was accumulated dominantly in roots and hypocotyls. Compared with the control, *CbPLD γ* was positively responded to the low temperature, salt, mannitol, and exogenous ABA. Subcellular localization analysis showed that the *CbPLD γ* was localized in the cell membrane. *CbPLD γ* -overexpression *Arabidopsis* under drought stress showed higher relative expression of superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD), as well as high content of proline, soluble protein and soluble sugar. However, H₂O₂, malonaldehyde (MDA) content and electrolyte leakage (EL) were lower than wild-type *Arabidopsis*. These indicated that *CbPLD γ* was involved in the drought tolerance, and overexpression of *CbPLD γ* enhanced the drought tolerance in *Arabidopsis*. 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*.

1. Introduction

Phospholipids are critical in cell membranes and also play a pivotal role in cell development. The phospholipase D (PLD) can hydrolyze phospholipids to produce phosphatidic acid (PA) and soluble head group. Each of the PLD gene superfamilies involves two copies of the conserved HxKxxxxD sequence, which is an active site is known as the HKD motif. Besides, the N-terminal domain contains the binding motif lipid and the C-terminal domain contains the catalytic motif. Different PLDs seem to have different functions but somewhat overlapping functions in cellular processes (Lein and Saalbach, 2001). *PLD* gene has been cloned from many species such as *rice bran*, *Maize* (Ueki et al., 1995), *tobacco* (Lein and Saalbach, 2001), *cowpea* (Ben Ali et al., 2007), *tomato* (Tiwari and Paliyath, 2011), *Arabidopsis thaliana* (Pappan et al., 1997). *PLD* genes are classified into six subfamilies in the *Arabidopsis* genome: α (3), β (2), γ (3), δ , ϵ and ζ (2) (Wang, 2005). The majority of PIP₂ and Ca²⁺ can influence the activity of *PLD γ 1* and *PLD γ 2* (Qin et al., 1997, Qin et al., 2006), and previous studies have shown that *PLD γ* is more tolerant to all stress in the *Arabidopsis* seedlings (Zhao et al., 2011).

Abiotic stresses trigger the physiological and molecular mechanisms to protect the plant from cell damage or death, such as increasing antioxidant activity, decreasing membrane permeability, and controlling the reactive oxygen homeostasis (Hong et al., 2008, Hong et al., 2010). Many abiotic stresses, such as salt, drought, salicylic acid (SA), abscisic acid (ABA), can influence the PLD expression (Hong et al., 2016, Guo et al., 2013, Kalachova et al., 2013). *Chorispora bungeana* (*C. bungeana*) is a cruciferae plant, which is a typical representative of the subnival alpine plant. It can adapt to the environment of low oxygen pressure, cold, drought, and strong ultraviolet radiation. As nature resistant plant, *C. bungeana* contains abundant stress-related genes. It is also closely related to *Arabidopsis*. It has a surface lint and

wax layer, and 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). Previous research also showed that *C. bungeana* has no special morphological characteristics. It is an ideal plant species to study the stress-related molecular mechanisms of *C. bungeana*.

PLDs are thought to be involved in plant responses to abiotic stresses and have multiple functions during plant growth and development (Pinhero et al., 2003). Water deficiency can trigger PLD activity in *Craterostigma plantagineum* (Frank et al., 2000). PLD participated in freeze stress response in *C. bungeana* callus (Yang et al., 2012). In ABA signaling, *PLDa1* and *PLD δ* have a synergistic effect in *Arabidopsis* guard cells (Kalachova et al., 2013). SA can activate the PLD and induce stomatal closure in *Arabidopsis* (Guo et al., 2013). And the stomata from *PLDa1* deficient *Arabidopsis* plants fail to close in response to ABA, whereas the external supply of PA, promoted the stomatal closure (Hong et al., 2010). Overexpression analyses suggested isoform *PLD δ 3* is activated in pollen tubes and PA promotes the binding of *PLD δ 3* to the plasma membrane in *tabacco* (Dreßler et al., 2017). Overexpression *TaPLDa* (*Triticum aestivum* L) plants can enhance tolerance to drought and osmotic stress in *Arabidopsis*. *TaPLDa* is a potential candidate gene to enhance stress tolerance in plant (Wang et al., 2014). Although there are many PLD reports, so far, there is no detailed study about the characterization and function of the *CbPLD γ* in *C. bungeana*.

In this work, the full length of the *CbPLD γ* cDNA sequence from *C. bungeana* was cloned and analyzed. The localization, expression pattern, and possible function of *CbPLD γ* were also investigated. It showed a better insight into the molecular mechanisms of *CbPLD γ* under drought stress and provided a theoretical basis for further research of the *PLD* gene family.

2. Material And Methods

2.1. Plant material

The plantlets of *C. bungeana* were obtained with some modification (Fu et al., 2006). *C. bungeana* seedlings were cultivated on Murashige and Skoog (MS) medium with 1 mg/L 6-benzyladenine (6-BA) and 3% (w/v) sucrose.

Arabidopsis 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 22°C light for 16 h and dark light for 8 h.

2.2. Stress treatment

The *C. bungeana* seedlings were grown according to 2.1. A week later, the seedlings in vitro were subjected to 0.3 M mannitol, 150 mM NaCl, 4°C, and 100 Mm ABA, for 72 h respectively. All the samples were immediately frozen in liquid nitrogen for further experiments. The *CbPLD γ* overexpressing

Arabidopsis seedlings were grown according to 2.1. Two weeks later, the seedlings were growing without watering for 1 week and rehydrated for five days.

2.3. 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 synthesized 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) (Supplement Table 1) corresponding to sequences of the conserved regions of *PLD* genes were used to amplify part of the *CbPLD γ* sequence. The amplified product was linked to the pMD19-T vectors (Takara, Dalian) for further sequencing. The primers (CbPLD γ 2-F and CbPLD γ 2-R) (Supplement Table 1) were designed from the 5' end of the first amplified fragment to amplify the middle fragment of *CbPLD γ* . The product was linked to the pMD19-T vectors (Takara, Dalian) for further sequencing. The three sequences were combined to obtain the middle part of *CbPLD γ* .

The 5' and 3' rapid amplification of cDNA ends (RACE) were performed using the SMARTer™ RACE cDNA Amplification (Invitrogen, USA). Based on the instruction manual, total RNA was 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 (Supplement Table 1) were designed to amplify the RACE products. And the nest PCR products were cloned into the pMD19-T vector and sequenced.

By comparing and aligning the sequences, the full length of the *CbPLD γ* gene sequence was obtained. The full length of the *CbPLD γ* gene was verified by PCR using Primer Star HS DNA polymerase (Takara, Dalian) and sequencing.

2.4. Cloning of the *CbPLD γ* promoter

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 *CbPLD γ* promoter was cloned by using Genome Walking Kit (Takara, Dalian). Adaptors were provided by the kit and SP primers (Supplement Table 1) were designed according to the sequence of *CbPLD γ* . The PCR products were purified using TIANGEN Midi Purification Kit (TIANGEN, Beijing), cloned into a pMD19-T vector, and sequenced.

2.5. 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 (Supplement Table 1) were subjected to RT-qPCR using TBGreen® Premix Ex Taq™ II (Tli RNaseH Plus) (Dalian Takala). *ACTIN* gene

(GenBank Accession No. AY_825362) was used as internal controls for normalizing gene expression levels. The results were displayed using the $2^{-\Delta\Delta ct}$ method.

2.6. Histochemical location of *CbPLDy*

The promoter of *CbPLDy* was cloned (Supplementary Table 1) by the Genomic DNA of *C. bungeana*, and the correct sequences were inserted into vectors. The final construct of pBIB-*CbPLDy*-GUS was introduced into *Arabidopsis*. The homozygous seedlings were screened by herbicide Basta and were used to detect the histochemical location of GUS activity. Plant tissues were incubated for 16 h at 37°C in X-Gluc solution, then were decolorized in destaining solution for approximately 10 h. The seedlings were washed and then observed under Olympus CX31 Microscope (Olympus, Japan).

2.7. Subcellular localization of *CbPLDy*

The coding region of *CbPLDy* was amplified from *C. bungeana* by RT-PCR using primer CDS-F and CDS-R (Supplementary Table 1), and the PCR products were sequencing. The cloned gene fragment was recombined into the pMDC83 vector with recombinase. Competent DH5α cells were transformed and plasmids were verified by sequencing. The correct plasmids containing the pMDC83-*CbPLDy*-GFP vector were introduced into *Agrobacterium tumefaciens* GV3101. The recombinant plasmids were transformed into *tobacco* for transient expression (Sparkes I A, et al. 2006), and the transformed tobaccos were cultured in darkness at 22°C for 48 h. The GFP fluorescence was captured by the confocal microscope (Leica, German).

2.8. Transformation of *Arabidopsis*

The *CbPLDy* promoter and coding regions were amplified by nested PCR using primer Promoter-F and Promoter-R (Supplementary Table 1). The cloned fragment was recombined into the pBIB vector with recombinase. Competent DH5α cells were transformed and plasmids were verified by sequencing. The correct plasmids containing the pro-pBIB-*CbPLDy*-GUS vector were introduced into *Agrobacterium tumefaciens* GV3101. Then, the recombinant plasmids were transformed into the *Arabidopsis* Col-0 according to the floral dip method and the transformed *Arabidopsis* was cultured in darkness at 22°C for 24 h. The transformants were selected by MS medium containing Basta. The fourth generation of transgenic *Arabidopsis* plant was used for further analyses (Hong et al., 2010). The seedlings of transgenic *Arabidopsis* which have been screened to the fourth generation were selected for RT-PCR identification and RT-qPCR analysis.

2.9. Determination of physiological indicators

Electrolyte leakage (Zhang et al., 2014), MDA, H₂O₂, and proline level (Zhu et al., 2020) were measured according to previous studies. The soluble sugar content was determined by anthrone-sulfuric acid colorimetry (Kaoru et al., 1991). The relative expression levels of *SOD*, *CAT*, and *POD* were measured according to the previous method (Liu et al., 2021).

2.10. Statistical analyses

All experiments were repeated at least three biological times. Data were analyzed by one-way ANOVA using SPSS 19.0 for windows (SPSS Inc. Chicago, IL, USA). Data were shown with the mean \pm SD of three biological replicates.

3. Results

3.1. Cloning of *CbPLD γ* gene

Based on the *PLD γ* gene sequence in other homologous species, the middle fragments of the *CbPLD γ* gene were cloned from *C. bungeana*. And the fragments about 480 bp, 712 bp, 524 bp were obtained in sequence. 5'GSP and 5'NGSP were designed beside the middle region sequence for 5'-RACE. A 160 bp 5' untranslated region (UTR) of the ATG codon upstream was obtained. Then the 3'GSP and 3'NGSP for 3'-RACE were designed, the 1373 bp was cloned which contained 197 bp 3'UTR in the TAA codon downstream (including a poly-A tail of 12 bp). By comparing and aligning, the 2937 bp full-length cDNA was deduced (Supplementary Fig. 2A).

Analysis by DNASTar, the full-length cDNA contained a 2580 bp ORF (GenBank accession No. MF951104), which encoded a protein of 859 amino acids with a calculated molecular weight of 96.3 kDa and with a pI of 7.88. Besides, *CbPLD γ* had the conserved motif "FIYIENQFF", which may be related to the hydrophobic interaction between the methyl groups in the choline group. The motif "FIYIENQFF" was as important as the HKD domain (Supplementary Fig. 2B). Phylogenetic analysis showed that the *CbPLD γ* gene had high homology with other plant *PLD γ* genes. Also, among the *PLD γ* subtypes, *C. bungeana* was closely related to *Arabidopsis PLD γ* gene (Supplementary Fig. 3).

3.2. The expression of *CbPLD γ* under abiotic stress

The expression of transcription level of *CbPLD γ* was analyzed to explore the *CbPLD γ* responding to low temperature, salt, mannitol, and exogenous ABA. The relative expression levels of the *CbPLD γ* gene fluctuated under different abiotic stresses. It peaked at 12 h under 0.3 M mannitol and 150 mM NaCl, and was 2.97 fold and 3.34 fold compared to the control, respectively (Fig. 1A&B). The relative expression levels of the *CbPLD γ* gene peaked at 24 h under 4°C and 100 mM ABA and was 4.3 fold and 6.5 fold compared to the control (Fig. 1A &B). This result showed that the *CbPLD γ* was up-regulated under low temperature, salt, mannitol, and ABA, and the *CbPLD γ* actively responded to different abiotic stress.

3.2. The expression patterns of *CbPLD γ* in different tissues

To explore the expression patterns of *CbPLD γ* in different tissues, the histochemical location was carried out. The GUS activity results confirmed that *CbPLD γ* was expressed in all the tissues, but with significant enrichment in root tips and anther (Fig. 2).

3.4. Subcellular localization of *CbPLD γ*

The recombinant plasmids were transformed into tobacco according to the transient expression method for epidermal cells. The results showed that the *CbPLD γ* was located in the cell membrane (Fig. 3).

3.5. Identification of transgenic *Arabidopsis*

Four transgenic plants (T₁-T₄) were selected from Basta screening to the fourth generation, and genomic DNA was extracted for RT-PCR identification (Fig. 4A). The successful heterologous expression of the *CbPLD γ* gene in *Arabidopsis* was confirmed. Take 18-day-old *Arabidopsis* seedlings (T₁-T₄) for RT-qPCR analysis (Fig. 4B). In subsequent experiments, select the two highest expression lines (T₁, T₃) for analysis.

3.6. Overexpression of *CbPLD γ* enhances the drought stress tolerance of *Arabidopsis*

To study the effect of *CbPLD γ* on the cell membrane and plant osmoregulatory substances, we measured the EL, MDA, proline, soluble protein, and soluble sugar in *CbPLD γ* transgenic *Arabidopsis* plants under drought stress. In this experiment, the EL and MDA of WT were significantly higher than those of transgenic plants under drought stress, indicating that overexpression of *CbPLD γ* decreased the membrane damage under stress (Fig. 5A & B). Compared with the WT, the transgenic plants had higher contents of proline, soluble sugar and soluble protein, suggesting that overexpression of *CbPLD γ* enhanced the plant osmoregulatory substances to protect the plant from drought stress (Fig. 5C, D & E).

Under normal conditions, there was no significant difference in *SOD*, *POD*, and *CAT* between WT and *CbPLD γ* transgenic lines. However, the drought significantly increased the relative expression levels of the antioxidant enzyme. The relative expression levels of *SOD*, *POD*, and *CAT* in transgenic lines were higher than that in WT significantly (Fig. 6A, B & C). The H₂O₂ contents in the transgenic plants were also lower than that in WT significantly (Fig. 6D).

4. Discussion

C. bungeana is a typical subnival alpine plant, its living environment is extremely harsh (Wu et al., 2008). In recent years, the research on *C. bungeana* has been reported from various levels, especially the anti-adversity response mechanism. Phospholipase D (PLD) is involved in different plant growth processes, including plant development, the responses to various stresses et al (Distéfano et al., 2015). In this study, a *PLD γ* gene, named *CbPLD γ* (GenBank accession No. MF951104), was cloned from *C. bungeana* for the first time. 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. The study showed that 4°C low temperature, salt, and mannitol stress can induce the expression of the *CbPLD γ* gene (Fig. 1), indicating that *CbPLD γ* is widely involved in the abiotic stress responses of *C. bungeana*. Plant growth regulators, participate in plant stress response and play an important role (Shaterian et al., 2005). In this study, exogenous ABA can also induce the expression of the *CbPLD γ* gene (Fig. 1).

The study of the expression pattern of PLD and the subcellular location may be of great significance for further understanding the function and regulatory mechanism of PLD. PLD ϵ was found in microsomes, but not in the soluble fraction (Yao et al., 2016). The GmPLD γ -GFP fusion protein is expressed in transgenic *Arabidopsis* roots and tobacco leaf mitochondria (Bai et al., 2020). The average expression level of *PLDa3* in buds, flowers, siliques, stems, old leaves, and roots was 1000 times lower than that of *PLDa1* in *Arabidopsis* (Hong et al., 2008). The subcellular localization indicated that the protein was significantly clustered on the cell membrane, which implied that the CbPLD γ protein mainly performed its functions on the cell membrane (Fig. 3).

The permeability and stability of plant cell membranes play an important role in plant growth and development, PLD is involved in the degradation of cell membrane lipids. The EL and MDA of WT were always higher than that of *CbPLD γ* transgenic lines under drought treatment (Fig. 5). This indicates that overexpression *CbPLD γ* enhanced the drought stress tolerance by alleviating the membrane damage.

As normal plant osmoregulatory substances, proline, soluble proteins, and soluble sugar are used for regulating the osmotic pressure of cell membranes and cell osmotic potential under various stresses (Chun et al., 2018). In this research, compared with the WT, the *CbPLD γ* transgenic plants had higher contents of proline, soluble sugar, and soluble protein, reflecting that overexpression *CbPLD γ* could increase the osmoregulatory substances to responding to the drought stress (Fig. 6).

Under environmental stress, the content of reactive oxygen species in plants erupts (Miller et al., 2010, Fichman and Mittler, 2020). ROS is a substance produced by plants under stress conditions and is an important medium for plants to respond to stress. H₂O₂ is the most stable marker of ROS. Plants have an array of antioxidant enzymes, such as *SOD*, *CAT*, and *POD*, which protect cells from oxidative damage. In this study, drought treatment significantly increased the H₂O₂ content and the relative expression of *SOD*, *POD*, and *CAT* in WT and *CbPLD γ* transgenic plants (Fig. 6A, B & C). The *CbPLD γ* overexpressed lines had higher antioxidant enzyme relative expression than WT. And the H₂O₂ contents in the transgenic plants were also lower than that in WT significantly (Fig. 6D). This indicates that *CbPLD γ* was involved in the regulation of H₂O₂ and antioxidant enzymes, overexpression *CbPLD γ* enhanced the drought stress tolerance by regulating the antioxidative system.

5. Conclusion

In this study, a novel *CbPLD γ* gene is amplification from *C. bungeana* and is identified. Compared with the control, the expression pattern of the *CbPLD γ* mRNA is up-regulated in response to low temperature, salt, mannitol, and exogenous ABA. *CbPLD γ* is expressed in all examined tissues and is located in the cell membrane. The overexpression of *CbPLD γ* enhances the drought stress tolerance by alleviating the membrane damage, increasing the osmoregulatory substances, and regulating the antioxidative system. These support that *CbPLD γ* is 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 *CbPLD γ* gene

from *C. bungeana*. It lays a foundation for further research and improvement of the PLD gene family of *C. bungeana* and represents a potential candidate gene to enhance stress tolerance in plants.

Declarations

Competing interests

The authors declared that they have no conflicts of interest to this work

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Author' Contribution

Ning Yang experiments; Bo Liu carried out subcellular localization assay and RT-qPCR and histochemical GUS assays, genetically transformed tobacco, Hui Li and Bo Liu study the resistance of transgenic plant and wrote manuscript and participated in the research on the resistance of transgenic plant; Wei Wang, Bianfeng Zhang and Ruirui Liu analyzed the data; Ning-Yang cloned the *CbPLD γ* gene.

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Supplementary Information

Supplementary Table 1 and Supplementary Figures 1-3 are not included with this version.

Figures

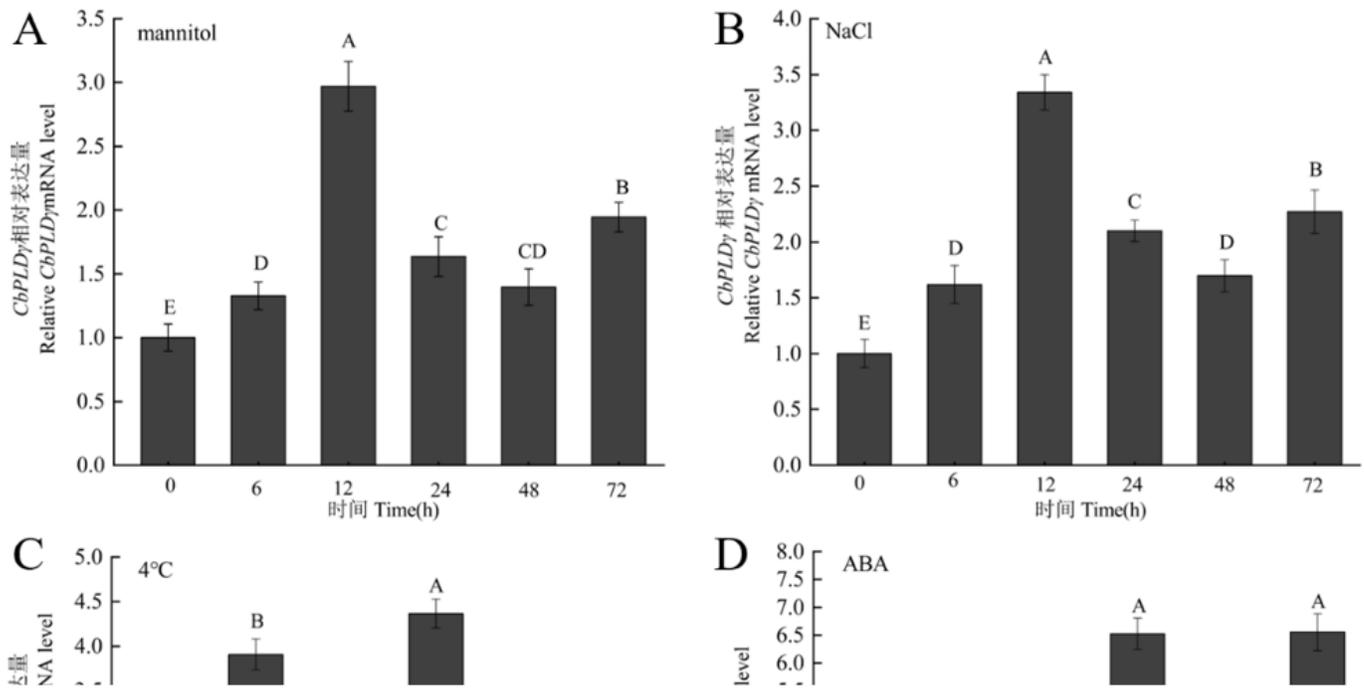


Figure 1

The relative expression levels of *CbPLD γ* in *C. bungeana* under different abiotic stress. (A) 0.3 M mannitol treatment. (B) 150 mM NaCl treatment. (C) 4 °C treatment. (D) 100 mM ABA treatment. Data are shown with the mean \pm SD of three biological replicates. Different letters the significant differences at $P < 0.05$.

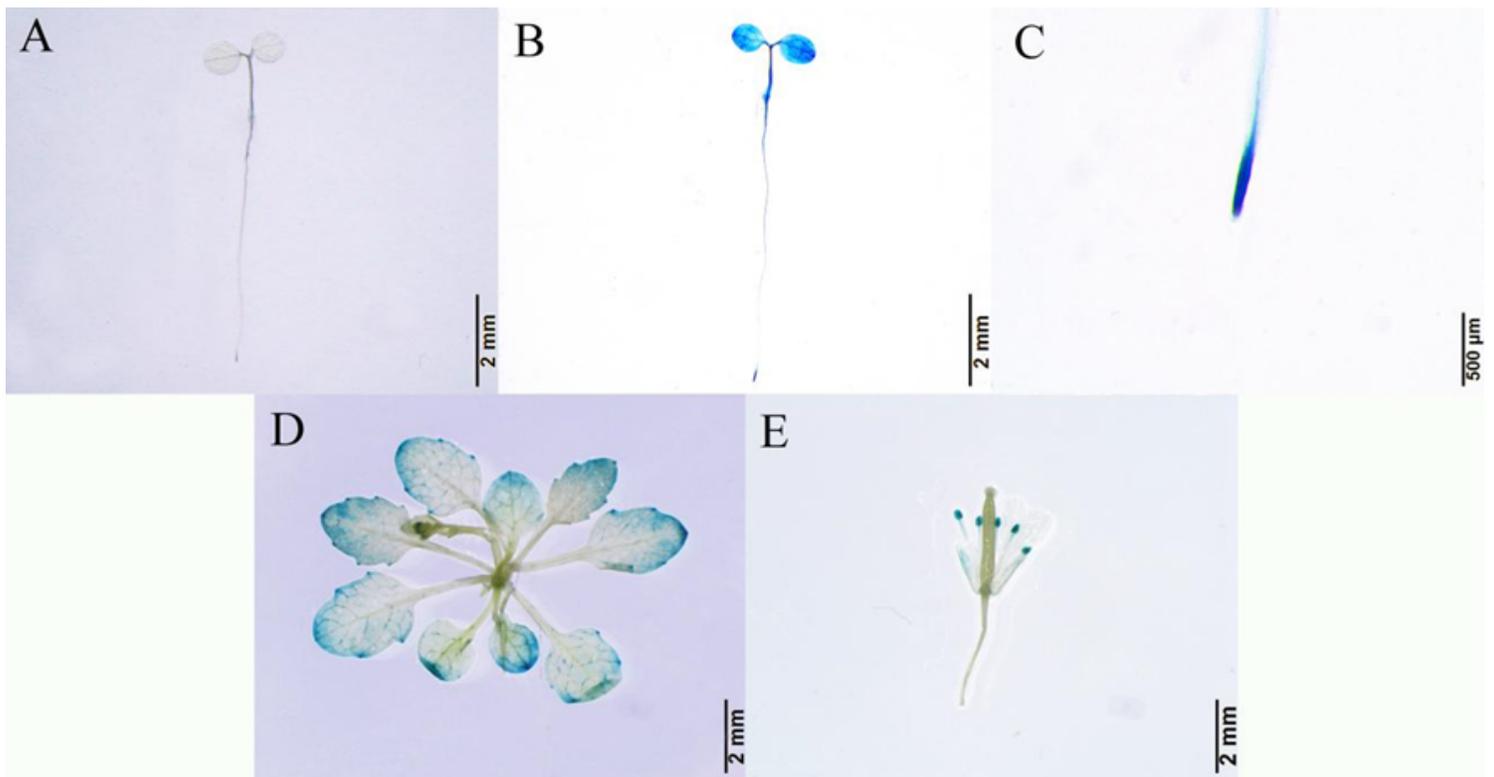


Figure 2

Expression patterns of CbPLD γ in different tissues. (A) 3-day-old Arabidopsis Col-0 seedlings. (B) Transgenic Arabidopsis seedlings were grown for 3 days. (C) Root tips of transgenic Arabidopsis seedlings were grown for 3 days. (D) Leaves of transgenic Arabidopsis plants were grown for 28 days. (E) Flowers of transgenic Arabidopsis plants were grown for 28 days. Data are shown with the mean \pm SD of three biological replicates. Different letters indicate the significant differences at $P < 0.05$.

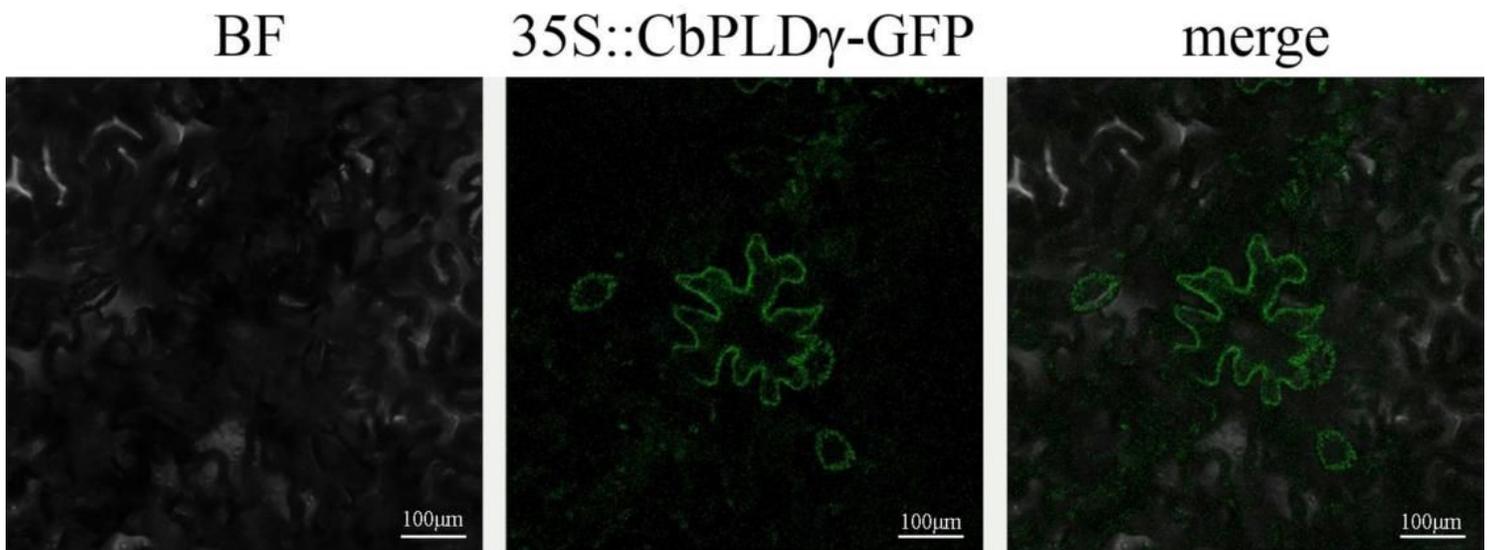


Figure 3

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

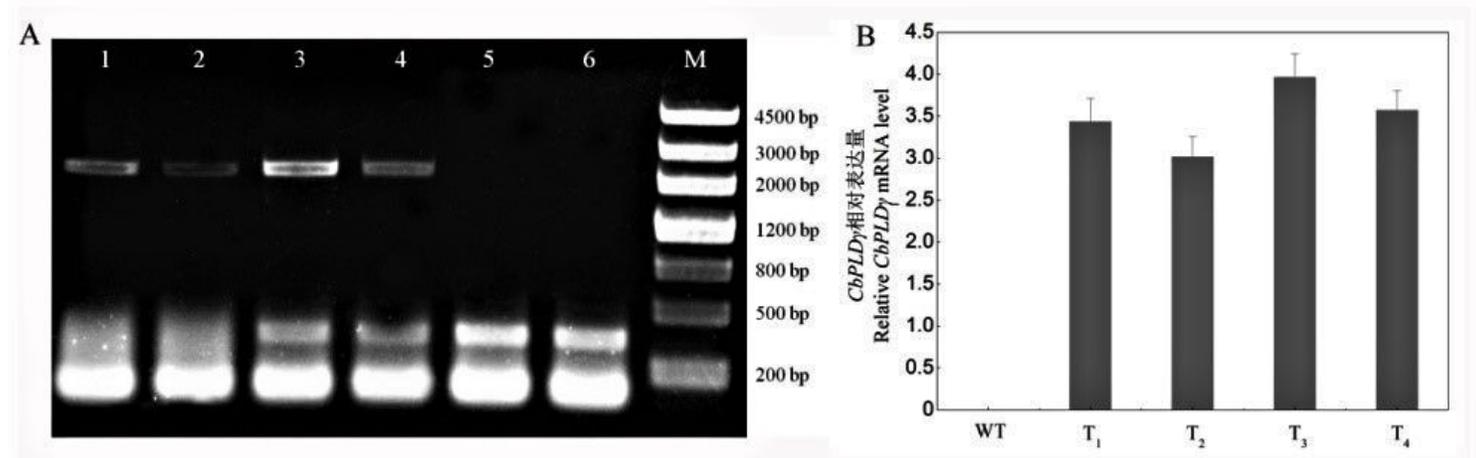


Figure 4

Identification of transgenic Arabidopsis. (A) PCR analysis of transgenic Arabidopsis. 1~4: transgenic lines; 5: non-transgenic line (negative control); 6: empty plasmid pBIB; M: Marker. (B) Quantitative analysis in transgenic Arabidopsis. Data are shown with the mean \pm SD of three biological replicates.

Figure 5

The effect of drought stress on the EL, MDA, and osmotic adjustment substances in WT and CbPLD γ transgenic plants. (A) Electrolyte leakage. (B) Content of MDA. (C) Content of Pro. (D) Content of SP. (E) Content of SS. Data are shown with the mean \pm SD of three biological replicates. Different letters the significant differences at $P < 0.05$.

Figure 6

The effect of drought stress on the antioxidant enzyme and H₂O₂ in WT and CbPLD γ transgenic plants. Data are shown with the mean \pm SD of three biological replicates. Different letters the significant differences at $P < 0.05$.