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Cloning and expression analysis of HSP70 gene from mangrove plant *Kandelia obovata* under cold stress

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Abstract Heat shock protein 70 (HSP70), the primary member of the HSPs that play various stress-protective roles in plants. In this study, a *hsp70* gene of *Kandelia obovata* (*KoHsp70*) was cloned by rapid amplification of cDNA ends (RACE). The full-length of *KoHsp70* was 2255 bp, consisting of a 5′-terminal untranslated region (UTR) of 118 bp, a 3′-terminal UTR of 178 bp, and an open reading frame (ORF) of 1959 bp. The ORF (KoHSP70) was predicted to encode a polypeptide of 652 amino acids with a theoretical molecular weight (MW) of 71.40 kDa and a pI of 5.16. The amino acid sequence analysis revealed that the KoHSP70 contained three conserved regions of HSP70 family, a bipartite nuclear localization signal sequences (NLS), an ATP/GTP-binding site motif and a cytoplasmic characteristic motif (EEVD). Homology analysis indicated that KoHSP70 shared 96.0 % identity with the known HSP70 (*Gossypium hirsutum*). Bioinformatics analysis indicated that the KoHSP70 was hydrophilic and had no signal peptide or transmembrane region. The mRNA expression of *KoHsp70* kept relatively stable at first and then increased significantly after 48 h cold stress, and reached the highest level at 168 h after cold treatment. The results indicated that the *KoHsp70* was a stress-inducible gene that might play a role in cold stress-protective response and in coping with environmental and biological stresses for *K. obovata.* This study provided a basis to further study the mechanism of anti-adverseness and expression characteristics under stress conditions of *K. obovata.*

Keywords Mangrove plant · *Kandelia obovata* · *KoHsp70* · Cold stress · RACE

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Introduction

Heat shock proteins (HSPs), also called heat stress proteins, are the most abundant and ubiquitous soluble intracellular proteins. HSPs were first described in *Drosophila busckii* (Ritossa 1962). These proteins are phylogenetically conserved in all organisms from procaryotes, yeasts and plants to eucaryotes (Sharp et al. 1999, Mosser and Morimoto 2004, Feng and Livi 2010, Al-Whaibi 2011). HSPs are also highly conserved both in structure and function. They can perform biological functions under both the normal and stressful conditions (Kiang and Tsokos 1998, Sorensen et al. 2003). HSPs primarily act as molecular chaperones, and also have been found to assist in the folding of nascent proteins and take part in protein metabolism, cell cycle regulation, apoptosis and other processes under normal conditions (Hightower 1991, Hendrick and Hartl 1993, Srivastava 2002, Robert 2003, Zmijewski et al. 2004). The HSPs' induction is generally regulated at the transcription level (Zhang et al. 2009). The mRNA expression levels of HSPs would significantly increase under unnormal conditions (such as cold and hot temperature, salinity, oxygen radicals, heavy metals, toxins, hunger, trauma, microbial infection and etc.), which might enable organisms to

modulate stress response and protect organisms from the environmental damages (Lindquist and Craig 1988, Gabai et al. 1997, Kiang and Tsokos 1998, Feder and Hofmann 1999, Yenari et al. 1999, Srivastava 2002, Sorensen et al. 2003).

According to the homology and molecular mass, HSPs have been classified into several main families, such as HSP90 (85–90 kDa), HSP70 (68–73 kDa), HSP60 and HSP47 (Al-Whaibi 2011). HSP70 is one of the most conserved and important protein families from eukaryotes to prokaryotes (Lindquist and Craig 1988, James et al. 1997, Feder and Hofmann 1999, Srivastava 2002, Tomanek and Sanford 2003, Fuertes et al. 2004, Franzellitti and Fabbri 2005, Mohanarao et al. 2014). HSP70 has a conserved amino (N)-terminal ATPase domain of approximately 44 kDa (Flaherty et al. 1990) and a carboxyl (C)-terminal peptide binding domain of approximately 25 kDa which is further subdivided into a *b*-sandwich subdomain of 15 kDa and a C-terminal-helical subdomain (Zhu et al. 1996). As molecular chaperones, HSP70s can participate in many important cellular processes, including protein synthesis, translocation, assembly and degradation (Sharma and Masison 2009, Pratt and Toft 2003). HSP70s were involved in stress protection by improving cell survival and raising the tolerance to environmental stress, such as heat, cold, heavy metal, water deficit, oxidative stress, wounding etc. (Heikkila et al. 1984, Dhankher et al. 1997, Chong et al. 1998, Uenishi et al. 2006, Agarwal et al. 2010). The gene expression of HSP70 has been reported in many different species and also was recognized to play an important role in regulating physiological and ecological regulation under changing environments (Hamdoun et al. 2003, Piano et al. 2005, Park et al. 2007). It was reported that HSP70 was an essential component in the INF1-mediated hypersensitive response, and could regulate upstream of the MAPK cascade in plants (Boutet et al. 2003). Although the *Hsp70* gene could be induced under cold or heat stress treatment (Li et al. 2013, Guo et al. 2014), there is no information about *Hsp70* from mangrove plants, such as *K. obovata.*

In this paper, a full-length cDNA of heat shock protein 70 gene (*KoHsp70*) from *K. obovata* was the first time cloned and analyzed. The expression patterns of *KoHsp70* in *K. obovata* under cold stress were also discussed.

Materials and methods

Plant material and cold treatment

K. obovata, which was collected from Shenzhen, China, was used throughout the study. Seeds were sown and germinated in clean sands. After incubation, the seedlings were kept in a controlled condition with 75 % humidity and under a 14 h light/10 h dark cycle at 25 °C for 3 months. Subsequently, the seedlings with two pairs of fully expanded leaves were transferred to a growth chamber at 5 °C, and leaves of seedlings were sampled at 1, 3, 6, 9, 12, 24, 48, 96 and 168 h. The leaves of seedlings collected at 25 °C were used as the control. Each leaf of samples was washed with distilled water. The harvested leaves were quickly frozen in liquid nitrogen and stored at −80 °C prior to extraction of total RNA.

Total RNA extraction and reverse transcription

Total RNA was extracted using the TIANGEN RNA plant Plus Reagent (TIANGEN BIOTECH (BEIJING), Cat. No. DP121221) as previously described (Song and Wang 2011). The integrity and purity of total RNA were evaluated with Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and 1.0 % agarose gel. The genomic DNA of total RNA was removed by DNaseI. First strand cDNA was synthesized using SMART^{TM} reverse transcription Kit (Clontech) according to the manufacturer's instruction.

Cloning of the 5′ and 3′ ends of cDNA

The 5′ and 3′ ends cDNA of *KoHsp70* were obtained with a forward primer (5′-CTTGGTGGGGATTGTTGTATTCC TCGGG-3′) and reverse primer (5′-GGCTGGAAACTGC TGGTGGTGTTAT-3′) by using the SMART^{TM} RACE cDNA Amplification Kit (Clontech), respectively. The primers were designed by Oligo 7 software according to the EST of *KoHsp70* (a Ko1012 sequence was annotated as putative *Hsp70* from our team, GenBank accession No. JZ585624). Touchdown polymerase chain reaction (PCR) was used for *KoHsp70* amplification. Each PCR reaction was performed in a PTC-200 thermo cycler (Bio-Rad, USA) with the following Program: 5 cycles of 95 °C for 30 s, 72 °C for 3 min; 5 cycles of 95 °C for 30 s, 70 °C for 15 s, 72 °C for 3 min; 27 cycles of 95 °C for 30 s, 68 °C for 15 s, 72 °C for 3 min; followed by 72 °C for 10 min. The PCR products were gel-purified and were cloned into T-Vector pMD20 (Takara, Japan). Five positive clones were sequenced by Life Technologies (Invitrogen, Carlsbad, CA, USA).

Sequence analysis

The full-length cDNA sequence was obtained by linking sequences through overlap fragments using DNAMAN software. The deduced amino acid sequence was analyzed with ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The molecular weight (MW) and theoretical isoelectric point (pI) of deduced protein were analyzed by

Compute pI/MW tool (http://web.expasy.org/compute_pi/). The motif sequences were searched using Motif Scan (http://myhits.isb-sib.ch/cgi-bin/motif_scan). The subcellular localization was predicted using PSORT (http://www.psort.org/). SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/) was used for prediction of the signal peptide. TMpred was used (http://www.ch.embnet.org/software/TMPRED_form.html) for transmembrane analysis. The similarity analysis of nucleotide and protein sequence was carried out by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple alignments of the HSPs were performed with the ClustalX software. A phylogenetic tree of amino acid sequences was computed by using MEGA 5.0 software according to the neighbor-joining method. 1000 bootstrap replicates were performed for the phylogenetic analysis. The molecular modeling of KoHSP70 was carried out by the SWISS-MODEL (http://swissmodel.expasy.org/).

qRT-PCR analysis

The quantitative real-time PCR (qRT-PCR) was performed on iCycler iQ5 real time PCR detection system (Bio-Rad, CA, USA). Oligo 7 Software was used to design specific primers for *KoHsp70*. The *rcbl* of *K. obovata* was used as an internal control. Each qRT-PCR reaction was performed with SYBR® Premix Ex TaqTM II (Takara). The reactions were subjected to 95 °C for 1 min, 40 cycles at 95 °C for 10 s, 55 °C for 30 s, and 72 °C for 40 s. The primers of *rcbl*: forward primer, 5′-ATAAAGCACAGGCGGAAAC-3′; reverse primer, 5′-CGACAATAATGAGCCAAGC-3′. The primers of *KoHsp70*: forward primer, 5′-ATCCTGA TGAGGCTGTTGC-3′; reverse primer, 5′-CTTGTTCTT TCTTGGTGGG-3′. Each reaction was done in triplicate and three non-template controls were included. Statistical analysis of gene relative expression level was calculated with the $2^{-\triangle\triangle CT}$ method (Pfaffl 2001) with rcbl gene for normalization.

Statistical analysis was performed with GraphPad Prism 5 (GraphPad Software). The statistical significances were determined with a one-way ANOVA followed by Duncan's test, and a Student's test. Significance was defined as $P < 0.05$.

Results

Isolation of the full-length cDNA of KoHsp70

Total RNA samples, which were isolated from *K. obovata* leaves at 1, 3, 6, 9, 12, 24, 48, 96 and 168 h under 5 °C, were equally mixed and used to synthesize first strand cDNA. Two cDNA fragments of 1175 and 794 bp were obtained by 5′ RACE and 3′ RACE, respectively, and sequenced. A fragment of 286 bp was the EST (GenBank accession No. AAG23797) using for RACE. DNAMAN software was used to combine the aboved three fragments into a 2255 bp consensus sequence through overlap fragments. BLASTx analysis showed that the 2255 bp sequence shared significant identity (96 %) to the HSP70 of *Gossypium hirsutum* (GenBank accession No. ACJ11745.1). The encoding gene was named for *KoHsp70* (GenBank accession No. KM878580).

Analysis of nucleotide and deduced amino acid sequences of KoHSP70

The full-length cDNA of *KoHsp70* was 2255 bp, containing a 5′-terminal untranslated region (UTR) of 118 bp, a 3′-terminal UTR of 178 bp, and an open reading frame (ORF) of 1959 bp. The ORF was predicted to encode a protein of 652 amino acids (Fig. 1) with a calculated MW of 71.40 kDa and pI of 5.16. EXPASy Molecular Biology Server was used to analyze the basic physical and chemical characters of the deduced protein KoHSP70. KoHSP70 included 83 positively charged residues (Arg and Lys) and 100 negatively charged residues (Asp and Glu), with a net negative charge. The Gly (8.7 %), Ala (8.4 %), Lys (8.0 %), Asp (7.8 %), and Glu (7.5 %) contents were high while that of Trp was low (0.5 %).

Bioinformatics analysis

The secondary structure of KoHSP70 was predicted by PSIpred, and the results showed that KoHSP70 included 44.33 % of α-helix, 18.40 % of β-sheet (extended strand), 6.44 % of β-turn and 30.83 % of random coil. Amino acid sequence analysis indicated that there were three conserved signatures of the HSP70 family (signature 1, IDLGTTYS, residues 12–19; signature 2, IFDLGGGTFDVSIL, residues 203–216; signature 3, IVLVGGSTRIPKME, residues 340–354) and a cytoplasmic characteristic motif EEVD (residues 649–652) (Fig. 1). A putative ATP/GTP-binding site motif A (P-loop) AEAYLGKT (residues 135–142) and a putative bipartite nuclear localization signal (NLS) KRKHKKDISDNKRAVRRL (residues 252–269) were also observed in KoHSP70 (Fig. 1). Interpro program showed that KoHSP70 included a substrate peptide binding domain from residues 392–549, and a C-terminus domain from residues 525–628. The conservation of the N-terminus was higher than the C-terminus in the whole amino acid sequence of KoHSP70. About 90 amino acids were poorly conserved in the C-terminal regions. However, the EEVD motif (the last four amino acids) was highly conserved in the HSP70 family of plants. The signal peptide sequence analysis indicated that there was no signal

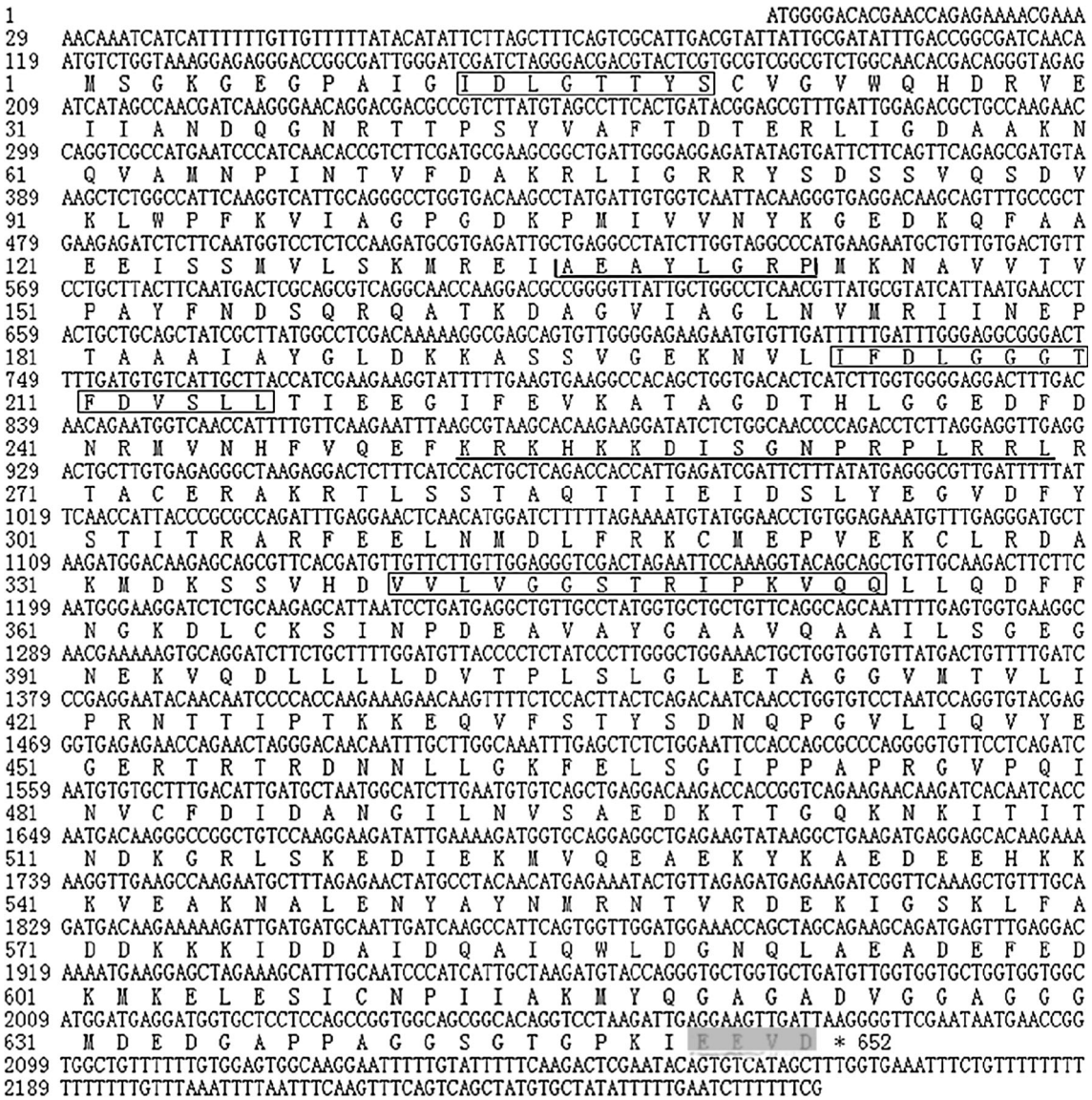
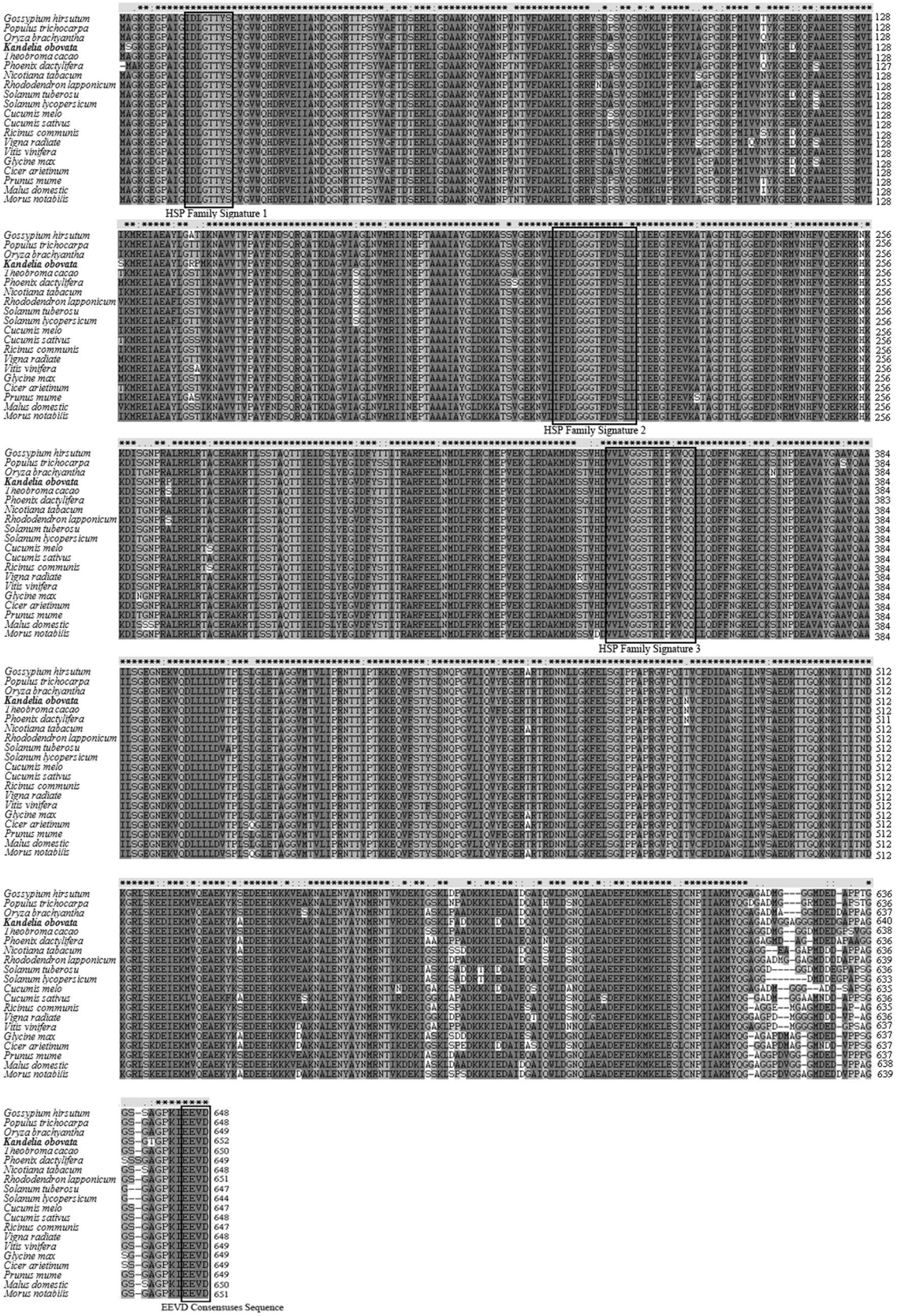


Fig. 1 The nucleotide sequence of *KoHsp70* and its deduced amino acid sequence. The nucleotides and amino acids are numbered along the *left margin*. An *asterisk* (*) below the last three nucleotides indicates a stop codon. Three classical HSP signature motifs are marked with *closed box*. ATP/GTP-Binding Site Motif A (p-loop) is marked with *opened box*; Bipartite Nuclear Targeting are shown with *underline*; Consensus sequence EEVD motif at the C-terminus is marked with *gray box*; This nucleotide and deduced amino acid sequence data of Ko-HSP70 was registered in the GenBank (No. KM878580)

peptide in KoHSP70. Transmembrane analysis showed that there was no apparent transmembrane region in KoHSP70. In addition, ESLpred program allowed the prediction of a chloroplast protein for KoHSP70.

BLAST program analysis showed that the amino acid sequence of KoHSP70 shared high similarity (96 % identity) with the other known HSP70s. Although the sequences of N and C terminal were different among KoHSP70 and other HSP70s, the sequence alignment revealed strong amino acid sequence conservation in KoHSP70 (Fig. 2). A neighbor-joining phylogenetic tree was constructed by MEGA 5.0 software (Fig. 3). The results indicated that

Fig. 2 Alignment of HSP70 protein sequences of *K. obovata* and other ▶ plants. The listed species names and their corresponding GenBank accession numbers are as follows: *Kandelia obovata*, KM878580; *Gossypium hirsutum*, ACJ11745.1; *Cucumis melo*, XP_008447699.1; *Vitis vinifera*, XP_002263599.1; *Theobroma cacao*, XP_007016460.1; *Nicotiana tabacum*, AAR17080.1; *Ricinus communis*, XP_002527736.1; *Populus trichocarpa*, XP_002311161.1; *Cucumis sativus*, XP_004142749.1; *Morus notabilis*, EXB58128.1; *Glycine max*, XP_003552691.1; *Solanum tuberosu*, XP_006349514.1; *Prunus mume*, XP_008233611.1; *Rhododendron lapponicum*, ADD71975.1; *Malus domestic*, XP_008371627.1; *Oryza brachyantha*, XP_006663128.1; *Solanum lycopersicum*, XP_004249574.1; *Phoenix dactylifera*, XP_008776656.1; *Cicer arietinum*, XP_004492118.1; *Vigna radiate*, AAS57913.1. HSP70 family signature sequences and the consensus sequence EEVD at the C-terminal are shadowed. The amino acids are numbered along the right margin. Asterisks (*) indicated the same amino acid sites among all sequences in the alignment


HSP Family Signature 1
HSP Family Signature 2
HSP Family Signature 3
EEVD Consensuses Sequence

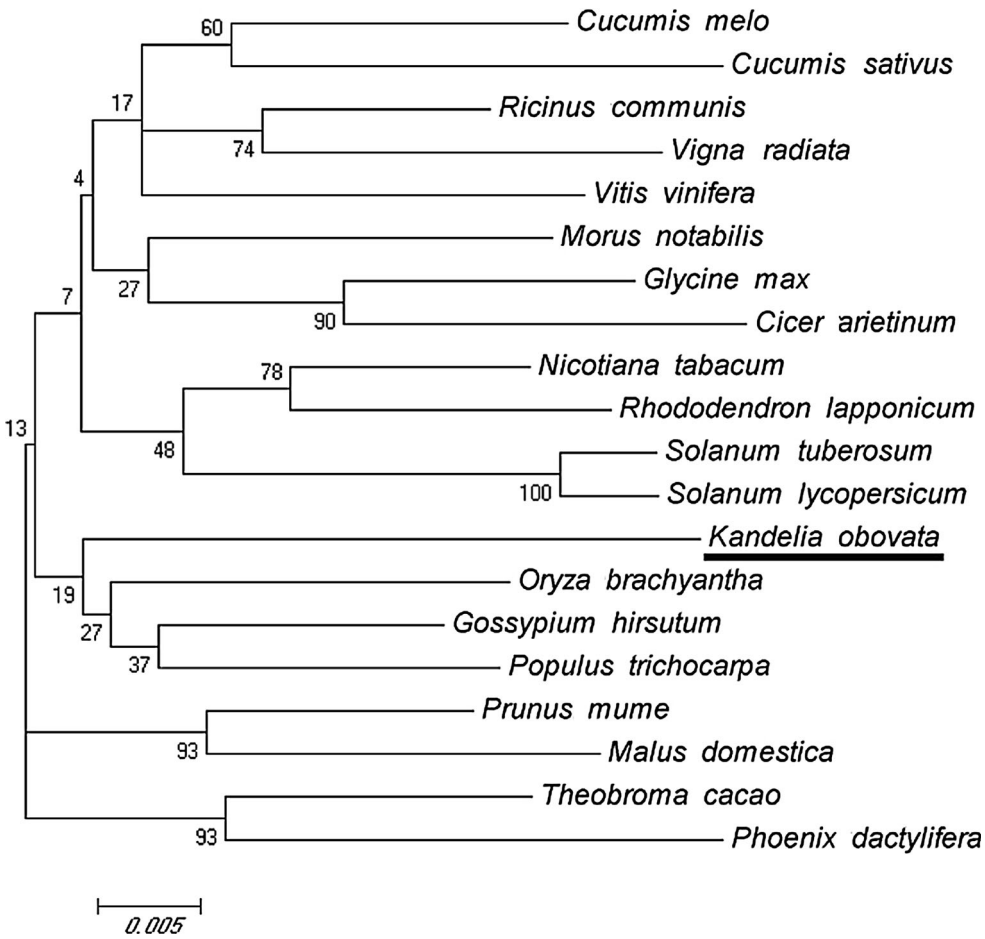


Fig. 3 Phylogenetic tree for KoHSP70 of *K. obovata.* The tree was constructed by MEGA 5 software using the neighbour-joining method. The *number at each node* indicates the percentage of bootstrapping after 1000 replications. The species names and the GenBank accession numbers are same as in Fig. 1

KoHSP70 represented different taxonomic status. KoHSP70 was more closely related to HSP70s of *Gossypium hirsutum*, *Populus trichocarpa* and *Oryza brachyantha*, forming a larger branch with other HSP70s. The function of any protein is determined by its formation and folding into three dimensional structure (Levitt et al. 1997). Molecular model of KoHSP70 was performed by SWISS MODEL as shown in Fig. 4. Formation of three dimensional structure requires 50 % of principle amino acids sequence (Dobson et al. 1998). The sequence identity is 81.49 % between KoHSP70 and the template (heat shock cognate 70 kDa protein, SMTL id: 4fl9.1.A). This confirmed that the 3D-model of KoHSP70 is reasonable and receivable.

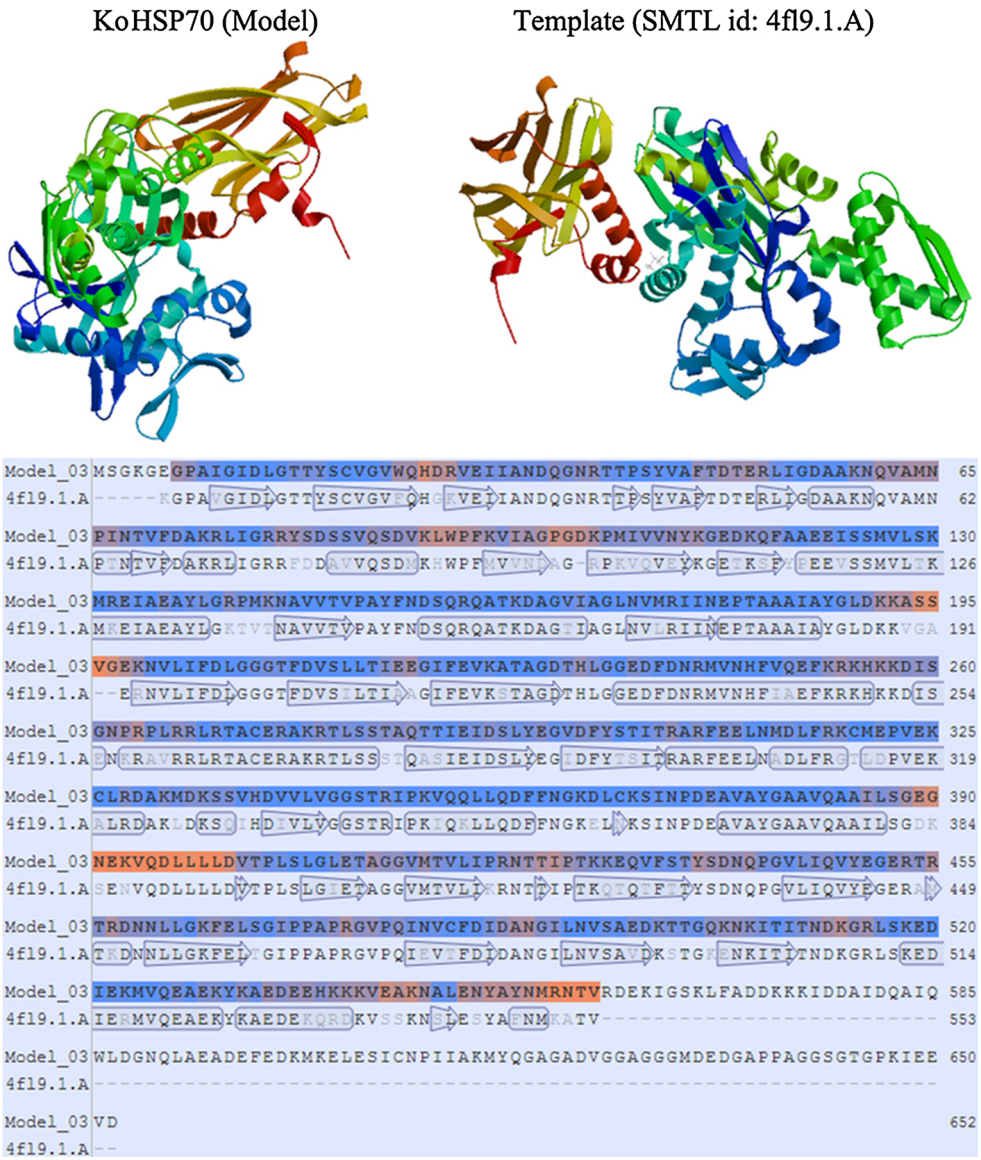
The mRNA expression analysis of KoHsp70 under cold stress

In order to determine whether *KoHsp70* is expressed in response to stress, it was determined for the level of *KoHsp70* mRNA at 1, 3, 6, 9, 12, 24, 48, 96, 168 h at 5 °C, and the control temperature was 25 °C. As shown in Fig. 5, the *KoHsp70* was expressed at different times under cold treatment. The cold induction was not distinct at first, while the accumulation of *KoHsp70* significantly increased after 48 h (about 6.90 fold) comparing with the control. And the maximum level (about 46.75 fold) of transcripts occurred at 168 h under cold treatment. The mRNA expression was clearly delayed. *KoHsp70* may play a role in later period under cold stress.

Discussions

The existence of the heat shock genes (especially the *hsp70* gene) involved in cold shock response has recently been documented in several organisms, including maize, Arabidopsis, pea etc. (Heikkila et al. 1984, Dhankher et al. 1997, Chong et al. 1998, Uenishi et al. 2006, Agarwal et al. 2010). At present, none of heat shock gene of *K. obovata* has been found.

In this experiment, the full-length cDNA sequence of *KoHsp70* from *K. obovata* was the first time cloned by using RACE technology. According to the structural and phylogenetic features and the high identity compared with the known HSP70s of plants, KoHSP70 can be suggested



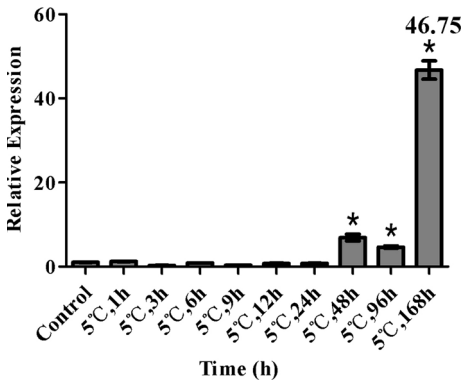


Fig. 5 Relative expression level of *KoHsp70* gene of *K. obovata* exposed to 5 °C cold stress by fluorescent real-time PCR. *Asterisks* above the *bars* represent significant differences ($P < 0.05$) of comparisons with the control

as a member of the HSP70 family. Since highly conserved sequences of HSP70 indicated similar functions and analogous protection of cells during or after stress (Kampinga and Craig 2010), KoHSP70 may have the similar function of HSP70s. As molecular chaperones, HSP70s function in the folding and refolding of nonnative proteins to prevent irreversible misfolding and aggregation (Wang et al. 2004, Mayer and Bukau 2005). HSP70s also play roles in protein transport and assembly processes (Marshall et al. 1990, Bush and Meyer 1996). HSP70 could interact with co-chaperones through the N-terminal ATPase domain and substrate at the C-terminal substrate-binding domain (Laufen et al. 1999). A highly conserved N-terminal ATPase domain and a C-terminal substrate binding region were included in KoHSP70. KoHSP70 perform probably the similar molecular chaperone functions by the conserved

ATPase domains under cold stress. HSP70s are also involved in interaction with signal transduction proteins (Pratt and Toft 2003), and this is not necessarily related to function as a chaperone (Gabai et al. 1997). The HSPs could be induced under low temperature (Swindell et al. 2007) and have an adaptation to tolerate under cold stress (Wang et al. 2014). KoHSP70 was increased on mRNA expression level in the later period under cold stress. This may enhance the adaptation to cold stress of *K. obovata*. The molecular model of KoHSP70 was performed by SWISS MODEL according to the template (heat shock cognate 70 kDa protein, HSC70). A highly conserved and diagnostic motif was existed in both HSP70s and HSC70 (Duan et al. 2011). This result was also consistented with study in spinach (Guy and Li 1998). It is also suggested that KoHSP70 may have similar function with HSC70.

The members of HSP70s from eukaryotes are located in major subcellular compartments, including the endoplasmic reticulum, mitochondria, cytoplasm and chloroplast. The various subcellular localizations imply both functional distinction and phylogenetic divergence (Zhang et al. 2009). KoHSP70 was predicted to locate in chloroplast. The highly conserved EEVD of HSP70s was reported as a predictive localization motif for cytosolic HSP70s (Al-Whaibi 2011). This may be of interest to determine its expression pattern in photosynthetic and nonphotosynthetic tissues (Barua et al. 2008, Horvath et al. 2012).

The mRNA expression level of the *KoHsp70* gene remained constant level at first and then significantly increased after 48 h, reaching the highest level at 168 h. The results indicated that the mRNA expression of *KoHsp70* was delayed under cold treatment. *KoHsp70* will be to play a stress-associated role in the later period under cold stress. There were similar responses under cold shock that observed in other organisms (Li et al. 2013, Nam et al. 2013, Guo et al. 2014, Jensen et al. 2014, Mohanarao et al. 2014).

In summary, *KoHSP70* was the first time cloned from *K. obovata* seedlings. The mRNA expression of *KoHsp70* was delayed under cold treatment, and the *KoHsp70* might play a role in cold stress-protective response and in coping with environmental and biological stresses for *K. obovata*. This research will give some information for anti-adverse mechanism and improvement of stress-tolerance of *K. obovata* in the future.

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Conflict of interest The authors declare that they have no conflict of interest.

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