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Expression, purification and molecular characterization of a novel transcription factor KcCBF3 from *Kandelia candel*

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ABSTRACT

Kandelia candel, a major species of mangrove in the tropical and subtropical area, is susceptible to low temperature in winter. *K. candel* was introduced into Zhejiang Province (the northern margin of South China) several decades ago, and suffered from low temperature causing growth retardation, in server cases, even death. To explore the molecular mechanisms of cold acclimation in *K. candel*, a novel C-repeat binding factor gene *KcCBF3* (Genbank accession no. KF111715) of 729 bp open reading frame (ORF) encoding a protein of 242 amino acid residues was isolated, expressed, purified and characterized. Multiple sequence alignment analysis revealed that KcCBF3 contained a highly conserved AP2/EREBP DNA-binding domain which consisting of 79 amino acid residues, as well as two CBF signature sequences. Phylogenetic analysis indicated that KcCBF3 belonged to the A-1 subgroup of DREB subfamily based on the classification of AP2/EREBP transcription factors in *Arabidopsis*. Semi-quantitative RT-PCR showed that *KcCBF3* transcripts were highly accumulated in roots and leaves, and could be induced by low temperature. Electrophoresis mobility shift assay (EMSA) demonstrated KcCBF3 could bind to the core sequence (CCGAC) of *cis*-acting element C-repeat (CRT)/dehydration-responsive element (DRE) *in vitro*. These results implied that *KcCBF3* might participate in the adaptation of *K. candel* to low-temperature stress by binding to CRT/DRE element.

1. Introduction

Abiotic stresses such as low temperature, drought and high salinity are important limiting factors affecting plant growth and development. In order to acclimate to the stress environment, plants have to regulate the expression of multiple genes and undergo a series of physiological and biochemical adjustments [1]. Stress-responsive genes can be divided into two major categories according to their functions: (1) functional protein-coding genes, including osmoregulatory genes and antioxidant genes, which mainly play a vital role in protecting cells from stress injury; (2) regulatory genes, including transcription factor genes and kinase genes, which are involved in signaling transduction and regulation of gene expression [2]. AP2/EREBP, bZIP, MYB, WRKY and NAC are the five most reported transcription factors coping with adversity in plants [3].

The signaling pathway mediated by the transcription factor CBF/DREB (C-repeat/dehydration-responsive element binding) is currently the most studied regulation network of gene expression response to low temperature. CBF/DREB belongs to the subfamily of AP2/EREBP (APETALA2/Ehylene responsive element binding protein), which is an important transcription factor of plants responding to abiotic stresses [4]. The proteins of CBF family can be further classified into six subgroups (A1-A6) based on the similarities of the AP2 structures in their sequences [5]. Among subgroups A1-A6, transcription factor A1 (also called subgroup DREB1/CBF, such as DREB1B/CBF1, DREB1C/CBF2 and DREB1A/CBF3 from *Arabidopsis*) plays a critical role in the response of plant to low temperature [6]. DREBl/CBF is widespread in plants and has been successively identified in many glycophytes, such as *Arabidopsis thaliana*, *Brassica napus*, *Medicago sativa*, *Glycine max*, *Nicotiana tabacum*, *Solanum tuberosum*, *Triticum aestivum*, *Oryza sativa*, as well as halophytes, including *Suaeda salsa* [7], *Aegiceras corniculatum* [8] *and Avicennia marina* [9].

Studies have shown that the N-terminal conserved nuclear localization signal (NLS) PKKR/PAGRxKFxETRHP consists of basic amino acid, and downstream DSAWR sequence, C-terminal LWSY conserved sequence as well, are known as the signature sequence (SS) of transcription factor DREB1/CBF [10]. In addition, the transcription factor CBF contains an AP2/ERF conserved domain of about 60 amino acid residues, which can form three antiparallel β-sheets and one α-helix. It has been demonstrated that the highly conserved valine (V) of position

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14 and glutamic acid (E) of position 19 in β-sheet played an essential role in binding of DREB1/CBF to *cis*-acting element C-repeat (CRT)/dehydration-responsive element (DRE) [5].

In recent years, the DREB1/CBF pathway of plants has been well studied, and the molecular mechanism of its function and regulation has been revealed. The CBF protein can specifically recognize the sequence of CCGAC as a core motif in *cis*-acting element CRT/DRE, which is often found in the promoter region of low-temperature-induced genes, such as *COR* (cold regulated), *LTI* (low-temperature induced), *DHN* (dehydrin)、*KIN* (cold inducible), *ERD* (*early responsive to dehydration*) and *RD* (responsive to dehydration) [11]. When plants are exposed to low temperature, the combination of transcription factor CBF and its promoter can trigger the expression of multiple low-temperature-induced genes downstream, causing many physiological and biochemical changes in plants and generating certain resistance to cold stress [4]. Therefore, the transcription factor CBF has received extensive attention in the research of plants acclimation to low temperature stress.

Overexpression of gene *AtCBF1*(*DREB1B*) from *Arabidopsis thaliana* enhanced the expression of a series of *COR* genes and increased the freezing tolerance in *Arabidopsis* [12], likewise, overexpression of *AtCBF1* also significantly improved the tolerance of *Populus* to low temperature [13]. Similarly, overexpression of *VrCBF1* or *VrCBF4* from *Vitis vinifera* significantly enhanced the freezing tolerance of *Arabidopsis* transgenic lines, accompanying a increase expression of cold-regulated genes *AtCOR15a*, *AtCOR6.6*, *AtCOR47* and *AtRD29*A [14]. Soltész et al. [15] also found that overexpression of *TaCBF14* or *TaCBF15* from *Triticum aestivum* improved the frost tolerance of barley transgenic lines, the expression of *HvCOR14*b, *HvDHN5* and *HvDHN8* were up-regulated as well. These observations suggested that *CBF* gene elevated the low-temperature tolerance of transgenic plants via up-regulating cold-regulated genes.

Mangroves are emerged woody plants that grow in the intertidal zone of tropical to subtropical estuaries and shorelines, which have the functions of providing biological habitat, maintaining the balance of wetland ecosystem, against typhoon winds and purifying the marine environment [16]. The habitat of mangroves is unique with the characteristics of soil salinization, poor ventilation, and periodic flooding. In recent years, mangroves have been increasingly exposed to the threat of heavy metals and organic substances pollution, sea-level rise and low temperature due to human activities and global climate change. Therefore, it is of great significance to research on the resistance mechanism of mangrove plant to stress environment [17].

Kandelia candel is a major species of mangrove in southern China. Although it has strong tolerance to many abiotic stresses such as flooding, high salt and heavy metals, temperature is an important limiting factor for its distribution and survival [18]. As the most cold-tolerant species of mangrove in the northern hemisphere, *K. candel* is nevertheless vulnerable to low temperature stress in winter in higher latitudes [19]. There have been many researches on the physiological ecology of chilling injury of *K. candel*, but its underlying molecular mechanism remains unclear. Here we cloned the gene of cold stress-related transcription factor *KcCBF3* from *K. candel* and expressed in prokaryotes, as well as explored its binding properties with *cis*-acting elements through electrophoretic mobility shift assay (EMSA), this paper will provide a basis for the further study of *K. candel* gene regulatory networks that contribute to low-temperature acclimation.

2. Materials and methods

2.1. Plant material, strains and plasmids

The healthy and mature propagules of *K. candel* picked from Yuhuan County (Zhejiang, China) mangrove wetland were planted in clean beach sand and fertilized with 1/2 Hoagland nutrient solution twice a week in a climate-controlled growth chamber, which was controlled as 300 μmol/(m^2·s), 28 °C/25 °C (day/night), 16 h/8 h (light/dark). For low temperature stress, 2-month-old plantlets were treated at 4 °C for 0, 1, 2, 4, 8, 16 and 32 h, respectively. The root, stem and leaf samples were collected and frozen in liquid nitrogen quickly and stored at −80 °C freezer for total RNA extraction.

Escherichia coli DH5α and Rosetta (DE3) (Invitrogen, Shanghai, China) were employed for recombinant plasmid construction and expression of the gene *KcCBF3*. The vector pUCm-T (Beyotime, Shanghai, China) and pET-28a plasmid (Novagen, Shanghai, China) were utilized for gene cloning, DNA sequencing, and protein expression of *KcCBF3*, respectively.

2.2. Cloning and identification of gene KcCBF3

Total RNA was isolated from the young leaves of *K. candel* by TRIzol™ reagent (TaKaRa, Dalian, China) following the manufacturer's instructions. cDNA synthesis was produced from the total RNA using PrimeScript™ One Step RT-PCR Kit (TaKaRa, Dalian, China). The gene *KcCBF3* (GenBank accession no. KF111715) was amplified by high fidelity KOD-Plus PCR polymerase (TOYOBO, Shanghai, China) with two primers: KcCBF3-F 5′-CCCAAGCTTGCATGAACAATAAAGACCCTT-3′ and KcCBF3-R 5′- CCGCTCGAGATAGCTCCATAAGGACACGTC -3’. The PCR products were purified and ligated into vector pUCm-T to form a recombinant plasmid pUCm-T-KcCBF3 according to the manufacturer's instructions (Dingguo, Beijing, China), followed by transforming into *E. coli* DH5a competent cells and spreading onto LB agar medium supplemented with 100 μg/mL ampicillin. White colonies grew over 12 h were selected and the positive clones were verified by colony PCR and DNA sequencing.

2.3. Bioinformatics analysis

Multiple amino acid sequences alignment analysis was performed with DNAMAN 7.0 (Lynnon Biosoft, Quebec, Canada). Phylogenetic tree of CBF proteins in different species analysis was carried out based on the classification of transcriptional factors AP2/ERF family in *Arabidopsis* proposed by Sakuma [5] with Mega 5.05 (Tempe, AZ, USA) by Neighbour-Joining (NJ) method. Prediction of the protein subcellular localization was carried out using server CELLO (http://cello.life.nctu.edu.tw/). Three-dimensional structure estimated by homology modeling using the online service SWISS-MODEL (http://swissmodel.expasy.org/) [20].

2.4. Expression profile analysis of gene KcCBF3

Semi-quantitative RT-PCR was employed to evaluate the expression profile in different tissues and expression dynamics of gene *KcCBF3* to low temperature in *K. candel* using 18S rRNA as an internal control. Specific primers for KcCBF3 were KcCBF3-q-F 5′-GGAGCCCAACAAGAAGTCGAGAATA and KcCBF3-q-R 5′-GCCTTGACTCATCCGCCGAAAC-3′, and for 18S rRNA were Kc-18S-F 5′-ACCATAAACGATGCCGACCAG-3′ and Kc-18S- R 5′-CCTTTAAGTTTCAGCCTTGCG-3’.

2.5. Construction of recombinant expression vector pET28a-KcCBF3

The recombinant plasmid pUCm-T-KcCBF3 verified by DNA sequencing were digested with restriction endonuclease *Hin*dIII and *Xho*I and inserted into the multiple clone site of vector pET28a to produce a recombinant plasmid pET28a-KcCBF3. The plasmid pET28a-KcCBF3 was then transformed into *E. coli* DH5α and plated on kanamycin (50 μg/mL) plates. Positive clones were confirmed by PCR using T7 promoter primer 5′-TAATACGACTCACTATAGGG-3′ and T7 terminator primer: 5′-TGCTAGTTATTGCTCAGCGG-3′, digestion analysis with restriction endonucleases *Hin*dIII and *Xho*I, and DNA sequencing of the isolated plasmids from these clones. The PCR product was detected by 1% agarose gel electrophoresis followed by ethidium bromide (EB)

staining. The confirmed recombinant plasmid pET28a-KcCBF3 was transformed into Rosetta (DE3) competent cells for further over-expression trials.

2.6. Expression and purification of recombinant protein KcCBF3

The *E. coil* Rosetta (DE3) transformed with pET28a-KcCBF3 was cultured in LB medium containing 50 mg/mL kanamycin at 37 °C and a shaking speed of 200 r/min until the value of OD_{600} reached 0.5–0.6. Protein expression was induced with isopropyl-D-1-thiogalactopyranoside (IPTG) at a final concentration of 0.5 mM at 18 °C for 18 h. The Rosetta (DE3) cells were harvested by centrifugation at 8000 r/min for 10 min at 4 °C. The pellet was re-suspended in ice-cold lysis buffer (50 mM Tris-HCl, pH 8.0) and lysed by sonication for 19.5 min (90 cycles of 8-s work and 5-s break interval) in an ice water bath. Whole cell lysates were centrifuged at 12,000 r/min for 30 min at 4 °C, both the supernatant and the precipitate were collected separately. A portion of the samples was prepared for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis, and the other was used for follow-up purification. Both the supernatant and the precipitate were added equal volume of 2 × SDS loading buffer, and boiled for 5 min in a boiling water bath, followed by separation on 12% SDS-PAGE and visualization by staining with Coomassie brilliant blue R-250.

For KcCBF3 purification, the supernatant was loaded onto a nickle nitrilotriacetic acid (Ni^{2+}-NTA) affinity resin column equilibrated with lysis buffer. The column was washed with 5 column volumes of lysis buffer, then non-specifically bound proteins were washed with lysis buffer supplemented with 30 mM imidazole, and the recombinant protein was subsequently eluted from the column with the same buffer containing 250 mM imidazole [21]. The purified protein was also analyzed by SDS-PAGE stained with Coomassie brilliant blue R-250.

The quantity of protein KcCBF3 on SDS-PAGE gel was determined by densitometry analysis using software ImageJ 1.51k (https://imagej.nih.gov/ij/), and the concentration of the purified KcCBF3 was measured by Bradford assay [22].

2.7. Western blot analysis of recombinant protein KcCBF3

Whole cell lysates were separated on SDS-PAGE and proteins were subsequently transferred to nitrocellulose membrane (Thermo Scientific, Waltham, MA, USA) using a Mini Tran-Blot® Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA, USA) at 100 V for 60 min. After blocking for 2 h in 5% skimmed milk in phosphate-buffered saline (PBS), membrane was incubated overnight at 4 °C with anti-6 × His rabbit polyclonal antibody (Sangon, Shanghai, China). The secondary antibody HRP-conjugated goat anti-rabbit IgG (Sangon, Shanghai, China) were employed to react with the primary antibody for 2 h at room temperature. The protein bands were then visualized using a HRP-DAB Detection Kit (Sangon, Shanghai, China).

2.8. MALDI-TOF/TOF analysis and protein identification

Specific protein band was excised directly from the SDS-PAGE gel, which had been stained with Coomassie brilliant blue. The sample was washed with ultrapure water and 50% acetonitrile containing 25 mmol/L ammonium bicarbonate for destaining, followed by dehydrated with acetonitrile, and then re-swollen in trypsin for digestion. The supernatant was extracted with 50% acetonitrile containing 0.1% trifluoracetic acid and dried by a freeze dryer. Purified peptides were eluted in CHCA matrix solution and analyzed using a 5800 matrix-assisted laser desorption/ionization-time of flight (MALDI TOF)/TOF analyzer (AB Sciex, Framingham, MA, USA) according to the method of Wang et al. [23]. Both MS and MS/MS data identification were processed using software of GPS Explorer™ (version 3.6, AB Sciex, USA) and Mascot search algorithm (v2.3, Matrix Science, London, UK).

2.9. Electrophoretic mobility shift assays (EMSA)

EMSA was performed using a 2nd generation DIG Gel Shift Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. The probe sequence (sense probe 5′-AAAAGATATACTACCGACATGAGTT-3′ and antisense probe 5′-AACTCATGTCGGTAGTATATCTTTT -3′) were chosen from the promoter region (−258 ~ −234) of *rd29A* gene of *Arabidopsis* [24,25]. Mutated probe (mutated sense probe 5′-AAAAGATATACTATTTTTATGAGTT-3′ and mutated antisense probe 5′-AACTCATAAAAATAGTATATCTTTT-3′) was obtained from the probe above, which changed the core sequence of CCGAC to TTTTT in the sense strand. Both probe and mutated probe were synthesized and annealed, respectively, and labeled subsequently at the 3′ ends with non-radioactive digoxigenin-11-ddUTP (DIG).

The DNA-binding reaction was performed in a total volume of 10 µl of TEN buffer (10 mM Tris, 1 mM EDTA, 0.1 M NaCl, pH 8.0) containing 0.1 µg of poly L-lysine, 30 fmol of labeled probe, and 2 µg of recombinant KcCBF3 proteins. For competition analysis, 1 µg poly (dI-dC) and 50-, 100-, 500-fold excess of unlabeled probe were added to the binding reactions. The mixture was incubated at 25 °C for 30 min, and then loading buffer with bromophenol blue was added. Protein-bound and free probe were separated on 6% native polyacrylamide gels and then transferred to positively charged nylon membranes by electroblotting. The membranes were baked at 120 °C for 20 min, and Dig-labeled DNA-protein complexes detected following the manufacturer's recommendations.

3. Results

3.1. Cloning and identification of gene KcCBF3

cDNA was synthesized from total RNA by RT-PCR and the gene *KcCBF3* was amplified using specific primers designed from the sequence information available in GenBank (accession no: KF111715). The amplification product was detected by 1% agarose gel electrophoresis. As shown in Fig. 1, a specific band of approximate 750 bp, which matched the size of the *KcCBF3* gene, was successfully amplified. The DNA fragment was ligated into the vector pUCm-T to generate a recombinant plasmid pUCm-T-KcCBF3, which was then transformed into *E. coli* DH5α. Five white colonies were picked up and verified by colony PCR and DNA sequencing. The results showed that five clones selected were all positive, and the coding sequence length of the target gene *KcCBF3* was 729 bp, which encoded a protein of 242 amino acid residues.

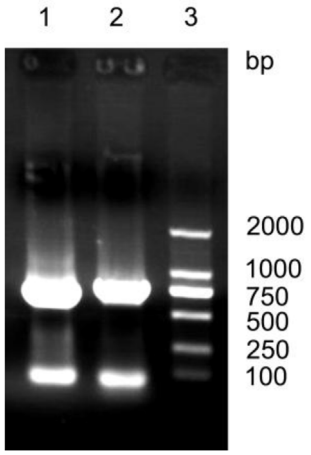

Fig. 1. PCR amplification products of gene *KcCBF3*. Lane 1–2: *KcCBF3* gene amplified using cDNA as template reverse-transcribed from leaf total RNA of *K. candel*; Lane 3: DNA ladder.

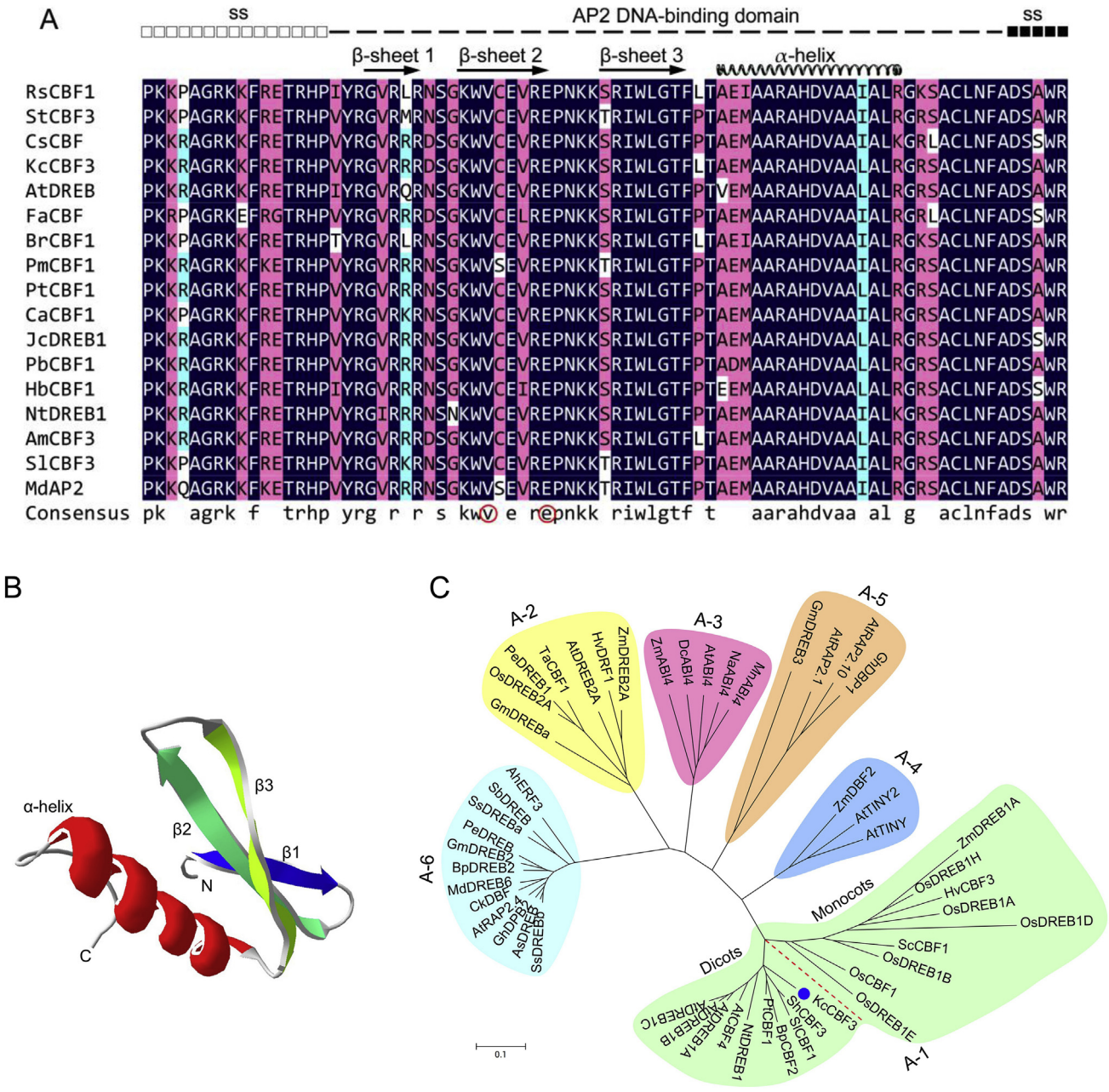

Fig. 2. Sequence analysis of protein KcCBF3. (A) Alignment of central region of different plant CBF proteins. The AP2 DNA-binding domain is marked by a broken line and the ss (signature sequences) PKK/RPAGRxKFxETRHP and DSAWR were indicated by white squares and black squares, respectively. Circles represent conserved valine (V) at position 14 and glutamate (E) at position 19 of AP2 domain. (B) Three-dimensional structure of AP2 DNA-binding domain in protein KcCBF3 was estimated from homology modeling using the crystal structure of AtERF1 (PDB ID: 1gcc.1. C) as a reference. (C) Phylogenetic analysis of CBF/DREB proteins using MEGA 5.05 by Neighbour-Joining method. The scale bar indicates branch lengths. A-1 ~ A-6 indicate subgroups of DREB subfamily proposed by Sakuma et al. [5]. The accession number of the each protein is: KcCBF3 (*Kandelia candel*, KF111715); TaCBF1 (*Triticum aestivum*, AF303376); ScCBF1 (*Secale cereale*, AF370730); HvCBF3 (*Hordeum vulgare* subsp. *vulgare*, AF239616); HvDRF1 (*H. vulgare*, AY223807); OsDREB1A (*Oryza sativa*, AF300970); OsDREB2A (*O. sativa*, AF300971); ZmDBF2 (*Zea mays*, AF493799); ZmABI4 (*Z. mays*, AY125490); AtABI4 (*Arabidopsis thaliana*, AF085279); AtTINY2 (*A. thaliana*, AY940160); GhDPB2 (*Gossypium hirsutum*, AY619718); SsDREBa (*Suaeda salsa*, KM365207); SsDREBa (*S. salsa*, KM365208); AhERF3 (*Arachis hypogaea*, JN613348); AsDREB (*Atriplex halimus*, JF451138); AtDREB1A (*A. thaliana*, NP_567720); AtDREB1B (*A. thaliana*, CP002687); AtDREB2A (*A. thaliana*, NP_196160); AtRAP2.1 (*A. thaliana*, AY086838); AtRAP2.10 (*A. thaliana*, NP_195408); AtRAP2.4 (*A. thaliana*, NP_177931); BpDREB2 (*Broussonetia papyrifera*, DQ211836); CkDBF (*Caragana korshinskii*, GU573848); GhDBP1 (*G. hirsutum*, AY174160); GmDREB2 (*Glycine max*, AY296651); GmDREB3 (*G. max*, DQ208969); GmDREBa (*G. max*, AY542886); MdDREB6 (*Malus domestica*, JQ669823); PeDREB (*Populus euphratica*, EF597499); SbDREB (*Salicornia bigelovii*, JF894301); ZmDREB1A (*Z. mays*, AF450481); ZmDREB2A (*Z. mays*, AB218832); AtTINY (*A. thaliana*, BT024923); DcABI4 (*Dendrobium catenatum*, KZ503118); NaABI4 (*Nicotiana attenuate*, XP_009619856); MnABI4 (*Morus notabilis*, XP_010105435); AtDREB1C (*A. thaliana*, AB013817); AtCBF4 (*A. thaliana*, CP002688); OsDREB1E (*O. sativa*, AY785896); OsDREB1D (*O. sativa*, AY785895); OsDREB1H(*O. sativa*, XP_015611305); NtDREB1 (*N. tabacum*, EU727155); PeDREB1 (*Phyllostachys edulis*, EU860441); ShCBF3 (*Solanum habrochaites*, EU365378); SlCBF1 (*S. lycopersicum*, AY034473); BpCBF2 (*Betula platyphylla*, HQ231406); PtCBF1 (*P. trichocarpa*, EF151456); OsDREB1B (*O. sativa*, AF300972); OsCBF1 (*O. sativa*, AP001168).

3.2. Sequence analysis of protein KcCBF3

The amino acid alignment of the sequences revealed that KcCBF3 shared some typical conserved motifs with other CBFs, which consisted of an AP2 DNA-binding domain and two CBF signature sequences (ss), PKKR/PAGRxKFxETRHP (nuclear localization signal, NLS) and DSAWR (Fig. 2A). Furthermore, three-dimensional structure analysis demonstrated that AP2 DNA-binding domains of KcCBF3 was similar to that of protein AtERF1 (PDB ID: 1gcc.1. C), which containing three continuous β-sheets and one α-helix (Fig. 2B). To illustrate the evolutionary relationship of a series of CBFs, a phylogenetic tree was constructed based on the classification of AP2/ERF transcriptional factors in *Arabidopsis* proposed by Sakuma. The phylogenetic analysis showed that KcCBF3 was classified into A-1 subgroup (Fig. 2C). Moreover, two major clades in A-1 group were distinguished in the phylogenetic tree, demonstrating that A-1 CBFs were conserved between monocotyledons and dicotyledons. Subcellular localization revealed that KcCBF3 should be a nuclear protein according to server CELLO.

3.3. Expression profile and dynamics analysis of gene KcCBF3

Semi-quantitative RT-PCR was performed to explore the expression abundance of gene *KcCBF3* in different tissues, the expression of *KcCBF3* was detected in roots, stems and leaves, however, the expression level in roots are much higher than in stems (Fig. 3A). To determine the response of *KcCBF3* to low temperature stress, 2-month-old plantlets were subjected to 4 °C for different intervals. Expression profiles showed that *KcCBF3* transcript level in both roots and leaves were sharply increased when treated with low temperature, furthermore, the expression level in leaves was dramatically increased after 1 h stress, and the abundance was much higher than that in roots at the first 16 h (Fig. 3B). The expression of gene *KcCBF3* was induced by low temperature stress rapidly and strongly, which suggested that *KcCBF3* participated in the early response to chilling.

3.4. Construction of recombinant expression vector pET28a-KcCBF3

Both the recombinant plasmid pUCm-T-KcCBF3 verified by DNA sequencing and vector pET28a were digested with restriction endonuclease *Hin*dIII and *Xho*I, and then ligated to produce the pET28a-KcCBF3 recombinant plasmid, followed by transforming into *E. coli* DH5α. The putative positive transformants were selected on LB medium containing kanamycin and confirmed by colony PCR using T7 promoter/terminator primers, the result of agarose gel electrophoresis showed the length of the amplified product was about 1000 bp, which was consistent with the theoretical size (Fig. 4A). Moreover, restriction digestion of the recombinant plasmid with *Hin*dIII and *Xho* I released a 750-bp fragment on agarose gel, which was the same as the predicted size (Fig. 4B). Sequencing analysis showed that gene *KcCBF3* was inserted into the expression vector pET28a correctly.

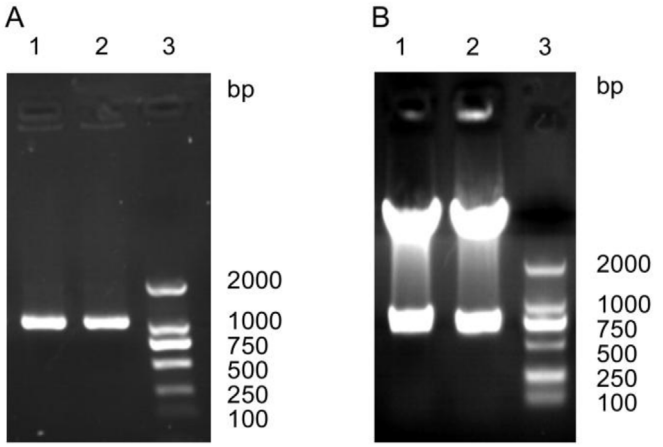

Fig. 4. Construction of recombinant expression vector pET28a-KcCBF3. (A) Confirmation of the recombinant vector pUCm-T-KcCBF3 by PCR. Lane 1–2: PCR product of recombinant plasmid using T7 primers; Lane 3: DNA ladder. (B) Verification of the recombinant vector by restriction enzyme analysis. Lane 1–2: recombinant vector were digestion by *Hin*dIII and *Xho*I; Lane 3: DNA ladder.

3.5. Induction and identification of the recombinant protein

E. coli Rosetta (DE3) harboring recombinant plasmid pET28a-KcCBF3 was cultured in LB liquid medium and the protein KcCBF3 was induced by IPTG. Both the supernatant and the precipitate were collected using sonication followed by centrifugation. The result of SDS-PAGE showed that there was an additional band of approximate 35 kDa in strain over-expressing KcCBF3, compared with the strain transformed with empty vector (pET28a), which is roughly in accordance with the predicted molecular weight of the fusion protein (Fig. 5A). Densitometric scanning of the SDS-PAGE gel analysis showed that 26.4% of the target protein KcCBF3 was expressed in soluble form, which offered an extremely convenient procedure for subsequent purification and identification. Western blot analysis of whole cell lysates over-expressing recombinant protein KcCBF3 indicated a clear band presented on the nitrocellulose membrane, which confirmed that fusion protein KcCBF3 was induced successfully in the *E. coli* Rosetta (DE3) (Fig. 5B). The recombinant soluble protein KcCBF3 was purified by Ni^{2+}-NTA affinity resin. The result of the purification revealed that a single protein band about 35 kDa was showed on the SDS-PAGE gel, which corresponded to the theoretical molecular weight of the fusion protein (Fig. 5C). The final yield of soluble purified recombinant KcCBF3 was 11.3 mg per liter culture of *E. coli.*

3.6. MALDI-TOF/TOF analysis

To confirm the sequence of the recombinant protein KcCBF3 purified by Ni^{2+}-NTA, the single protein band stained by Coomassie brilliant blue was excised from SDS-PAGE gel, followed by destaining with acetonitrile, digestion with trypsin and MALDI-TOF-TOF tandem MS analysis. MS/MS data showed three oligopeptide sequences (peak 1,

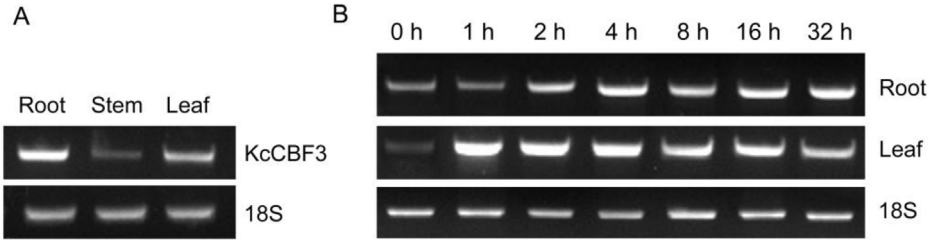

Fig. 3. Gene expression level of *KcCBF3* using semi-quantitative RT-PCR. (A) Expression abundance of gene *KcCBF3* in different tissues of *K. candel.* (B) Expression of gene *KcCBF3* in roots and leaves of *K. candel* under low temperature (4 °C) stress. 18S rRNA was used as an internal control.

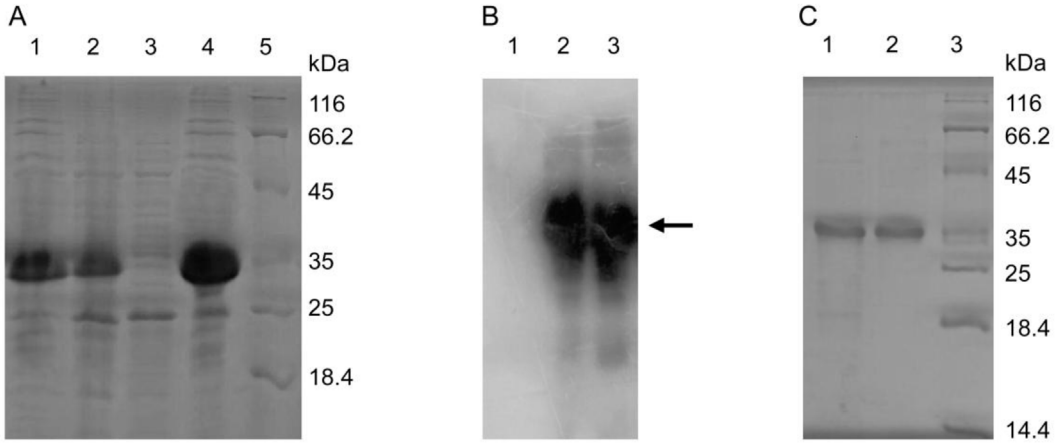

Fig. 5. Expression, identification and purification of the recombinant protein KcCBF3. (A) Expression of the recombinant protein KcCBF3 in *E. coli* Rosetta (DE3). Lane 1: precipitate of the whole cell lysates of Rosetta (DE3) harboring plasmid pET28a-KcCBF3 induced by IPTG. Lane 2: supernatant of the whole cell lysates of Rosetta (DE3) harboring plasmid pET28a-KcCBF3 induced by IPTG. Lane 3: whole cell lysates of Rosetta (DE3) transformed empty plasmid pET28a (control) induced by IPTG. Lane 4: whole cell lysates of Rosetta (DE3) transformed recombinant plasmid pET28a-KcCBF3 induced by IPTG. Lane 5: protein molecular weight marker. (B) Western blot analysis of recombinant protein KcCBF3 with anti-6 × His rabbit polyclonal antibody and HRP-conjugated goat anti-rabbit IgG. Lane 1: whole cell lysates of Rosetta (DE3) transformed empty plasmid pET28a (control) induced by IPTG. Lane 2–3: whole cell lysates of Rosetta (DE3) transformed recombinant plasmid pET28a-KcCBF3 induced by IPTG. The arrow indicated the position of the recombinant protein KcCBF3. (C) Purification of recombinant protein KcCBF3 by Ni^{2+}-NTA resin. Lane1-2: purified recombinant protein KcCBF3. Lane 3: protein molecular weight marker.

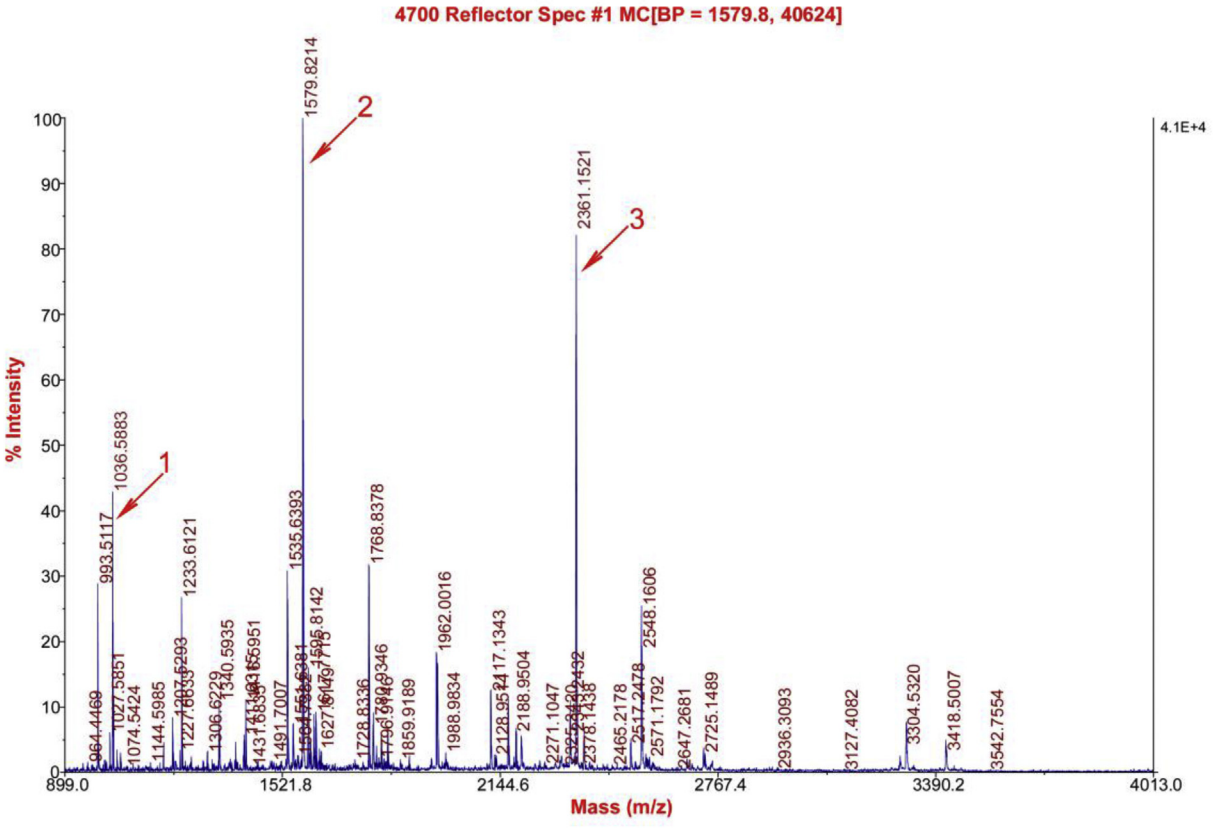

Fig. 6. Identification of the recombinant protein KcCBF3 by MALDI-TOF/TOF analysis. Peak 1 matched the oligopeptide of "IWLGTFLTAEMAAR"; peak 2 matched the oligopeptide of "AHDVAALALR"; peak 3 matched the oligopeptide of "AALEAAEAFRPAGLETVSADESR". The three oligopeptides corresponded to amino acid residue sequences of 102–115, 116–125 and 154–176 of protein KcCBF3, respectively.

peak 2, and peak 3) were acquired after processing with GPS Explorer and Mascot search (Fig. 6). They were IWLGTFLTAEMAAR, AHDVAALALR and AALEAAEAFRPAGLETVSADESR, which corresponding to amino acid residue sequences of 102–115, 116–125 and 154–176 of protein KcCBF3, respectively. MALDI-TOF/TOF analysis verified that the recombinant protein KcCBF3 was successfully expressed in *E. coli* Rosetta (DE3).

3.7. DNA binding activity of the KcCBF3 fusion protein

To determine whether protein KcCBF3 could bind to the *cis*-acting element DRE/CRT in gene *RD29A*, EMSA was performed with purified

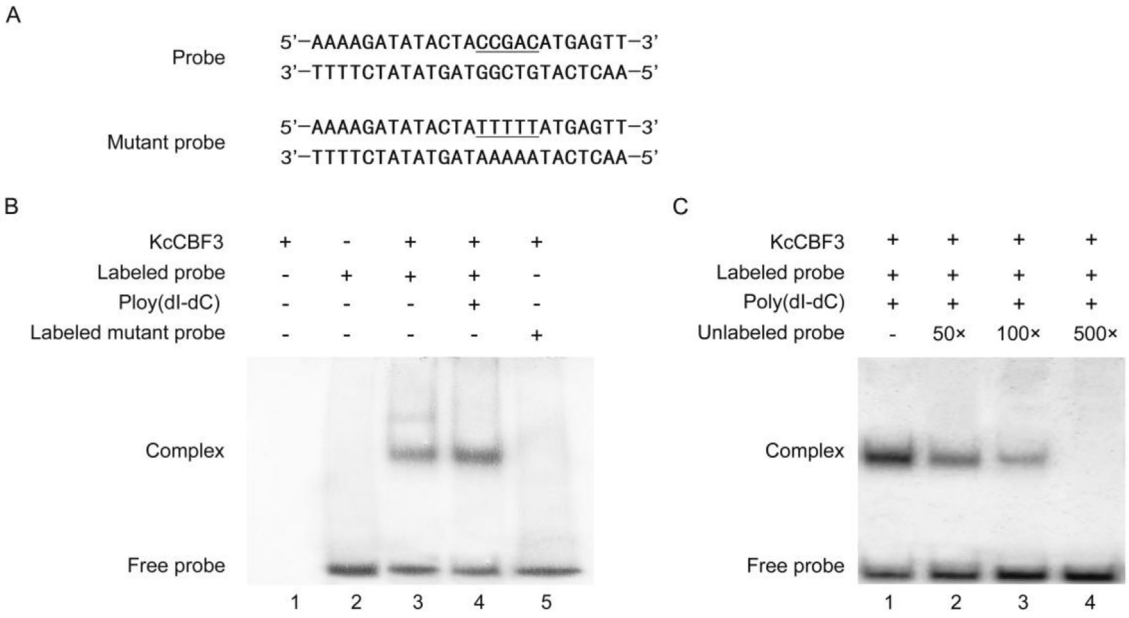

Fig. 7. The binding ability of protein KcCBF3 to the element DRE/CRT of *RD29*A promoter *in vitro*. (A) DRE/CRT probe and its mutant probe (−178 to −154 region of *RD29*A gene in the *Arabidopsis*). (B) EMSA analysis of binding affinity of protein KcCBF3 to DRE/CRT element. Lane 1: only protein KcCBF3; Lane 2: only labeled probe; Lane 3: protein KcCBF3 and labeled probe; Lane 4: protein KcCBF3, labeled probe and nonspecific competitor poly (dI-dC); Lane 5: protein KcCBF3 and labeled mutant probe. (C) Specific binding analysis of protein KcCBF3 to DRE/CRT element performed by EMSA. Protein KcCBF3, labeled probe and poly (dI-dC) were added to each lane. Lane 1: without unlabeled probe; Lane 2–4: amounts of unlabeled probe, which corresponds to 50-, 100- and 500-fold excess of labeled probe, respectively.

recombinant protein KcCBF3 and digo-labeled DRE/CRT probe. The sequences of the labeled probe (core sequence: CCGAC) and labeled mutant probe were shown in Fig. 7A. The EMSA results showed that there was a clear DNA-protein complex retardation in the migration when protein KcCBF3 incubated with labeled probe but not labeled mutant probe, which indicated KcCBF3 could recognize and bind to DRE/CRT element of gene *RD29*A (Fig. 7B). It seemed some impurity protein introduced during Ni^{2+}-NTA purification also was able to bind to labeled probe, however, this DNA-binding complex was abolished when the nonspecific competitor poly (dI-dC) was added (Fig. 7B). Furthermore, the extent of KcCBF3 binding to labeled probe was reduced or abolished with the addition of increasing concentrations of unlabeled probe (Fig. 7C). These results strongly revealed that protein KcCBF3 was capable of binding to the *cis*-acting element DRE/CRT in the promoter of *RD29*A *in vitro*.

4. Discussion

K. candel, a member of the Rhizophoraceae, is the dominant coastal mangrove species in south China. It is capable of adjusting to multiple stress factors, such as flooding, high salt, heavy metals, organic substances, and low-temperature. Therefore, *K. candel* is considered as a model for the study of plant ecological adaptation [26]. However, like many other mangroves, *K. candel* is still vulnerable to low-temperature, showing a high mortality during the attack of the extreme cold in January to February 2008 in southern China [27]. Fei et al. [18] has identified 143 unique cDNAs of *K. candel* subjected to low-temperature stress (5 °C) using suppression subtractive hybridization (SSH), including transcription factor WRKY and Zinc finger family protein. In this study, the ORF of a cold-responsive gene, *KcCBF3*, was cloned and characterized.

Studies on multiple plant species indicate that the signaling pathway regulated by the transcription factor DREB/CBF may be the most important pathway for inducing the improvement of cold tolerance in plants. The CBF protein is a subfamily of AP2/EREBP transcription factor families that contains a conserved AP2/EREBP domain. CBF1 [28], CBF2, CBF3 [29] and CBF4 [30] belong to the DNA-binding protein AP2/ERFBP family and both contain the AP2 motif. Although the amino acid sequence of CBF1 was only 81%, 84%, and 64% homologous to CBF2, CBF3 and CBF4, the sequence similarity of the AP2 motifs was over 91% [30]. The results of this study show that the transcription factor KcCBF3 also contains a highly conserved AP2/EREBP domain, which contains 79 amino acid residues (Fig. 2A). At the same time, there is low homology among the amino acid sequence of KcCBF3 and CBF proteins of other plants as a whole, which is consistent with the previous results [30]. KcCBF3 protein possesses the typical feature of transactivator of AP2/EREBP transcription factor family. The AP2/EREBP domain has a nuclear localization signal peptide at the N terminus and a PKKRAGRKKFRETRHP sequence at the N terminus. In addition, valine (14 V) and glutamic acid (19 E) in the AP2 structure of KcCBF3 protein may determine the binding activity of KcCBF3 to the *cis*-acting element CRT/DRE core sequence [31]. Both Arabidopsis transcription factors CBF1, CBF2 and CBF3 have these two similar short polypeptide sequences (characteristic sequence of CBF protein) [32] and conservative valine (14 V) and glutamic acid (19 E) It can be deduced that *KcCBF3* gene may have similar functions with *AtCBF1*, *AtCBF2* and *AtCBF3* genes and play an important role in cold signal transduction of *K. candel*.

The DREB/CBF subfamily of Arabidopsis genome is subdivided into six subgroups (A-1 to A-6), among which A-1 and A-2 were the two largest subgroups [5]. Most members of A-1 subgroup including DREB1A, DREB1B and DREB1C are mainly induced by low temperature [33]; A-2 subgroup are associated with drought and high salt resistance, such as DREB2A and DREB2B [34]. It can be seen from the phylogenetic tree that the KcCBF3 protein belongs to the A-1 subgroup (Fig. 2C). Semi-quantitative RT-PCR analysis revealed that the transcripts of *KcCBF3* in both roots and leaves of *K. candel* were induced by low temperature stress (Fig. 3B), but not by drought treatment (data not shown). Therefore, it is apparently that *KcCBF3* gene plays a role in cold resistance when it is subjected to low temperature stress. Similarly, the expression of gene *DREB1A*/1 B (A-1 subgroup) from *Arabidopsis* [35] and *OsDREB1A*/1 B (A-1 subgroup) from *O. sativa* [36] could be

induced by low temperature rather than by drought; furthermore, overexpression of *DREB1A* or *DREB1B* promoted the expression of gene *COR* and improved the cold tolerance of transgenic *Arabidopsis* [35]. However, the transcripts of gene *ZmDBP4* (A-1 subgroup) from *Z. mays* were activated by both cold and drought [37]. These inconsistent results implied the functional diversity of DREB1s homologs in A-1 subgroup among different species.

Escherichia coli is widely used for the expression of foreign recombinant proteins due to its relative simplicity, well-known genetic background, short growth cycle and low cost of cultivation. The pET expression system (Novagen) with the advantage of high-efficiency translation and one-step affinity purification, which is one of the most frequently used strategies for the expression of heterologous proteins in *E. coli* for decades [38]. To determine whether there is a DNA binding function of KcCBF3 to *cis*-acting element CRT/DRE, KcCBF3 was expressed in *E. coli* Rosetta (DE3) with expressing plasmid pET28a. The results of western blot and MALDI-TOF/TOF analysis showed that 6 × His-tagged fusion protein was successfully expressed in Rosetta (DE3) (Figs. 5B and 6). The 6 × His-tag is commonly used for its low molecular weight and convenient for protein purification by Ni^{2+}-NTA affinity chromatography, however, some studies demonstrated that the addition of 6 × His-tag to the N or C terminus influenced the structure, properties, and yields of some target proteins expressed in *E. coli* [39,40]. On the other hand, quite a few reports revealed that His-tag had no effect on the enzyme activities and structure of carboxylesterase [41] and chondroitinase [42], even the DNA-binding affinity of BRPF proteins [43].

When it came to studying the DNA-binding ability of transcription factor DREB/CBF to *cis*-acting element using EMSA *in vitro*, His-tag could be coexpressed in either N or C-terminus of the recombinant protein DREB/CBF [27, 48], which indicated that His-tag had null effect on the protein DREB/CBF. In this study, 6 × His-tag in the C-terminus was not removed from the recombinant protein KcCBF3 yet, and the EMSA results demonstrated that KcCBF3 was capable of binding to the *cis*-acting element CRT/DRE core sequence CCGAC rather than the mutant sequence TTTTT (Fig. 7B). In addition, the DNA-binding complex was reduced when a 50-fold or 100-fold excess of unlabeled probe was included as a competitor, or even abolished with the addition of a 500-fold excess of unlabeled probe (Fig. 7C). EMSA results showed that purified protein KcCBF3 was able to bind to the DRE/CRT motif in the promoter of *RD29A in vitro*, which was in accordance with the results reported in *Gossypium hirsutum* [24], *Setaria italica* [44], and *Malus sieversii* [45].

As a core motif in *cis*-acting element CRT/DRE, the conserved sequence CCGAC located in the promoter region of many genes, such as *COR* (cold regulated), *RD* (responsive to dehydration), *LTI* (low-temperature induced) [11], *LEA* (late embryogenesis abundant) as well, which participating in the response to cold, drought, and salinity stresses. Overexpression of *CBF3/DREB1A* in Arabidopsis enhanced the transcript level of *COR15a*, *COR6.6*, and *P5CS* (a gene coding key biosynthetic enzyme for proline), as well as content of proline and soluble sugars, thus the freezing tolerance of transgenic plants were increased significantly [46]. However, whether *KcCBF3* indeed plays the same role in plants remains unclear; more researches should be focused on the functional study of *KcCBFs* and its target genes in *K. candel*, the crosstalk between CBF signaling pathway and other transcriptional regulatory networks as well.

5. Conclusions

In the present study, a A-1 subgroup gene of DREB/CBF subfamily, *KcCBF3*, encoding a C-repeat/dehydration-responsive element binding transcription factors were cloned from *K. candel*. *KcCBF3* was expressed in roots, leaves and stems of 2-month-old plantlets, and could be strongly induced by low temperature (4 °C) in roots and leaves. The results of EMSA indicated that KcCBF3 expressed in *E. coli* Rosetta (DE3) could bind to *cis*-acting element CRT/DRE in gene *RD29A* (core sequence, CCGAC). Overall, this study provided an insight into the molecular mechanisms of adaptation to abiotic stresses in *K. candel*.

Acknowledgments

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