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Short communication

Molecular cloning and characterization of acyl-CoA binding protein (ACBP) gene from shrimp *Penaeus monodon* exposed to salinity stress

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ABSTRACT

Acyl-CoA binding protein (ACBP), a protein present ubiquitously in wide range of organisms play significant role in transport of acyl groups for macromolecular biosynthesis involved in various functional and regulatory processes. In crustaceans, ACBP has functional role in growth, reproduction and temperature tolerance. In the present study, two suppression subtractive hybridization (SSH) cDNA libraries were performed using gut tissues of shrimp *Penaeus monodon* exposed to low (3ppt) and high (55ppt) salinity stress conditions. SSH library resulted in identification of differentially expressed genes that belonged to various functional classes such as the nucleic acid regulation and replication, defence proteins, allergen protein, signal transduction pathways, apoptosis, energy and metabolism, cell cycle regulation and hypothetical proteins. ACBP was identified as one of the differentially expressed gene in both the SSH libraries of shrimp *P. monodon* subjected to low and high salinity stress. The full-length cDNA of *P. monodon* ACBP gene was isolated and the sequence revealed 273 bp open reading frame encoding 90 amino acids with molecular mass of 10 kDa and pI 6.8. The ORF showed presence of four phosphorylation sites, with absence of signal peptide sequence and glycosylation sites. The deduced amino acid sequence of ACBP exhibited high sequence identity (92%) with ACBP class of protein identified from *Fenneropenaeus chinensis*. Real time PCR analysis of shrimps subjected to 3ppt salinity conditions after 2 weeks revealed an increase in expression of ACBP transcripts, in the gut (28.08-folds), gills (11.71-folds) and in the muscle tissues (1.70-folds). Whereas, shrimps exposed to 55ppt salinity conditions after 2 weeks exhibited increased ACBP transcript levels in the gut (11.95-folds), gills (1.052-folds) and muscle tissues (7.35-folds). The significant increase in expression levels of ACBP in various tissues of shrimps suggests a functional role of this gene in salinity stress tolerance and adaptation.

1. Introduction

Acyl-CoA binding protein (ACBP) has been identified as a highly conserved 10 kDa cytosolic protein. The protein is known to function as a neuropeptide involved in inhibiting of diazepam (DBI)/endozepine (EP) binding to GABA receptor system (Guidotti et al., 1983; Kragelund et al., 1999). ACBP belongs to a multigene family of protein that is conserved from yeast to mammals (Elholm et al., 2000). ACBP is involved in multiple functions such as modulation of fatty acid biosynthesis, enzyme-regulation, regulation of the intracellular acyl-CoA pool, donation of acyl-CoA esters for β-oxidation, vesicular trafficking and gene regulation (Burton et al., 2005). ACBP is suggested to be involved in the transport of newly synthesized acyl-CoA esters from the fatty acid synthetase to acyl-CoA-consuming processes in yeast (Schjerling et al., 1996), as acyl-CoA transporter (Elholm et al., 2000) and in m-calpain regulation process (Melloni et al., 2000) in rats. Different types of ACBPs are reported to be encoded by set of genes in plants *Arabidopsis thaliana* (Xiao and Chye, 2009) such as the membrane-associated proteins ACBP1 and ACBP2 (Chye, 1998; Chye et al., 1999, 2000; Li and Chye, 2003), the extracellularly targeted ACBP3 (Leung et al., 2006) and the cytosolic proteins ACBP4, ACBP5 and ACBP6 (Chen et al., 2008; Xiao et al., 2008). *Arabidopsis* over expressing ACBP2 showed improved tolerance to heavy metal (Cd) stress suggesting the role of ACBP2 in membrane repair (Gao et al., 2008; Gao et al., 2010). Transgenic *A. thaliana* with high expression of ACBP6 along with decrease in phosphatidyl choline and accumulation of phosphatic acid displayed an enhanced freezing tolerance (Chye et al., 1999) and ACBP1 in transgenic *A. thaliana* has been reported to be specifically playing a role in freeze stress (Du et al., 2010). *Saccharomyces cerevisiae* with depleted levels of ACBP resulted in severe distortions in membrane assembly, organization and trafficking suggesting its function in maintenance of cellular integrity (Gaigg et al., 2001).

In invertebrates such as shrimp *Fenneropenaeus chinensis*, studies have shown that ACBP might possess antibacterial activity against *Vibrio anguillarum* (Ren et al., 2009). ACBP was identified

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as differentially expressed gene from white spot syndrome virus resistant shrimp *Litopenaeus vannamei* suggesting its role in shrimp immune response against viral infections (Zhao et al., 2007).

However, the functional role of ACBP in shrimp in response to biotic and abiotic stress remains poorly understood. In the present study, as an approach to identify genes that play critical role in salinity tolerance, suppression subtractive hybridization (SSH) libraries were constructed from *Penaeus monodon* gut tissues. In the present study ACBP has been identified as one of the differentially expressed gene in shrimp subjected to salinity stress. The gene has been cloned and characterized for the first time from shrimp *P. monodon* and the transcription profiles in various tissues during low (3ppt) and high (55ppt) salinity induced stress has been analyzed and its relevance towards salinity stress is discussed.

2. Materials and methods

2.1. Collection of shrimps, salinity conditions and tissue samples

P. monodon shrimps (10–15 g) were procured from the shrimp farms located in Chennai, India. The shrimps (intermolt stage) used in the experiment were divided into three groups of six numbers each and were acclimatized to three different salinity conditions. Low salinity levels (3ppt) were achieved by reducing the salinity of sea-water by 2ppt per day by adding fresh water. High salinity levels (55ppt) were achieved by increasing the salinity by 2ppt per day using brine. The third group of shrimps were maintained in normal sea-water (28ppt) as control group, without altering the salinity conditions. The gills, gut and muscle tissue samples from six shrimps maintained for a period of 2 weeks at low (3ppt), high (55ppt) and 28ppt salinity conditions were collected in RNA*later* (Qiagen, USA) and stored in −80 °C till further use.

2.2. Construction of SSH cDNA library

Two SSH cDNA libraries were constructed separately using gut tissues of the 3ppt and 55ppt salinity induced shrimp samples. Pooled gut tissues (six numbers in each group) collected from the experimental (3ppt or 55ppt) and control shrimp served as tester and driver respectively. The SSH cDNA libraries were constructed following the procedure described in PCR-Select cDNA subtraction kit (Clontech, USA) and as reported previously (Rajesh et al., 2012).

2.3. Screening of SSH cDNA library

The PCR products obtained by amplification of SSH clones were ligated into pGEM-T Easy vector (Promega, USA) and transformed into competent *Escherichia coli* DH5α cells. The recombinant clones were screened by colony PCR for the insert cDNAs with the vector primers (T7 forward and SP6) and sequenced (Scigenom technologies, India). The sequences were assembled using KEGG (Kyoto Encyclopedia of Genes and Genomes) software (www.genome.jp/kegg) and were grouped into contigs and singletons. Sequence analysis by BLASTX, BLASTN or TBLASTX (www.ncbi.nlm.nih.gov/blast) was used to identify differentially expressed genes in cDNA libraries constructed from low and high salinity shrimp groups.

2.4. Amplification and cloning of P. monodon ACBP gene

The ACBP gene amplification of *P. monodon* was carried out using gene specific forward and reverse primers (ACBP-F and ACBP-R) that were designed based on the reported *F. chinensis* ACBP gene (Ren et al., 2009) (Supplementary data, Table 1). The PCR reaction conditions included initial denaturation at 94 °C for 3 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min and final extension at 72 °C for 7 min. The cloned ACBP gene was confirmed by sequencing.

2.5. Rapid Amplification of cDNA ends (RACE) of P. monodon ACBP

Total RNA was extracted and mRNA was purified using NucleoSpin RNA II kit and NucleoTrap mRNA mini kit respectively from gut tissues of shrimps following the manufacturer's instructions (Macherey Nagel, Germany). 5′ and 3′ RACE were performed using FirstChoice RLM – RACE kit following manufacturer's instructions (Ambion, USA). The sequences of primers used in RACE are shown in Supplementary data, Table 1.

2.6. Sequence analysis of P. monodon ACBP gene

Nucleotide sequence analysis of *P. monodon* ACBP was performed by BLASTN (http://www.ncbi.nlm.nih.gov/blast) (Altschul et al., 1997) and the sequence was submitted in GenBank database. ACBP sequence was translated using the TRANSLATE tool in Expasy proteomics server (www.expasy.org). The multiple sequence alignment of ACBP protein was performed by CLUSTALW method (Higgins et al., 1992) in Expasy proteomics server (http://www.ebi.ac.uk/tools/msa/clustalw2/). Phylogenetic tree was constructed (PAM250) using MEGA 5 software (Tamura et al., 2011) with the Neighbour-Joining method and bootstrap analysis was performed for 1000 replicates. Protein sequence of *P. monodon* ACBP was analyzed for phosphorylation sites (www.cbs.dtu.dk/services/NetPhos), potential glycosylation sites (http://www.cbs.dtu.dk/services) and signal peptide cleavage site (http://www.cbs.dtu.dk/services/SignalP). The tertiary structure of *P. monodon* ACBP was developed with the Geno3D software tool (http://geno3d-pbil.ibcp.fr) using armadillo ACBP (2FDQ) as template.

2.7. P. monodon ACBP mRNA expression analysis by Real-time PCR

Total RNA extracted using NucleoSpin RNA II kit (Macherey–Nagel, Germany) from gills, gut and muscle tissues of the control, low and high salinity stressed shrimps were converted to cDNA using Protoscript M-MuLV first strand cDNA synthesis kit (New England Biolabs, USA). The cDNA was used to analyze the relative expression of ACBP transcripts by real-time PCR using the Power SYBR green PCR master mix (Applied Biosystems, UK). The gene specific primers for ACBP (ACBP RT-F and ACBP RT-R) were used to generate 89 bp PCR products for real time analysis. The shrimp β-actin gene was amplified with primers (β-actin RT-F and β-actin RT-R) to generate 124 bp product which was used as an endogenous control (Pongsomboon et al., 2009) (Supplementary data, Table 1). The relative quantification results were expressed as the fold change in levels of the gene expression and statistical analysis of the data for comparison between groups was carried out by one-way ANOVA and the values with $p < 0.05$ were considered significant.

3. Results and discussion

The SSH cDNA library generated multiple colonies, representing genes up-regulated in gut tissues of low and high salinity stressed *P. monodon* shrimps. An approximate of 500 clones was obtained from low and high salinity stressed shrimps with the insert size ranging from 200 to 1.5 kb. Clustering of ESTs using KEGG software generated 19 contigs and 32 singletons from low salinity SSH cDNA library; 10 contigs and 39 singletons from high salinity group respectively. SSH clones were grouped into different classes based on the predicted functional category by BLAST analysis (Table 1) (Supplementary data, Fig. 1A and B).