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Full Length cDNA Cloning and Expression Analysis of Calmodulin Gene from Deer Antler Tissue

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Abstract.- Calmodulin (*CaM*) is an important signal transduction molecules in organisms, it plays important regulatory roles in various cellular physiological activities, especially the regulation in cell proliferation. In the present study, full length cDNA of *CaM* gene was isolated from full-length cDNA library of velvet antler tip tissue of sika deer (*Cervus nippon hortulorum*); the amino acid sequence and gene expression was analyzed by using bioinformatics method and real-time RT-PCR technique. Nucleotide sequence analysis reveals that the full-length cDNA of *CaM* gene comprised of 703 bp, containing 462 bp in the open reading frame (ORF), its relative molecular weight was 16.9 kDa, isoelectric point was 4.09. Sequence analysis indicates that the protein, like most animals and plants *CaM*, includes two basic "EF-hand" structures. Expression analysis by real-time quantitative RT-PCR reveals that *CaM* gene has a higher expression level in antler skin layer than in reserve mesenchyme layer, precartilage layer and cartilage layer, indicating that this gene may play a regulatory role in rapid growth of antler skin.

Key words: Antler, calmodulin (*CaM*) gene, cDNA library, real-time quantitative RT-PCR, cDNA cloning.

INTRODUCTION

Antlers are male secondary sexual characteristic and used for female attraction and fighting (Brockes and Kumar, 2002; Molnár *et al*., 2007). The annual regeneration cycle of deer antlers represents a unique model of epimorphic regeneration in adult mammals (Han *et al*., 2005; Lord *et al*., 2007; Kierdorf and Kierdorf, 2011).

Growing antlers are enveloped in a hair-covered skin that presents several peculiarities, including lack of sweat glands and arrector pili muscles and the presence of abundant sebaceous glands (Li and Suttie, 2000). Antlers maximal elongation rate can be around 2 cm per day (Brockes and Kumar, 2005; Stéger *et al*., 2010). This extremely rapid growth localizes at the tip of the velvet antlers, and includes multiple tissues: skin, mesenchyme, cartilage, bone, blood vessels and nerves (Price and Allen, 2004).

The development of antlers is a modified endochondral ossification process (Rucklidge *et al*., 1997), which is co-regulated by various biomolecules, including IGF-I, FGFs, TGF-β etc. (Li *et al*., 1995; Mescher, 1996; Odelberg, 2005). Antlers differentiation showed a sequential development from the tip to the base. The growing antlers become hardened gradually by progressive mineralization and occlusion of blood vessels**.** At the end of the summer, antlers become calcified (Pita-Thomas *et al*., 2010), the velvet skin is shed; the antler is polished and ready for the rutting season.

Calmodulin (*CaM*) is a Ca^{2+}-receptor protein that regulates about 20 enzymes, including adenylate cyclase, protein kinase C, phosphorylating kinase etc. (Means *et al*., 1991). *CaM* in combination with Ca^{2+} can activate many downstream signal transduction pathways such as protein kinase C (PKC) and mitogen-activated protein kinase (MAPK1/2), which subsequently plays important regulatory roles in various cellular physiological activities, including cell proliferation, differentiation, nerve conduction, muscle contraction and relaxation, gene transcription (Vogel, 1994; Soderling *et al*., 2001). In the present study, the *CaM* gene in the constructed cDNA library of velvet antler tip tissue of sika deer belongs to high abundance expression gene, indicating that it may be an important factor regulating antler development.

To date, there is not any record about deer-derived *CaM* gene sequences in GenBank. In this study, we successfully cloned the full-length cDNA

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of *CaM* genes from full-length cDNA library of velvet antler tip tissues of sika deer, further studied the gene structure and their expression levels of different tissue layers of the antler tip by using bioinformatics and real-time quantitative RT-PCR technique. These results provide an important basis for further study of the *CaM* genes' biological function and mechanism in regulating antler development.

MATERIALS AND METHODS

Velvet antler tissue

Antler grown for 80 days were collected from adult anaesthetized sika deer (*Cervus nippon hortulorum*) stags. The distal 4 cm of the tips was removed. Different tissue layers (skin, reserve mesenchyme, precartilage, cartilage) of the growing antlers were determined as described by Li *et al.* (2002). After being harvested, all samples were immediately preserved in liquid nitrogen and kept at −80 °C until they were used for isolating the RNA.

Methods obtaining full length cDNA of CaM genes

Total RNA extraction and cDNA library construction were based on the protocols of SV Total RNA Isolation System reagen (Promega) and Creator^{TM}SMART^{TM}cDNA Library Construction Kit (Clontech), respectively. Using M13 primer, single colonies randomly selected from velvet antler tip tissue original library were conducted large-scale 5'-end EST sequencing by using 3730XL sequencer (Applied Biosystems, Foster City, CA, USA). High-quality ESTs were clustered and spliced by using Phrap software, the consensus sequence spliced was programmed by BLASTX and BLASTN, then sequence homology comparison and function notes of the obtained genes were conducted with non redundant proteins and nucleic acid database in GenBank. *CaM* genes corresponding positive colonies were selected to carry out PCR identification, bacterial amplification, and plasmid extraction, and then two-way sequencing for the plasmid DNA.

Nucleotide sequence analysis

The *CaM* gene sequence was analyzed and compared using the BLAST P and ORF search programs with GenBank database search. The multiple sequence alignment of *CaM* gene was created by Clustal W analysis program, the signal-peptide site was predicted by Signal P3.0, and the *CaM* protein MW and p*I* were computed by ProtParam **tool**. A phylogenetic tree based on evolutionary distances was constructed from amino acid sequences using the njplotWIN95 program.

Quantification of CaM gene expression by real-time RT-PCR

Total RNAs using SV Total RNA Isolation System reagen (Promega) were isolated from different tissues including skin, mesenchyme, precartilage and Cartilage. The residue of DNA were removed by DNase I digesting, at 37 °C for 30 min. Four microgram of the total RNA were used in each lane and electrophoresed in a 0.8% agarose gel, at 100 V/12 cm for 15 min. First-strand cDNA synthesis was performed using M-MLV reverse transcriptase (TaKaRa Biotechnology (Dalian) Co., Ltd. Japan) to transcribe poly $(A)^+$ RNA with oligo-d(T)18 and random six as the primers, reaction conditions were recommended by the manufacturer's instructions. The cDNA was used for the assay of quantitative real-time PCR. The SYBR Green I real-time RT-PCR assay was carried out in an Option-II Sequence Detection System (MJ Research, U.S.).

In a real-time RT-PCR study, specific primers (*CaM*-F: 5'-GGTCAGAACCCAACAGAAG-3' and *CaM*-R: 3'-CTTCACTGTCGGTGTCTTTC-5') were used to amplify a 130 bp fragment with cDNA from skin, reserve mesenchyme, precartilage and cartilage, organs using 18S as a positive control. Quantitative real-time PCR primers were designed on the basis of EST sequences of CaM gene and 18S rRNA gene from deer antler tip tissue by using Primer Premiers 5.0 and Oligo 6.0 software. The EST sequences of the two genes were obtained by large-scale EST sequencing of full-length cDNA library from deer antler tip tissue constructed by our lab. The amplifications were performed in a 96-well plate in a 25 μL reaction volume containing 12.5 μL of 2 × SYBR Green Master Mix (TARAKA), 2.5 μL (each) *CaM*-F and *CaM*-R primers (10 mM), 1 μL of template, and 9 μL of DEPC-water. The thermal profile for SYBR Green real-time PCR was 95 °C

for 2 min, followed by 45 cycles of 94 °C for 12 s, and 57 °C for 30 s. In a 96-well plate, each sample was conducted in triplicate. DEPC-water for the replacement of template was used as negative control. The relative expression was calculated as $2^{-\Delta\Delta C\,t}$ (Livak and Schmittgen, 2001; Ingham *et al*., 2001), and the refer gene was 18s rRNA.

Statistical analysis

A multiple comparisons (Duncan's) test was conducted to compare significant differences in *CaM* expression between skin, mesenchyme, precartilage and cartilage, using the SPSS13.0 software. A significant level of $p = 0.05$ was chosen.

RESULTS AND DISCUSSION

Sequencing and bioinformatics analysis of CaM

Computer analysis, using the BLAST algorithm, confirmed that the selected sequence corresponded to calmodulin. The full-length calmodulin cDNA comprised of 703 bp, containing 105 bp in the 5'-untranslated region (UTR); 462 bp in the open reading frame (ORF); and 99 bp in 3'-UTR with poly (A) tail (Fig. 1). The ORF encodes a polypeptide of 149 amino acids. The calculated molecular mass of the mature protein (149 amino acids) is 16.9 kDa, with an estimated p*I* of 4.09. *CaM* proteins do not have signal peptides, so they belong to non-secretory proteins. Sequence analysis indicates that the *CaM* protein, like in most others animal and plant *CaM*, contains two basic "EF-hand" structures that are from amino acids 1 to 63 and from amino acids 85 to 147 (Fig. 2). EF-hand structure can bind Ca^{2+}ion and activate the function of *CaM*. Each EF-hand structure can bind two Ca^{2+} ions, thus a *CaM* can bind four Ca^{2+} ions. *CaM* amino acids are composed of 33.3% acidic amino acids, therefore, it can provide negatively charged carboxyl groups that are in reversible binding of Ca^{2+}. *CaM* protein does not contain easily oxidized, peptide-chains fixed components such as cysteine, hydroxyproline, and tryptophane.

Homology comparison of CaM

The comparison of the ORFs with other known *CaMs* indicates that the *CaM* shows homology: Identities = 148/149 (99%); with *Homo sapiens*: Identities = 146/149 (98%); with *Gallus gallus*: Identities = 146/149 (98%); with *Ictalurus punctatus*: Identities = 145/149 (97%); with *Procambarus clarkii*: Identities = 145/149 (97%); with *Schistosoma mansoni*: Identities = 140/149 (94%); with *Zea mays*: Identities = 138/149 (93%); with *Arabidopsis thaliana* and so it continues.

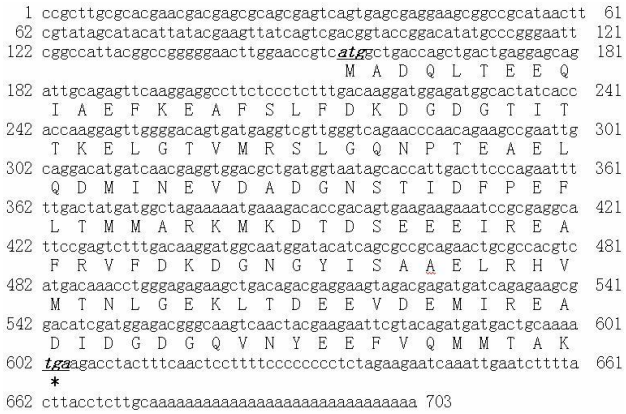

Fig. 1. Nucleotide and deduced amino acid sequences of *CaM* cDNA of velvet antler tip tissue from sika deer (*Cervus nippon hortulorum*). ATG is the initiation codon; * is the stop codon.

Homological analysis shows that *CaM* proteins derived from different organisms have high conservation in evolution (Fig. 3), there are high similarity in amino acids composition and physicochemical properties in the process of biological evolution from plants to invertebrates and vertebrates. Moreover, unhomological *CaM* has an immunologic crossreactivity (Laoudj *et al*., 1994; Gonda *et al*., 2000). Recent finding showed that peptide fragment similar as that of *CaM* in plants and/or animals occurred at bacterium. High conservation of *CaM* in the structure may play functions in maintaining the interactions with *CaM*-combined protein family (Crivici and Ikura, 1995). It implies that *CaM* is an important stabilizing agent for cell functions. Cladogram can be divided into two branches: one branch is animal, the other branch is plant. Tree diagram shows that there is the closest genetic relationship in the gene locus between sika deer and terrestrial vertebrates such as *Homo sapiens* and *Gallus gallus* (Fig. 4). This result

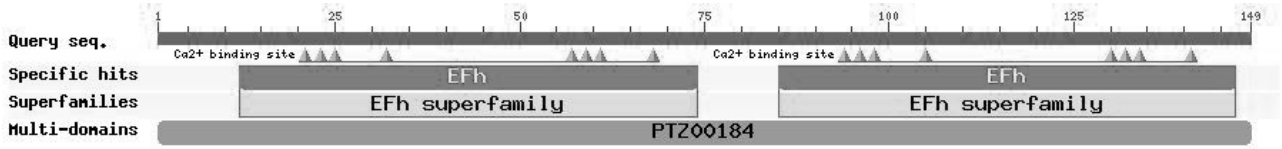

Fig. 2. Search for the conserved domains in deduced amino acid sequence of *CaM* cDNA.

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                ******:***:*************** **********************************
Cervus          MADQLTEEQIAEFKEAFSLFDKDGDGTITTKELGTVMRSLGQNPTEAELQDMINEVDADG 60
Gallus          MADQLTEEQIAEFKEAFSLFDKDGDGTITTKELGTVMRSLGQNPTEAELQDMINEVDADG 60
Homo            MADQLTEEQIAEFKEAFSLFDKDGDGTITTKELGTVMRSLGQNPTEAELQDMINEVDADG 60
Ictalurus       MADQLTEEQIAEFKEAFSLFDKDGDGTITTKELGTVMRSLGQNPTEAELQDMINEVDADG 60
Procambarus     MADQLTEEQIAEFKEAFSLFDKDGDGTITTKELGTVMRSLGQNPTEAELQDMINEVDADG 60
Schistosoma     MADQLTEEQIAEFKEAFSLFDKDGDGTITTKELGTVMRSLGQNPTEAELQDMINEVDADG 60
Zea             MADQLTDEQIAEFKEAFSLFDKDGDGCITTKELGTVMRSLGQNPTEAELQDMINEVDADG 60
Arabidopsis     MADQLTDEQISEFKEAFSLFDKDGDGCITTKELGTVMRSLGQNPTEAELQDMINEVDADG 60

                *.******:*. :*:**********::********* **:******************:
Cervus          NSTIDFPEFLTMMARKMKDTDSEEEIREAFRVFDKDGNGYISAAELRHVMTNLGEKLTDE 120
Gallus          NGTIDFPEFLTMMARKMKDTDSEEEIREAFRVFDKDGNGYISAAELRHVMTNLGEKLTDE 120
Homo            NGTIDFPEFLTMMARKMKDTDSEEEIREAFRVFDKDGNGYISAAELRHVMTNLGEKLTDE 120
Ictalurus       NGTIDFPEFLTTVARKMKDTDSEEEIREAFRVFDKDGNGYISAAELRHVMTNLGEKLTDE 120
Procambarus     NGTIDFPEFLTMMARKMKDTDSEEEIREAFRVFDKDGNGFISAAELRHVMTNLGEKLTDE 120
Schistosoma     NGTIDFPEFLTMMARKMKDTDSEEEIREAFRVFDKDGNGFISAAELRHVMTNLGEKLTDD 120
Zea             NGTIDFPELLNLMARKMKDTDSEEELKEAFRVFDKDQNGFISAAELRHVMTNLGEKLTDE 120
Arabidopsis     NGTIDFPEFLNLMAKKMKDTDSEEELKEAFRVFDKDQNGFISAAELRHVMTNLGEKLTDE 120

                :*:*****:*:*****:*:****::* :
Cervus          EVDEMIREADIDGDGQVNYEEFVQMMTAK 149
Gallus          QVDEMIRESDIDGDGQVNYEEFVQMMTAK 149
Homo            EVDEMIREADIDGDGQVNYEEFVQMMTAK 149
Ictalurus       EVDEMIREADIDGDGQVNYEEFVQMMTA- 148
Procambarus     EVDEMIREADIDGDGQVNYEEFVRMMTSK 149
Schistosoma     EVDEMIREADIDGDGQVNYEEFVKMMTAK 149
Zea             EVDEMIREADVDGDGQINYEEFVKVMMAK 149
Arabidopsis     EVEEMIREADVDGDGQINHEEFVKIMMAK 149
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Fig. 3. Multiple alignment of *CaM* protein sequences.

is in agreement with their traditional taxonomic position for the tested species, according with animal evolution relationship.

Tissue expression of CaM gene

The real-time RT-PCR showed that the *CaM* gene was detected in skin, reserve mesenchyme, precartilage and cartilage. However, the gene has a higher expression level in skin layer than in other three layers reserve mesenchyme 0.34, precartilage 0.16, and cartilage 0.27) (Fig. 5), indicating that this gene may play a regulatory role in rapid growth of antler skin.

As an important Ca-dependent regulation protein, *CaM* plays a central role in cell generation cycle (MacNeil *et al.*, 1988). A study showed that *CaM* plays a crucial role in the transition from DNA pre-synthesis phase (G1-phase) to DNA synthesis phase (S-phase). A high expression of *CaM* gene can shorten G1 phase and accelerate cells into S

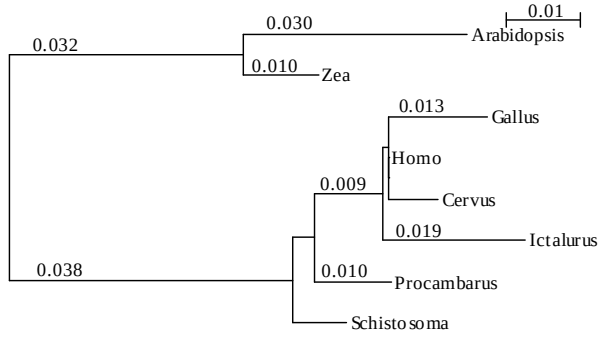

Fig. 4. The phylogenetic tree of *CaM* from animals. Clustal W was used to establish the phylogenetic tree, and the result was displayed using Treeview software.

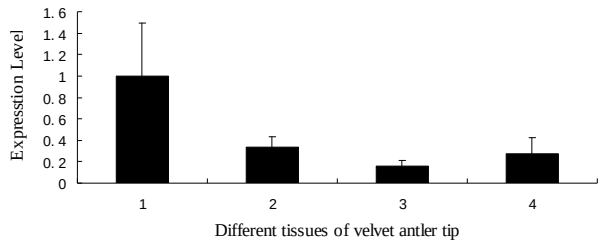

Fig. 5. Expression of *CaM* in different tissues of velvet antler tip. 1-skin; 2-reserve mesenchyme; 3-precartilage; 4-cartilage

phase, therefore it can shorten cell period and promote cell proliferation (Baitinger *et al*., 1990; Poovaiah *et al*., 1999); whereas *CaM* antagonist can delay significantly G1 phase and inhibit cell generation. Deer antler growth is divided into two phases: the first phase, from germination of new antler to dropping of antler skin; the second phase, from dropping of antler skin to the formation of ossification and then dropping. In the first phase, growth rate of antler skin is faster than that of endochondral ossification; while, in the second phase, endochondral ossification rate is faster, and then antler skin begins to drop to form bone antler. In the present study, real time RT-PCR testing results indicated that *CaM* may play an important role in regulating rapid growth of antler skin in the first phase.

Currently, our laboratory is conducting studies on biological functions of *CaM* genes. The results obtained has the significance to a better understanding of biological functions of *CaM* gene, meanwhile, it provides basic data for further researching intrinsic regulation mechanism of antler growth in the gene level.

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