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Cloning and Tissue Expression Analysis of Calcium-dependent Protein Kinase Gene *BsCDPK1* in *Bletilla striata*

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Abstract Calcium-dependent protein kinases (CDPKs) play vital roles in the regulation of plant growth, development, and the response to adversity stress. Studying the sequence information and expression pattern of CDPK gene could lay the foundation for the protein structure, gene functions and signal network of plant stress resistance. The full-length cDNA of *BsCDPK1* gene was obtained by RT-PCR and RACE approaches in this study. The physicochemical properties and structural domains of BsCDPK1 protein were analyzed by bioinformatics tools; In addition, amino acid sequence alignment and phylogenetic analysis of BsCDPK1 were performed. The expression pattern of *BsCDPK1* gene in the seedling stage of *Bletilla striata* was detected by real-time fluorescence quantitative PCR. The result showed that the full cDNA of the cloned *BsCDPK1* was 1 981 bp, encoding 496 amino acids. The molecular weight of encoded protein was 55.997 KD, isoelectric point 5.38, aliphatic index 87.50 and instability index 43.54. The BsCDPK1 protein included the typical kinase domain of CDPKs in other plants, autoinhibitory domain, ATP binding site and the four EF-hands were highly conserved, which was more homologous with CDPKs of *Dendrobium candidum* and *Phalaenopsis equestris*, clustered on the same evolutionary branch. The results of qRT-PCR indicated that *BsCDPK1* gene was expressed in the roots, stems and leaves in Bletilla striata seedlings. The cloning and sequence characterizing and tissue expression pattern analysis of *BsCDPK1* provided experimental basis for elucidating the function of *BsCDPK1* gene in plant growth and development and adversity stress responses.
Keywords *Bletilla striata*; Calcium-dependent protein kinase; Gene cloning; Expression profiling

Bletilla striata is a perennial herb of the genus *Bletilla* Rchb. f. in Orchidaceae. It is used in medicine with dried tubers, which is a rare Traditional Chinese Medicine with the effect of invigorating lung and promoting granulation, promoting blood circulation, and removing blood stasis. Modern pharmacology has found that *Bletilla striata* plays an important role in promoting wound healing, treating ulcerative colitis, antibacterial, antiviral, and antitumor activities (Shuangguan et al., 2019). Meanwhile, Bletilla polysaccharide and its derivatives can be used as preparation materials and drug carriers to play a role in the delivery of antitumor drugs (Guan et al., 2017). With the development and utilization of *Bletilla striata* resources, *Bletilla striata* is widely used in industrial fields such as high-end cosmetics, food, daily chemical products, and has high ornamental value. In recent years, to meet the market demand, the seed direct seeding technology and tissue culture technology of *Bletilla striata* have been continuously developed, and its planting scale has been gradually expanded. However, due to the slow growth of *Bletilla striata* and the harsh requirements for the living environment, the abiotic stress factors in the environment such as high and low temperature, drought, waterlogging and salinity seriously affect its normal growth and the accumulation of effective components in the artificial cultivation of *Bletilla striata*, which is an urgent problem to be solved in its industrialization development.

Calcium-dependent protein kinase (CDPK) family genes play an important role in plant growth, development and response to stress. At present, CDPK family genes have been found in *Arabidopsis thaliana* (Cheng et al., 2002), *Oryza sativa* (Asano et al., 2005), *Triticum aestivum* L. (Li et al., 2008), *Zea mays* (Kong et al., 2013), *Vitis vinifera*×*Vitis Lubrusca* L. cv Kyoho (Yu et al., 2006), *Lycopersicon esculentum* (Chico et al., 2002), *Capsicum annuum* (Cai et al., 2015) and other plants, and confirmed that they play an important role in response to low

temperature, drought, high salt, low osmotic and other environmental factors. CDPK gene family has typical Ser/Thr protein kinase activity, which plays a role in Ca^{2+} mediated signaling pathway. As a calcium signal receptor and transmitter, CDPK gene family transmits the signal to the downstream regulatory network, thereby regulating plant growth and development and stress response (Boudsocq and Sheen, 2013; Atif et al., 2019). However, the cloning and functional identification of CDPK family genes in Orchidaceae is still in its infancy. It has been found that *DoCDPK1*, *DoCDPK2*, *DoCDPK6* and *DcCDPK8* genes of *Dendrobium candidum* may be involved in the response of abiotic stresses such as low temperature (Sheng et al., 2016; Sheng et al., 2017; Wang et al., 2019). The expression of *PaCDPK1* of *Phalaenopsis equestris* could respond to low temperature, mechanical damage, and pathogen infection (Tsai et al., 2007). *CmCDPK* gene of *Cypripedium macranthos* has also been successfully cloned (Fu, 2019), but the *CDPKs* gene of the genus *Bletilla* Rchb. f. has not been reported.

In this study, the *BsCDPK1* gene of purple flower *Bletilla striata* was cloned, and its protein characteristics and expression patterns of different tissues at seedling stage were analyzed, which laid the foundation for elucidating the biological function of *BsCDPK1* gene. At the same time, it also provided data support for the breeding of new *Bletilla striata* varieties with stress resistance, and the improvement of stress resistance and industrial development of *Bletilla striata*.

1 Results and Analysis

1.1 Cloning of *BsCDPK1* gene

By comparing the conserved regions of CDPK homologous genes in known species, degenerate primers were designed, and the cDNA of *Bletilla striata* seedlings was used as a template for PCR amplification to obtain a specific band with a length of 979 bp (Figure 1a). Primers were designed according to the obtained conserved sequence. After 3'-RACE amplification, a specific band of 768 bp was obtained (Figure 1b). After 5'-RACE amplification, a specific band of 723 bp was obtained (Figure 1c). The product sequence was spliced to obtain a cDNA sequence of 1 981 bp. ORF Finder found that the sequence had a complete ORF. The length of 5'-UTR was 112 bp, 3'-UTR was 378 bp, and the coding region was 1 491 bp.

The splicing sequence was verified. Primers CDPK-F and CDPK-R were designed to amplify the full-length sequence of *BsCDPK1*, and a band with a length of 1 938 bp was obtained (Figure 1d). The sequence obtained after sequencing was consistent with that after splicing, which contained a complete ORF and could encode 496 amino acids. Through the search of NCBI database, it is shown that the sequence belongs to the gene of *CDPK* gene family, and the sequence is named *BsCDPK1*.

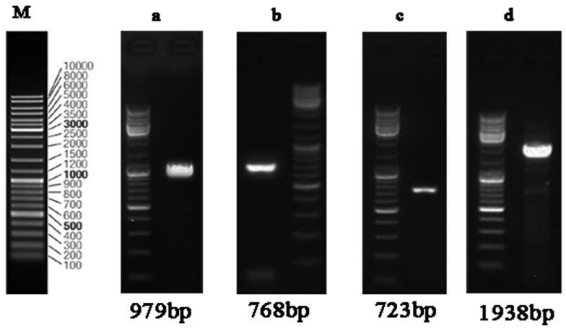

Figure 1 Cloning of *BsCDPK1* gene

Note: M: GeneRuler DNA Ladder Mix; a: Conserved fragment of *BsCDPK1*; b: 3'-RACE amplification fragments; c: 5'-RACE amplification fragments; d: The cDNA full-length amplication of *BsCDPK1*

1.2 Characteristic analysis of BsCDPK1 encoding protein in *Bletilla striata*

Using the online tool ProtParam to analyze the protein, the molecular formula was $C_{2481}H_{3898}N_{676}O_{752}S_{24}$, the molecular weight was 55.998 KD, encoding 496 amino acids (Figure 2A), isoelectric point (PI) was 5.36, aliphatic index was 87.50, and the instability index II was 43.54. It belongs to unstable protein, and the total average hydrophilic coefficient was -0.339, which was hydrophilic protein.

SOPMA predicted the secondary structure of BsCDPK1 protein and found that its secondary structure was composed of α-helix, β-turn, extended strand and random coil, accounting for 47.78%, 9.07%, 10.69% and 32.46%, respectively (Figure 2B). The SWISS-MODEL online tool was used to model the homology of BsCDPK1 in *Bletilla striata*, and the tertiary structure of *Bletilla striata* was predicted and analyzed (Figure 3). The protein was mainly composed of α-helix and random coil, which was consistent with the prediction of secondary structure.

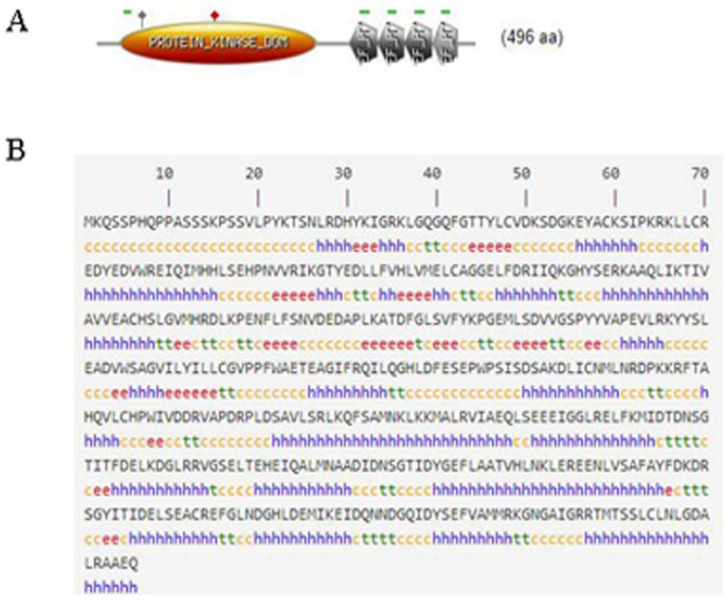

Figure 2 The protein structure prediction of BsCDPK1

Note: A: Protein structure of BsCDPK1; B: Secondary structure prediction of BsCDPK1; h: Alpha helix; e: Extended strand; t: Beta turn; c: Random coil

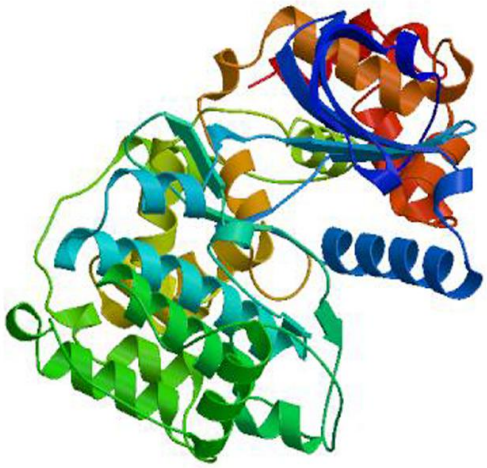

Figure 3 Tertiary structure prediction of BsCDPK1

1.3 Multiple sequence alignment analysis of BsCDPK1 encoding protein in *Bletilla striata*

To further analyze the structure of BsCDPK1 and speculate its biological function, the amino acid sequences of BsCDPK1 were compared with those of other identified CDPK plants (Figure 4). The results showed that the amino acid sequence homology of *BsCDPK1* with *Phalaenopsis equestris* (XP_020588939.1) and *Dendrobium catenatum* (XP_020685368.1) reached 98.39%. The amino acid sequence homology of *BsCDPK1* with *Cypripedium macranthos* (QEV88430.1), *Apostasia shenzhenica* (PKA62509.1), *Ananas comosus,* (XP_020106824.1), *Sorghum_bicolor* (XP_002450385.1), and *Zea mays* (NP_001105752.1) was 95.36%, 91.89%, 88.08%, 84.02% and 83.61%, respectively. By amino acid sequence alignment with known CDPK proteins, it was found that the amino acid composition of CDPKs from different sources was different to varying degrees, but the

BsCDPK1 sequence included the typical kinase domain of CDPKs in other plants, autoinhibitory domain, ATP binding site and the 4 EF-hand structures at C-terminal were highly conserved. At the same time, online software SMART analysis also showed that BsCDPK1 in *Bletilla striata* had 4 EF-hand structures bound to Ca^{2+} and Ser/Thr protein kinase sequences that played a catalytic role, suggesting that BsCDPK1 showed similar kinase activity.

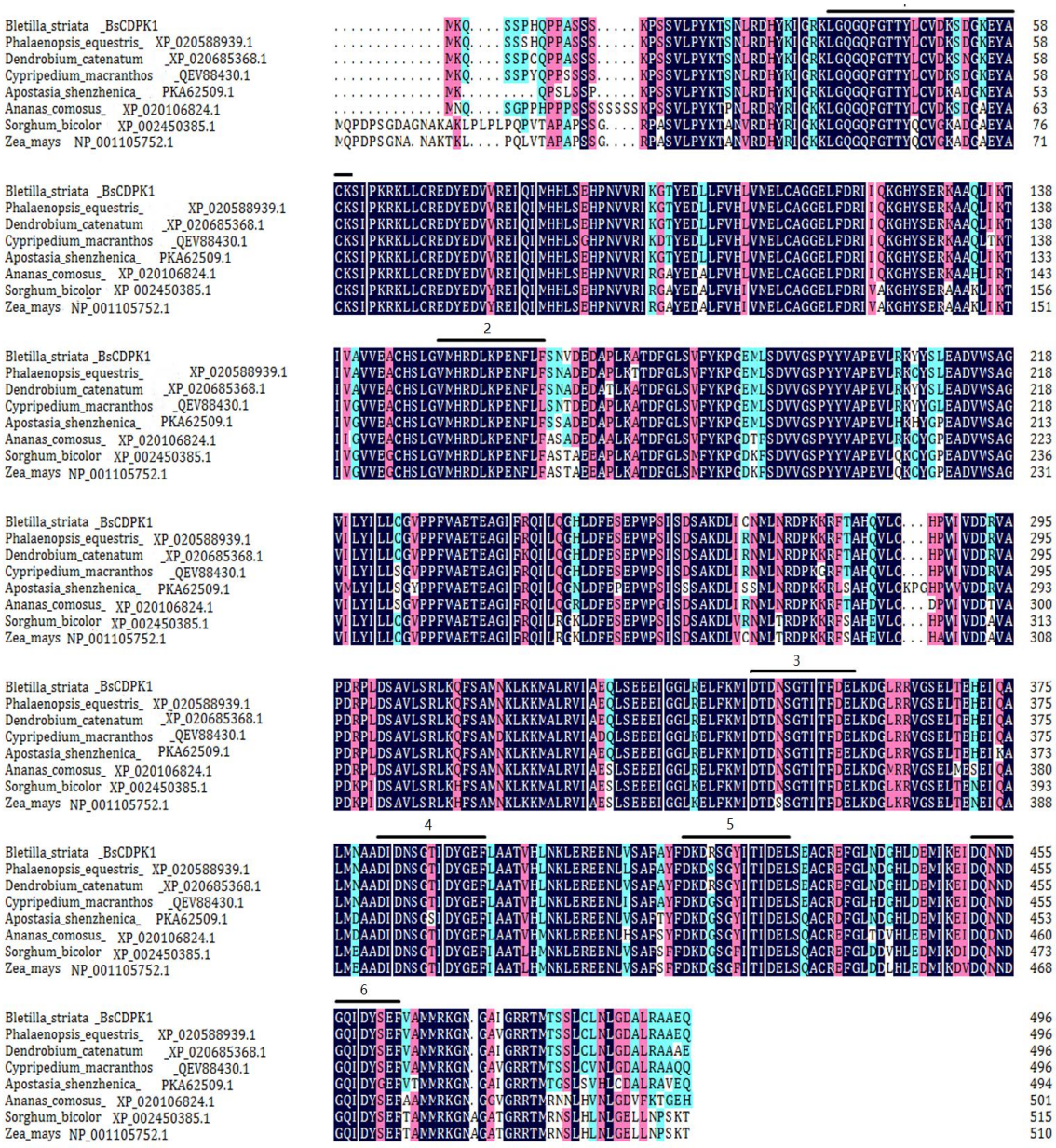

Figure 4 Amino acid sequence alignments of CDPKs

Note: Conserved sequences were indicated by black solid lines; 1: ATP-bindig region; 2: Serine/Threonine protein kinases active-site; 3~6: The EF-hand structures

1.4 Phylogenetic tree of BsCDPK1 protein in *Bletilla striata*

Phylogenetic tree was constructed to further analyze the relationship between BsCDPK1 and CDPKs in other plants (Figure 5). BsCDPK1 of *Bletilla striata* was more homologous with CDPKs of *Dendrobium candidum* and *Phalaenopsis equestris*, clustered on the same evolutionary branch, which was consistent with their evolutionary and taxonomic status.

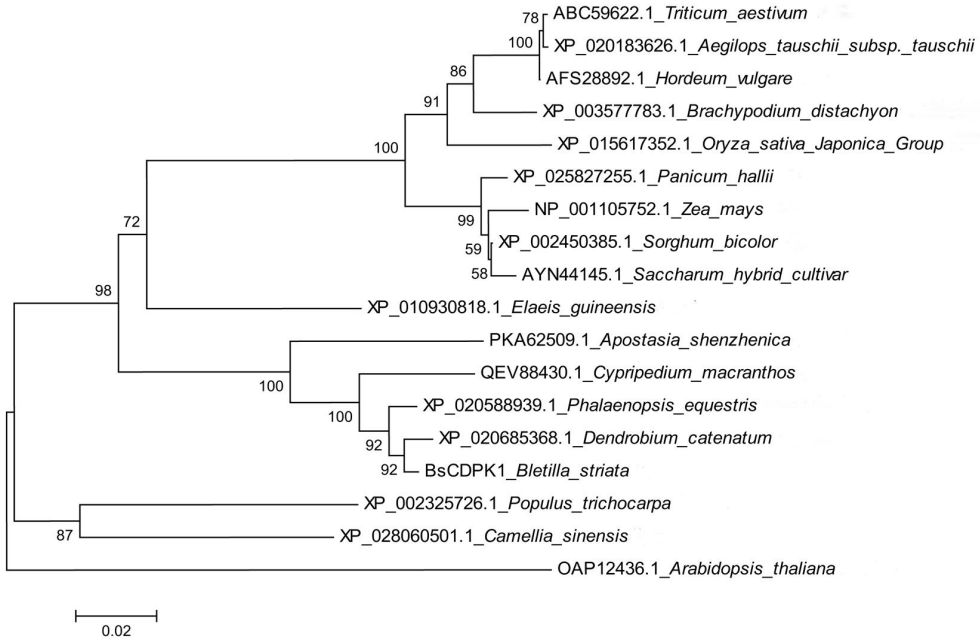

Figure 5 Phylogenetic tree of CDPKs from different plants

1.5 Tissue expression analysis of *BsCDPK1* gene in *Bletilla striata*

qRT-PCR was used to analyze the expression of *BsCDPK1* in different tissues of *Bletilla striata* (Figure 6). The results showed that the *BsCDPK1* was expressed in roots, stems, and leaves of *Bletilla striata* seedling.

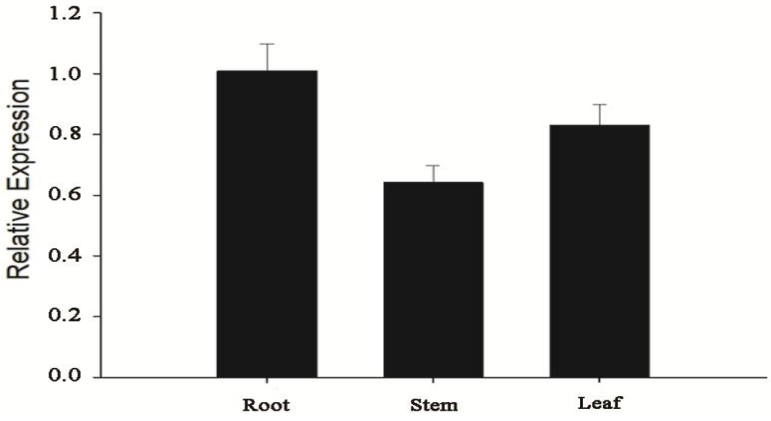

Figure 6 Relative expression of *BsCDPK1* in different tissues of *Bletilla striata*

Note: Data shown in the form of mean ± SE (n=3)

2 Discussion

Bletilla striata, as a precious Chinese herbal medicine, is widely used. However, due to the growth characteristics and environment conditions, the improvement of yield and quality of *Bletilla striata* are limited. Therefore, researchers mainly focus on the growth environment of *Bletilla striata* to study its growth and physiological characteristics. That is, starting from the external factors affecting the normal growth and development and the accumulation of effective components, but few studies have been carried out on the internal factors. It has been found that *CDPKs* were involved in the signal transduction of a variety of Ca^{2+} signaling pathways and played an important role in the growth and development process and stress response of various plants. Partial sequence information of CDPK gene family members was also obtained in Orchidaceae plant such as *Dendrobium candidum*, *Cypripedium macranthos* and *Phalaenopsis equestris*, and their expression patterns were preliminarily analyzed, but the function and regulation mechanism were not clear. As a rare traditional Chinese medicinal material, *Bletilla striata* has not seen the relevant information of *CDPK* gene family so far. In this study, the sequence information of *BsCDPK1* gene was cloned from purple flower *Bletilla striata*, and the structural

characteristics of protein were predicted by bioinformatics analysis. It was found that *BsCDPK1* had high homology with the *CDPK* family genes of other plants reported, which had typical kinase domain, autoinhibitory domain, ATP binding site and the 4 EF-hand structures at the C-terminal and showed high uniformity. Among them, *BsCDPK1* was more homologous with *Dendrobium candidum* and *Phalaenopsis equestris*, clustered on the same evolutionary branch, and had the closest genetic relationship. It was known that BsCDPK1 was a typical calcium-dependent protein kinase family gene. At present, 4 members of CDPK gene family *DoCDPK1*, *DoCDPK2*, *DoCDPK6* and *DcCDPK8* obtained from *Dendrobium candidum* have been found to respond to a variety of abiotic stresses such as low temperature and salt (Sheng et al., 2016; 2017; Wang et al., 2019). It is speculated that *BsCDPK1* may also play a role in the stress response of *Bletilla striata*.

The expression pattern of gene tissue is closely related to gene function. Studies have found that the member genes of plant CDPKs family have tissue expression specificity. Some members participated in the regulation of plant growth and development, and some members participated in the regulation of calcium signal and hormone signal transduction in plant cells, and respond to stress (Atif et al., 2019). At present, the cloned genes *DoCDPK1*, *DoCDPK2* and *DoCDPK6* of *Dendrobium candidum* are mainly expressed in leaves and are involved in the response to abiotic stresses such as low temperature and salt. 40 *CDPK* genes were identified in maize CDPK family, and most of them were specifically expressed in different tissues and developmental stages (Kong et al., 2013). Among them, the gene *ZmCPK11*, which has 83.61% homology with *BsCDPK*, is highly expressed in seeds and seedlings, and lowly expressed in stems, roots, and leaves, and participates in the regulation of multiple stresses such as injury, phospholipids, ABA and MeJA signal transduction (Szczegielniak et al., 2012). Through qPCR analysis, it was found that the expression of *BsCDPK1* in *Bletilla striata* was higher in the seedling stage, and it was expressed in the roots, stems, and leaves of the seedlings, but the expression pattern of *BsCDPK1* in different development stages and seedlings was still unknown. At the same time, there are many worthy of in-depth discussion about the *CDPK* family genes, such as *BsCDPK* family genes involved in the response to stress and hormone signal transduction process and expression patterns, the verification of the function of gene members and the molecular mechanism of *BsCDPK* family regulating *Bletilla striata* stress resistance.

3 Materials and Methods

3.1 Experimental materials

Experimental materials of the capsule of purple flower *Bletilla striata* were collected from Shibao Township, Chishui City, Guizhou Province (106°9′E, 28°38′N), treated with asepsis. The seeds were sowed in the medium with the formula of MS+6-BA (1.5 mg/L)+NAA (1.0 mg/L)+sucrose 30 g/L+agar 6.5 g/L, and cultured in the culture room for about 5 months, with the temperature of 25°C, and the light cycle of 14 h/10 h. The samples were taken for further use when the seedling height reached about 5 cm.

3.2 RNA extraction and cDNA synthesis

RNA was extracted from the whole *Bletilla striata* seedling, and different tissues roots, stems, and leaves, respectively. Trizol produced by Shanghai Sangon Biotech Co., Ltd. was used to extract the total RNA of *Bletilla striata* according to the instructions. RNA was reversely transcribed into cDNA by RevertAid Premium Reverse Transcriptase of Thermo Fisher according to the instructions.

3.3 *BsCDPK1* gene amplification in *Bletilla striata*

Amplification of intermediate fragment: cDNA first strand synthesis: adding reagents (total RNA 5 μL, random primer 1 μL, ddH_2O 1 μL) into 0.2 mL PCR tube, 70°C warm bath for 5 min, ice bath for 2 min. Centrifuge adding reagent: 5× First-Strand Buffer 2.0 μL, 10 mmol dNTP 0.5 μL, RNase inhibitor 0.25 μL, Reverse Transcriptase 0.25 μL, Total volume 10.0 μL, 42°C warm bath for 60 min, 72°C warm bath for 10 min. Using the obtained cDNA as template, the conserved intermediate fragment of *BsCDPK1* was amplified by degenerate primer CDPK-cF/CDPK-cR (Table 1). Amplification PCR reaction system was as follows: 2×GC Buffer I 12.5 μL, F (10 μmol/L) 0.5 μL, R (10 μmol/L) 0.5 μL, dNTP (10 mmol/L) 0.2 μL, template 1 μL, La *Taq* enzyme (5 U/μL) 0.2 μL, added ddH_2O to 25 μL. The PCR procedure was as follows: 95°C for 3 min, 94°C for 30 s, 58°C for 30 s, 72°C for 90 s, 33 cycles. Finally, extension at 72°C for 7 min.

Table 1 Primers used for gene cloning

Primers	Sequences
5'adaptor	GCTGTCAACGATACGCTACGTAACGGCATGACAGTGGGIIGGGIIGGGIIG
3'adaptor	GCTGTCAACGATACGCTACGTAACGGCATGACAGTGTTTTTTTTTTTTTTTT
5.3'outer	GCTGTCAACGATACGCTACGTAAC
5.3'inner	GCTACGTAACGGCATGACAGTG
CDPK-cF	CAGAAGGGGCAYTACAGCGA
CDPK-cR	TGCCCATCATTATYTTGATCWATYT
CDPK-F3	CTGACTGAGCATGAGATTCAGGCTCTAATGA
CDPK-F4	TGAATAAGTTGGAGAGGGAAGAGAACCTGGT
CDPK-R3	AGTACTTCAGGTGCAACATAGTAGGGGCTT
CDPK-R4	TAGCATTTCACCTGGCTTATAGAATACCGAGA
CDPK-RT1	GCGATAAAACAGCAGAATCTAATG
CDPK-RT2	TCTGGAGCCACTCTGTCATCAA
CDPK-F	CCTATTTCACCACCATAATCCGA
CDPK-R	TCATGGCACTTACAATGTTTTGATAC
CDPK-qF	TTGGGCAGAAACTGAAGCG
CDPK-qR	TGGAGCCACTCTGTCATCAAC
ACTIN-F	AATCCCAAGGCAAACAGA
ACTIN-R	CACCATCACCAGAATCCAG

3'-RACE amplification: Nested PCR was performed using cDNA with 3'adaptor as reverse primer as template. The PCR reaction system was as follows: 2×GC Buffer I 12.5 μL, F (10 μmol/L) 0.5 (CDPK-F3)/(CDPK-F4), R (10 μmol/L) 0.5 (5.3'outer)/(5.3' inner), dNTP (2.5 mmol/L) 4 μL, template 1 μL (cDNA)/(PCR dilution products of first round), *Taq* enzyme (5 U/μL) 0.2 μL, added ddH_2O to 25 μL. PCR procedure was as follows: 95°C for 3 min, 94°C for 30 s, 58°C for 30 s, 72°C for 60 s, 33 cycles. Finally, extension at 72°C for 7 min. The PCR products were detected, and the target bands were recovered and purified. The positive clones were sent to Shanghai Sangon Biotech Co., Ltd. for sequencing.

5'-RACE amplification: cDNA was obtained by reverse transcription with specific primers CDPK-RT1/ CDPK-RT2. After RNase H and TdT treatment, nested PCR was performed (For steps, refer to Invitrogen 5' -RACE system manual). The PCR reaction system was as follows: 2×GC Buffer I 12.5 μL, F (10 μmol/L) 0.5 (5'adaptor)/(5.3'outer), R (10 μmol/L) 0.5 (CDPK-R3)/(CDPK-R4), dNTP (2.5 mmol/L) 4 μL, template 1 (cDNA)/ (PCR dilution products of first round), *Taq* enzyme (5 U/μL) 0.2 μL, added ddH_2O to 25 μL. PCR amplification condition was as follows: 95°C for 3 min, 94°C for 30 s, 68°C for 30 s, 72°C for 60 s, 33 cycles. Finally, extension at 72°C for 7 min. The PCR products were detected and sequenced as above.

The full-length sequence of *BsCDPK1* gene with complete open reading frame (ORF) was obtained by splicing the 3' and 5' end sequences. The specific primers CDPK-F/CDPK-R were designed to amplify the full length of the gene. The target fragment was purified, recovered, and sequenced. The results were compared with the splicing sequence.

3.4 Bioinformatics analysis of *BsCDPK1* encoding protein in *Bletilla striata*

Based on online bioinformatics software, the basic physical and chemical properties, secondary structure, hydropathicity/hydrophobicity, and structural functional domain of BsCDPK1 were predicted and analyzed.

The domain, physicochemical properties, and secondary structure of BsCDPK1 protein were analyzed by online software SMART (http://smart.embl-heidelberg.de/), PROSITE (https://prosite.expasy.org/), Protparam (https://web.expasy.org/protparam/), and SOPMA (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html), respectively. SWISS-MODEL software (https://swissmodel.expasy.org/) was used to construct protein three-dimensional structure. DNAMAN

software was used to compare the amino acid sequence of BsCDPK1 and CDPK protein in other plants. MEGA6.0 software was used to construct phylogenetic tree.

3.5 Tissue expression analysis of *BsCDPK1* gene in *Bletilla striata*

The fluorescent quantitative primers CDPK-qF and CDPK-qR were designed with the housekeeping gene *BsACTIN* in *Bletilla striata* as internal reference, ACTIN-F/R as primer and the cDNA of roots, stems and leaves of *Bletilla striata* seedlings obtained by reverse transcription as templates. qRT-PCR was used to detect the expression level of *BsCDPK1* gene in different tissues of *Bletilla striata* at seedling stage. Total volume of reaction system was 20 μL: 2×SG Fast qPCR Master Mix 10 μL, primers (10 μmol/L) 0.4 μL each, template 1 μL (cDNA sample diluted 5 times), DNF Buffer 2 μL, added PCR-grade water to 20 μL. 3 repeats and 3 biological repeats in each reaction. PCR cycle conditions was as follows: 95°C for 3 min, 95°C for 3 s, 60°C for 30 s, 72°C for 20 s, 40 cycles. The instrument used in this study was CFX96 real-time fluorescence quantitative PCR (Bio-Rad). SigmaPlot 12.5 software was used to draw and analyze.

Authors' contributions

WSS designed and carried out the study. ZG and DQL participated in the data collation and the writing of the first draft of the manuscript. ZG, DQL, ZBL, and TQ participated in the part of the experiment. WSS conceived of the project, directed the design of the study, data analysis, draft, and revision. All authors read and approved the final manuscript.

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