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# Characterization and expression analysis of three CBF/DREB1 transcriptional factor genes from mangrove *Avicennia marina*

Ya-Lan Peng[a], You-Shao Wang[a,b,*], Hao Cheng[a], Cui-Ci Sun[a,b], Peng Wu[a], Li-Ying Wang[a], Jiao Fei[a]

[a] State Key Laboratory of Tropical Oceanography, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou 510301, China
[b] Daya Bay Marine Biology Research Station, Chinese Academy of Sciences, Shenzhen 518121, China

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ABSTRACT

Three *CBF/DREB*1 (C-repeat binding factor/dehydration responsive element-binding factor 1) homologues were isolated from mangrove *Avicennia marina* and designated *AmCBF*1, 2 and 3. Multiple sequence analysis showed that the three deduced proteins all contain an AP2 DNA-binding domain and two CBF signature sequences. According to the phylogenetic analysis, these proteins belong to the A-1 subgroup of the DREB subfamily. Expression analyses based on quantitative real-time PCR revealed that the *AmCBF*2 displayed relatively high expression under normal conditions, with the highest level in stems, while both *AmCBF*1 and 3 were weakly expressed without stress. The three genes also showed different responses to various environmental stimuli. The *AmCBF*2 was inducible by cold, drought, high salinity, heavy metals, as well as abscisic acid (ABA), and exhibited much stronger induction by cold, drought, $Pb^{2+}$ or $Zn^{2+}$ than by NaCl, ABA or $Cd^{2+}$. In contrast, both *AmCBF*1 and *AmCBF*3 displayed insignificant changes under these stimuli. These results indicate that the three *AmCBF* genes play different roles in *A. marina* and the *AmCBF*2 might be involved in the signaling pathway of cold, drought and heavy metal stress response.


## 1. Introduction

Salinity, low temperature, drought and heavy metals are main environmental factors that limit plant growth and development. Recent studies on stress-resistant mechanisms of plants have revealed that transcription factors play essential roles in stress signaling pathways and are involved in the cross-talk among different stresses (Agarwal et al., 2006; Maksymiec, 2007; Qin et al., 2011). Mangroves are ever-green plant communities distributed along the tropical and subtropical coasts of the world, and of great ecological importance and socio-economic significance (Tomlinson, 1994). Mangrove plants constitute the most well adapted halophytes surviving in harsh environmental conditions (e.g., high salinity, anaerobic soil, strong tides and anthropogenic pollution) (Kathiresan and Bingham, 2001). So far, many studies were aimed to reveal the stress-tolerant mechanism of mangroves (Tanaka et al., 2002; Jithesh et al., 2006; Mehta et al., 2009; Huang and Wang, 2010a) and have identified multiple stress-regulated genes, such as the genes encoding metallothionein (*MT*) and dehydrin (*DHN*). However, till now few researches have reported about the transcription factors in mangroves. The signaling/regulatory pathways of stress response in mangrove plants remain unclear.

The CBF/DREB1 (C-repeat binding factors/dehydration response element binding factors 1) proteins are the most studied transcription factors since they play critical role in plant stress response and tolerance (Novillo et al., 2012; Qin et al., 2011). They belong to the superfamily of AP2/EREBP (APETALA2/ethylene response element binding protein) transcription factors. The AP2/EREBP transcription factors are part of gene regulatory networks and play a variety of roles throughout the plant life cycle (Dietz et al., 2010). The CBF/DREB1 proteins can bind specifically to the C-repeat (CRT) elements or dehydration responsive elements (DRE) in the promoters of multiple stress-responsive genes, and thus activate their expression (Yamaguchi-Shinozaki and Shinozaki, 2009). In *Arabidopsis thaliana*, about 12–20% of cold-induced genes can be activated by AtCBF1-3, including several cold-regulated (*COR*) genes (e.g., *COR6.6*, *COR47* and *COR78*), genes encoding the key enzymes for osmolyte biosynthesis (e.g., *P5CS2*), and genes encoding transcription factors (e.g., *RAP2.1* and *RAP2.6*) (Fowler and Thomashow, 2002; Van Buskirk and Thomashow, 2006). Over-expression of *AtCBF* genes in transgenic *Arabidopsis* could induce the expression of multiple stress-responsive genes under normal conditions, and therefore confer freezing, drought, and salt tolerance to the corresponding transgenic plants (Jaglo-Ottosen et al., 1998; Gilmour et al., 2000). Highly conserved in plants, *CBF* homologues have also

* Corresponding author at: State Key Laboratory of Tropical Oceanography, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou 510301, China. Tel.: +86 20 89023102; fax: +86 20 89023102.
*E-mail address:* yswang@scsio.ac.cn (Y.-S. Wang).

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been identified from many other plant species, including herbaceous species such as *Triticeae aestivum* (Skinner et al., 2005), *Oryza sativa* (Dubouzet et al., 2003), and *Atriplex hortensis* (Shen et al., 2003), as well as some woody plant such as *Populus trichocarpa* (Zhuang et al., 2008) and *Eucalyptus gunnii* (Navarro et al., 2009). Despite these *CBF* homologues all exhibit conserved CBF characteristics, including an AP2 domain and two CBF signature sequences (PKKR/PAGRxKFxETRHP and DSAWR), some differences were detected in their expression patterns in response to stresses. For example, the *TaCBF*Ia gene from *T. aestivum* displayed much weaker induction by cold than its paralogous (*TaCBF*IIId, IVc, and IVd) (Badawi et al., 2007). Another work on *E. gunnii* showed that several *EguCBF*1 genes (*EguCBF*1a, b and d) were preferentially induced by cold, whereas another one (the *EguCBF*1c) was more responsive to salt (Navarro et al., 2009). Besides, the *BjDREB1B* gene from *Brassica juncea* was found to be inducible by multiple abiotic stresses (e.g., cold, drought, salt, and heavy metals), as well as some exogenous phytohormones (abscisic acid and salicylic acid) (Cong et al., 2008). These results indicate that different CBF proteins play diverse roles in stress response pathways.

*Avicennia marina* is one of the most widespread mangrove species in the world, and is highly tolerant to salt (Tanaka et al., 2002) and heavy metal exposure (Huang and Wang, 2010a). Till now, none CBF homologues have been identified from *A. marina*. The present work reported the isolation and characterization of three CBF-like genes from *A. marina*. Quantitative real-time PCR (qPCR) method was used to quantify the basal expression levels of the three genes and to determine the expression patterns of these genes in response to several stimuli, including cold, salt, drought, heavy metals and ABA. The results of this study will enable better understanding the regulatory mechanisms of stress response in mangroves.

## 2. Materials and methods

### 2.1. Plant material and stress treatments

Propagules of *A. marina* (collected from Dongchong mangrove wetland, Shenzheng, China) were planted in clean sand and fertilized with 1/2 Hoagland nutritive medium twice a week. Three-month-old plantlets with two pairs of fully expanded leaves (Fig. A1) were grown in controlled environment chamber at 25 °C day/22 °C night, with a long-day (LD) photoperiod (16 h/light = 200 μmol $m^{-2}$ $s^{-1}$).

All the results were obtained from at least two independent experiments on three plantlets. To determine the basal expression levels and tissue specificity of the three genes, different tissue samples (leaf, stem, and root) were randomly harvested on three plantlets and immediately frozen in liquid nitrogen until RNA extraction. The time-course expression patterns of *AmCBF* genes in response to cold were studied at 15 min, 2 h, 12 h, 24 h, 48 h and 120 h, on three plantlets grown at 5 °C in continuous light (100 μmol $m^{-2}$ $s^{-1}$). For high salinity, drought or ABA treatments, the plantlets were irrigated with solutions containing 200 mM NaCl, 12.5% PEG6000 or 100 μM ABA, respectively. Leaves were collected at 2 h, 24 h and 48 h after each treatment. These doses were established according to previous studies (Navarro et al., 2009; Cong et al., 2008). Plantlets irrigated with water were used as control. For the experiment of heavy metal exposure, plantlets were transferred to liquid nutritive medium containing $Pb(NO_3)_2$ (50 μM), $CuSO_4$ (500 μM) or $CdCl_2$ (10 μM) for 12 h, 2 d and 5 d. These doses were established based on previous studies (Deng et al., 2004; Huang and Wang, 2010a). Harvested leaves were dropped immediately into liquid nitrogen and stored at −80 °C until RNA extraction.

### 2.2. Identification of the full length cDNA of AmCBF1, 2 and 3

Three CBF cDNA fragments from *A. marina* were amplified using degenerated primers Dg CBF-F and Dg CBF-R (Table A1), designed based on the identified nucleotide sequence of CBFs from other plant species. The isolation of the full length cDNA sequences was carried out using the SMARTer™ RACE Kit (Clontech, USA). The cDNA pools for 3′ and 5′ RACE were generated on the total RNA extracted from leaves of *A. marina* exposed to cold for 6 h according to the manufacturer's protocol. The first PCR was performed on the cDNA pool using an *AmCBF* gene-specific primer (GSP) and the adaptor primer UPM (Table A1). The second PCR amplification was performed on the products of the first PCR using another *AmCBF* nest gene-specific primer (NGSP) and the adaptor primer NUP (Table A1). Finally, the amplicons were cloned into the pMD-18T vector (Takara, Dalian, China) and sequenced. The high efficiency thermal asymmetric interlaced polymerase chain reaction (hiTAIL-PCR) method (Liu and Chen, 2007) was employed to amplify the 5′ end of *AmCBF*2 because the 5′-RACE PCR was failed to amplify the 5′ end of *AmCBF*2. The pre-amplification PCR was performed on the cDNA pool using a specific primer (AmCBF2-0a) and the adaptor primer LAD1-1/2/3/4 (Table A2), respectively. The primary TAIL-PCR was performed on the products of the pre-amplification PCR using a specific primer (AmCBF2-1a) and the adaptor primer AC1 (Table A2). The secondary TAIL-PCR was performed on the products of the primary TAIL-PCR using a specific primer (AmCBF2-2a) and the adaptor primer AC1 (Table A2). Amplicons were cloned into the pMD-18T vector (Takara, Dalian, China) and sequenced. The full-length cDNA sequences were obtained by combining the 5′ and 3′ end sequences with an overlap fragment using DNAMAN software.

The molecular weight (MW) and theoretical isoelectric point (pI) of deduced proteins were analyzed by Prot Param tool (http://www.expasy.ch/tools/pi_tool.html). Homolog search was conducted by the NCBI BLAST server (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The molecular modeling of each AmCBF AP2/ERF domain was carried out by the SWISS-MODEL (http://swissmodel.expasy.org) using the AtERF1-DNA-binding domain (1gcc) as template. The subcellular localization was predicted using ESLPred (http://www.imtech.res.in/raghava/eslpred/). Multiple sequence alignment was performed using DNAMAN software. The phylogenetic tree was constructed by MEGA 5.1 using neighbor-joining method. The reliability of the branching was tested using bootstrap re-sampling with 1000 pseudo-replicates.

### 2.3. Gene expression analysis by real-time quantitative PCR

Total RNA was extracted from 0.1 g harvested leaves using total RNA extraction kit (Invitrogen, USA). Using PrimeScript® RT reagent Kit (Takara, Dalian, China), cDNAs were produced on 1 μg of each RNA sample according to the manufacturer's instructions. The gene specific primers for qPCR (Table A3) were designed using Oligo 7.0 program (MBI Inc, Cascade, CO). The amplification reactions were performed in a 15 μl of 2× SYBR® Premix Ex Taq™ II (Takara, Dalian, China) mixture, with 200 ng of cDNA and 350 nM of each primer. Three replicates of each PCR were run in iCycler iQ5 real-time PCR detection system (Bio-Rad, CA, USA) using a program as the following: 1 min denaturation at 95 °C, then 45 cycles of 5 s at 95 °C, 15 s at 57/60 °C and 20 s at 72 °C. To assess the specificity of the PCR amplification, melting curve analysis (Fig. A2) was performed at the end of the reaction by 30 min of slow ramp from 65 °C to 95 °C. Two reference genes (18 S rRNA and *Actin*) were used as the internal control for the normalization of the RNA expression level. The relative changes in expression of genes were quantified using $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001) by the qBase$^{plus}$ software (http://www.biogazelle.com). The results of gene expression level are presented as a mean value of the three assay replicates