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RESEARCH ARTICLE - ANTS

Molecular Cloning, Characterization and Expression Analysis of Calreticulin Gene in the Ant *Polyrhachis vicina* Roger (Hymenoptera: Formicidae)

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Abstract

Calreticulin (CRT) as a ubiquitous and highly conserved calcium-binding protein that exists in endoplasmic reticulum (ER), which possesses a variety of biological functions in the regulation of cell calcium homeostasis, molecular chaperoning and innate immunity. In our research, the calreticulin homologous gene (refered as PvCRT) was cloned from the ant *Polyrhachis vicina* Roger (Hymenoptera: Formicidae), the full-length cDNA of PvCRT is 1584bp base pairs (bp), contains a 5'-untranslated region of 87bp and a 3'-untranslated region of 246bp. The open reading frame (ORF) of 1251bp encodes 416 amino acids. Using real-time quantitative RT-PCR to study PvCRT mRNA expression levels indicates that this gene was expressed in different developmental stages of castes of *P. vicina*. The mRNA expression level in both embryos and adults shows that PvCRT gene may play some essential roles in the ant's development.

Introduction

Calreticulin (CRT) is located both inside and outside of the endoplasmic reticulum, and also distributed on the cell surface (Ostwald et al., 1974). CRT belongs to a multifunctional calcium-binding protein, which can regulate calcium balance, assist the protein folding, processing and presenting the antigen. Usually, the gene of CRT composition and the amino acid sequence are highly conservative. Many studies show that the CRT protein may play a critical role in the maintenance of cell functions (Lee et al., 2001; Lynch et al., 2006).

CRT was initially purified from hare sarcoplasmic reticulum of muscle cells by Ostwald and MacLennan in 1974. However, CRT gene's cDNA sequence was cloned from the liver of mice by Smith and Koch in 1989. Currently, CRT gene's cDNA sequence was cloned from vertebrates, invertebrates and higher plants. At the same time, the molecular structure and related functions of CRT were studied. In insects, CRT was detected as an encapsulative protein. CRT may be relevant to the identification of foreign objects in the cell defense response, since higher amounts of CRT can be found around the foreign substance. In *Drosophila melanogaster* Meigen CRT named DmCRT acts as a marker for phagocytosis of apoptotic cells by *D. melanogaster* phagocytes (Kuraishi et al. 2007). In .addition, DmCRT plays an important role in odor-guided behavior (Stoltzfus et al., 2003) and also is involved in mediating anesthetic sensitivity (Gamo et al., 2003). In *Anopheles gambiae* Giles AgCRT is known to interact with malaria parasite surface protein (Rodriguez-Mdel et al., 2007).

Here, the full-length cDNA sequences of the calreticulin gene were cloned from the ant *Polyrhachis vicina* Roger and the homologs were named PvCRT. A phylogenetic tree was constructed using MEGA 5.0. The results of the phylogenetic analysis indicated that PvCRT shares high identity with *Pteromalus puparum* L and *Nasonia vitripennis* Walker calreticulin. The expression pattern of PvCRT mRNAs was studied by using real-time RT-PCR. Our data indicate that this gene is expressed differentially in distinct development stages and different castes.

Material and Methods

Experimental insects

Polyrhachis vicina, which belongs to genus *Polyrhachis* (Hymenoptera:Formicidae), is a species of typical eusocial insect with the characteristic of castes differentiated in females, males and workers. It is a good material for studying the mechanisms of insect development and behavior. *P. vicina* as a complete metamorphosis insect, is mainly distributed in east and south of China, Burma, Cambodia, Japan, Australia and Papua New Guinea.

The colonies of *P. vicina* were bought from Hongfa Edible Ant Research Center in Nanning City, Guangxi Province, in People's Republic of China. Then, the ants were cultured in boxes at the laboratory, under a controled temperature of 28ºC, relative humidity of 40% and natural light-dark periods. The ants were usually reared on fruits, meat and honeydew. Egg, larvae, pupae and adults (workers, winged males and winged females) were collected from the two colonies, 6 individuals were used respectively for each caste, immersed immediately in liquid nitrogen, stored at -80°C for RNA extraction (Lv et al., 2008; Guo et al., 2010).

RNA preparation and cDNA synthesis

Total RNA was extracted from pooled samples of 12 frozen larvae selected randomly with RNAiso Plus (Takara Bio Inc., Shiga, Japan), and then immediately reverse-transcribed in order to generate cDNA using the First-Strand cDNA Sythesis Kit with oligo (dT) primer (Fermentas Life Sciences, Burlington, Ontario; http://www.fermentas.com/). All steps were followed through the manufacturer's instructions.

Molecular cloning and sequencing of PvCRT

The full-length cDNA of CRT from *P. vicina* named PvCRT was cloned based on the scheme shown in Table 1. First, a partial cDNA fragment of PvCRT was gained using degenerated primers (S1 and A4), according to the above fragment, primers P1 and L4 were designed and used these pair primers, and then Seq2 fragment was obtained. The four primers were designed on the basis of the conserved motifs of published PvCRT of other insect species (*Pteromalus puparum* L., *Bombyx mori* L, *Culex quinquefasciatus* (Say), *Nasonia vitripennis* Walker, *Galleria mellonella* L. PCR products were purified from agarose gel using Gel Extraction Kit (Qiagen, Germany http://www.qiagen.com/) and purified products were cloned into PMD19-T Vector (Takara Bio Inc.), then PCR positive clones were obtained for sequencing. After that, using 3' and 5' rapid amplification of cDNA ends (RACE) to get the full length of PvCRT, the specific primers of RACE are shown in table 1. All the primers were designed using PrimerPremier 5.0 and Oligo 6.0.

Structural and phylogenetic analysis of PvCRT

The ORF of PvCRT was obtained using the National Center for Biotechnology Information ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Signal peptide prediction was completed using the SingalP program (http://www.cbs.dtu.dk/services/SignalP/ Bendtsen J.D. et al. 2004; Center for Biological Sequence Analysis;). The secondary structure of the PvCRT protein was predicted usingSOPM (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_hnn.html).

The tertiary structure of *P. vicina* CRT was also pre-

Table1. Oligonucleotide primers used for cDNA cloning and real-time quantitative RT-PCR.

Target	Name	Primer Sequence 5′-3′	Expected size(bp)	Tm(°C)
CRT Fragment	S1	GAYTCVTGGGAAMACMATTGG	873	52
	A4	CTTGCCAYAAATCVAGWCCDAC		
	P1	TGAGGATAAAAAACCAGAGGAC	606	52
	L4	CATCRTGCTCTTCAATHTCAGG		
3'RACE	3outer	GCTGTCAACGATACGCTACGTAACG	310	60
	F1	CATTTGTGGCCCAGGAATTAGG		
	3inner	CGCTACGTAACGGCATGACAGTG		
	Q4	TACCCGATAGCGACGATGAT		
	3'oligo(dT)	GCTGTCAACGATACGCTACGTAACGGCATGACAGTG(T)18		
5'RACE	5outer	CATGGCTACATGCTGACAGCCTA	300	56
	H9	TCGCCATCCATTTCGTCATCC		
	5inner	CGCGGATCCACAGCCTACTGATGATCAGTCGATG		
	M1	ATCATTTTCTGGGTCATTCC		
Real time	E13	CGACAACGAGGAAGTAGAGT	224	60
	G13	ATTCGCCATCCATTTCGTCATC		
B-actin	B1	TGAACAGGAGATGGCTACAG	80	60
	B2	CCGATGGTGATGACTTGAC		

Note: Y: C or T; V: A or G or C; M: A or C; W: A or T; D: A or G or T; R: A or G; H: A or C or T; Tm: Annealing temperature.

dicted using Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index). Potential functional motifs were analyzed using PROSITE database (Expert Protein Analysis System, Swiss Institute of Bioinformatics, Basel; http://myhits.isb-sib.ch/cgi-bin/motif_scan). Sequence alignments based on the amino acid sequences of published CRT genes were performed with Clustal X 2 and DNAman. From alignments, a phylogenetic tree of PvCRT was generated by MEGA 5.0, based on the neighbor-joining method with a bootstrap test calculated with 500 replicates and a Poisson correction model.

Real-time quantitative RT-PCR

Real-time quantitative RT-PCR was used to quantify the mRNA expression levels of PvCRT from two colonies at different development stages and different castes including egg, wholly body of one to four instars larvae's, pupae and wholly body of caste adult ants (ergate, winged male ant and winged female ant) of *P. vicina* were used in this experiment. For each of these developmental stages, samples were taken from 6 individuals.

Reactions volume, reactions temperature are 25.5ul and 60ºC, respectively. Such reactions were performed in ABI Stepone with a SYBR Premix Ex Taq Kit (Takara Bio Inc). β-actin was used as an endogenous control to research the gene expression. Primers (B1, B2) of β-actin and primers (E13, G13) of PvCRT were used in real-time RT-PCR (Table 1). The amplified fragments of two pairs of primers are 80bp and 224bp, respectively. The detailed protocol was as follows: 95ºC for 10min, 40 cycles of 95ºC for 15s and 60ºC for 1min, followed by a melting-curve program from 59 to 95ºC with a heating rate of 0.3ºC every step and continuous-fluorescence acquisition. All RT-PCR reactions were completed in triplicate.

The relative expression quantification of PvCRT was determined by the formula F=10ΔCt,t/At-ΔCt,r/Ar (Zhang et al., 2005). One of cDNA samples was chose to construct standard curve of PvCRT gene. In this study, we chose the third instar as calibrator to analyze PvCRT expression at different development stages and different castes. The relative expression levels of PvCRT were analyzed using one way analysis of variance (ANOVA) with Dunnett's multiple comparision test.

Results

Cloning and characterization of PvCRT cDNA

The full-length cDNA of PvCRT was obtained by RT-PCR and RACE. The full-length cDNA of PvCRT is 1584bp. The PvCRT cDNA includes a 5'-untranslated region of 87bp, an ORF of 1251bp and a 3'-untranslated region of 246bp (Fig.1). A putative polyadenylation signal (AATAAA) was found upstream from the 18-nucleotide poly (A) tail, which

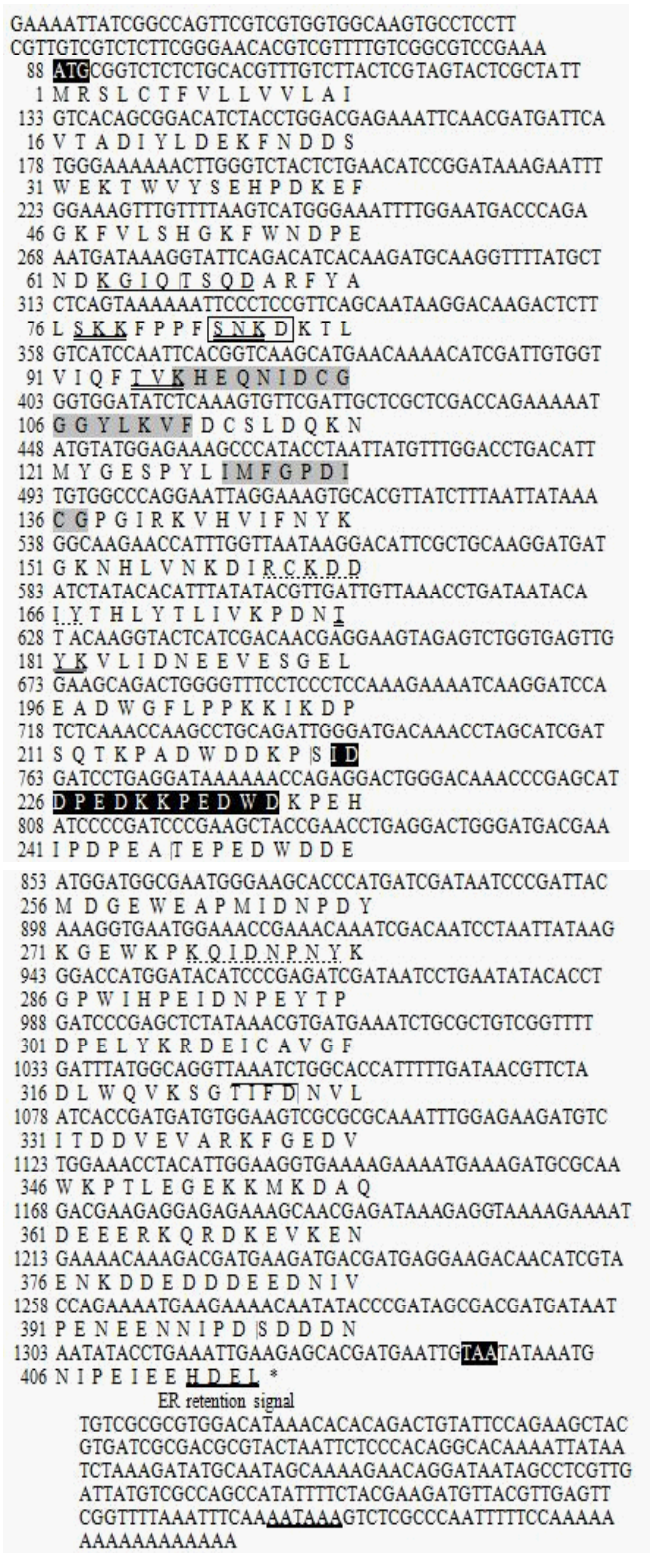

Fig. 1. The nucleotide and deduced amino acid from the PvCRT. The full sequence of PvCRT is 1584bp, which encodes 416 amino acid. The start codon ATG and the stop codon TAA are underlined and shaded, and the polyadenylation signal (AATAAA) is underlined. A lot of important functional motifs of PvCRT protein are marked. Six casein kinase II phosphorylation sites at 67-70 (TSQD), 84-87 (SNKD), 223-226 (SIDD), 247-250 (TEPE), 324-327 (TIFD), and 401-404 (SDDD); one N-myristoylation site at 64-69 (GIQTSQ); four protein kinase C phosphorylation sites at 77-79 (SKK), 84-86 (SNK), 95-97 (TVK), and 180-182 (TYK); two tyrosine kinase phosphorylation sites at 161-167 (RCKDDIY), and 277-284 (KQIDNPNY); calreticulin family signature 1 at 97-112 (KHEQNIDCGGGYLKVF); calreticulin family signature 2 at 129-137 (IMFGPDICG); calreticulin family repeated motif signature at 224-236 (IDDPEDKKPEDWD); endoplasmic reticulum targeting sequence at 413-416 (HDEL).

coincides with the fact that the polyadenylation signal is most often present 11-30 nucleotide upstream from the poly (A) tail (Fitzgerald et al., 1981). The deduced proteins of PvCRT have a calculated molecular mass of 48.5kDa and an isoelectric point (PI) of 4.37. Nucleotide sequence of PvCRT has been deposited into the GenBank database and obtained the accession number of JQ783054.

Protein structure prediction

The ORF of PvCRT encodes 416 amino acid, and the predicted protein shares 99% similarity with *N. vitripennis* and 88% with *Plutella xylostella* L. Analysis with the SignalP program showed that there was N-terminal signal sequence, and the cleavage site between 18 and 19 amino acid, so the protein was determined secreted protein. A series of predicted functional motifs were found by PROSITE program, including six casein kinase II phosphorylation sites at 67-70 (TSQD), 84-87 (SNKD), 223-226 (SIDD), 247-250 (TEPE), 324-327 (TIFD), and 401-404 (SDDD); one N-myristoylation site at 64-69 (GIQTSQ); four protein kinase C phosphorylation sites at 77-79 (SKK), 84-86 (SNK), 95-97 (TVK), and 180-182 (TYK); two tyrosine kinase phosphorylation sites at 161-167 (RCKDDIY), and 277-284 (KQIDNPNY); calreticulin family signature 1 at 97-112 (KHEQNIDCGGGYLKVF); calreticulin family signature 2 at 129-137 (IMFGPDICG); calreticulin family repeated motif signature at 224-236 (IDDPEDKKPEDWD); endoplasmic reticulum targeting sequence at 413-416 (HDEL).

The predicted secondary structure of the PvCRT protein contains 13.70% alpha helix, 14.66% extended strand and 71.63% random coil (Fig. 2). The prediction of tertiary structure is shown in Fig.4.

Alignment analysis and phylogenetic-tree construction in insects

A multiple alignment of the deduced amino acid sequence of PvCRT with other known calreticulin homologues was performed by Clustal X 2 and DNAman (Fig.3). A phylogenetic tree was constructed using MEGA 5.0 based on the neighbor-joining method (Tumura et al., 2007) in Fig. 5. The results of the phylogenetic analysis indicated that PvCRT shares high identity with *P. puparum* and *N. vitripennis* calreticulin (Fig.5).

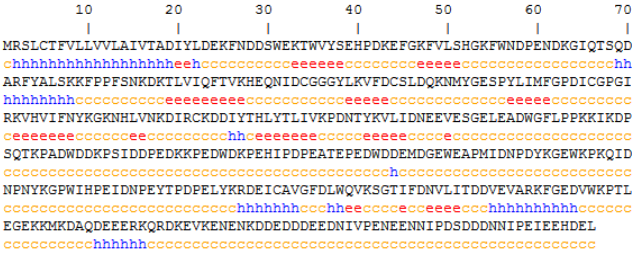

Fig. 2. Predicted secondary structure of the PvCRT protein, where c is random coil; h is alpha helix; e is extended strand; t is beta turn.

Expression analysis of PvCRT mRNA

The expression level of PvCRT mRNA at different developmental stages and in different castes was analyzed by means of real-time quantative RT-PCR. Previous studies revealed that the analyzed β-actin mRNA levels remain constant in tissues of insects regardless of their developmental and physiological condition (Simonet et al., 2004). The analyzed results indicated that PvCRT was expressed in all templates (Fig.6). During the developmental stages, the highest expression level was found in the third instars and the lowest expression level was found in embryos. Among the three adult's castes, the PvCRT gene expression was highest in workers and lowest in males.

Discussion

In this study, the full-length cDNA of calreticulin gene was cloned and characterized from *P. vicina*. The cDNA sequence of PvCRT is highly conserved with calreticulin homologs found from other insects, which indicated that this sequence is indeed calreticulin of *P. vicina*. The results of predicted functional motifs found that in PvCRT protein exists an ER targeting sequence HDEL, which is closely related to the cellular localization of CRT in ER. This result may shows that the PvCRT possesses function of calregulin in ER mainly involved in directing proper conformation of the proteins, controlling calcium level, and participating in immune responses (Wang et al., 2012; Byung-Jae Park et al, 2001;) including: regulation of intracellular Ca2+ homeostasis chaperone activity, steroid-mediated gene regulation, and cell adhesion (Mesaeli et al., 1999).

The phylogenetic tree (Fig.5) was analyzed and found that PvCRT is most closely related to that of *P. puparum* and *N. vitripennis*. Usually, *P. civica* is similar to *P. puparum* and *N. vitripennis* in classification status. All of them belong to hymenoptera insects, which may indicate that the calregulin possesses conservative properties. Khanna et al. (1987) demonstrated that"the physicochemical and structural properties of calregulin from a single tissue have been highly conserved during vertebrate evolution after comparison of calregulins from bovine, rabbit and chicken livers"Luana et al. (2007) cloned the full length cDNA of calreticulin from *Fenneropenaeus chinensis* Osbeck (FcCRT). disply that Calreticulin belong to a highly conserved calcium-binding protein. The deduced amino acid sequence of FcCRT showed high identity with those of *B. mori* (88%), *Drosophila melanogaster* Meigen (83%), *Mus musculus* L. (82%) and *Homo sapiens* L.(82%).

In this paper the analysis of real-time RT-PCR indicated that PvCRT gene is expressed in each template, but the expres-

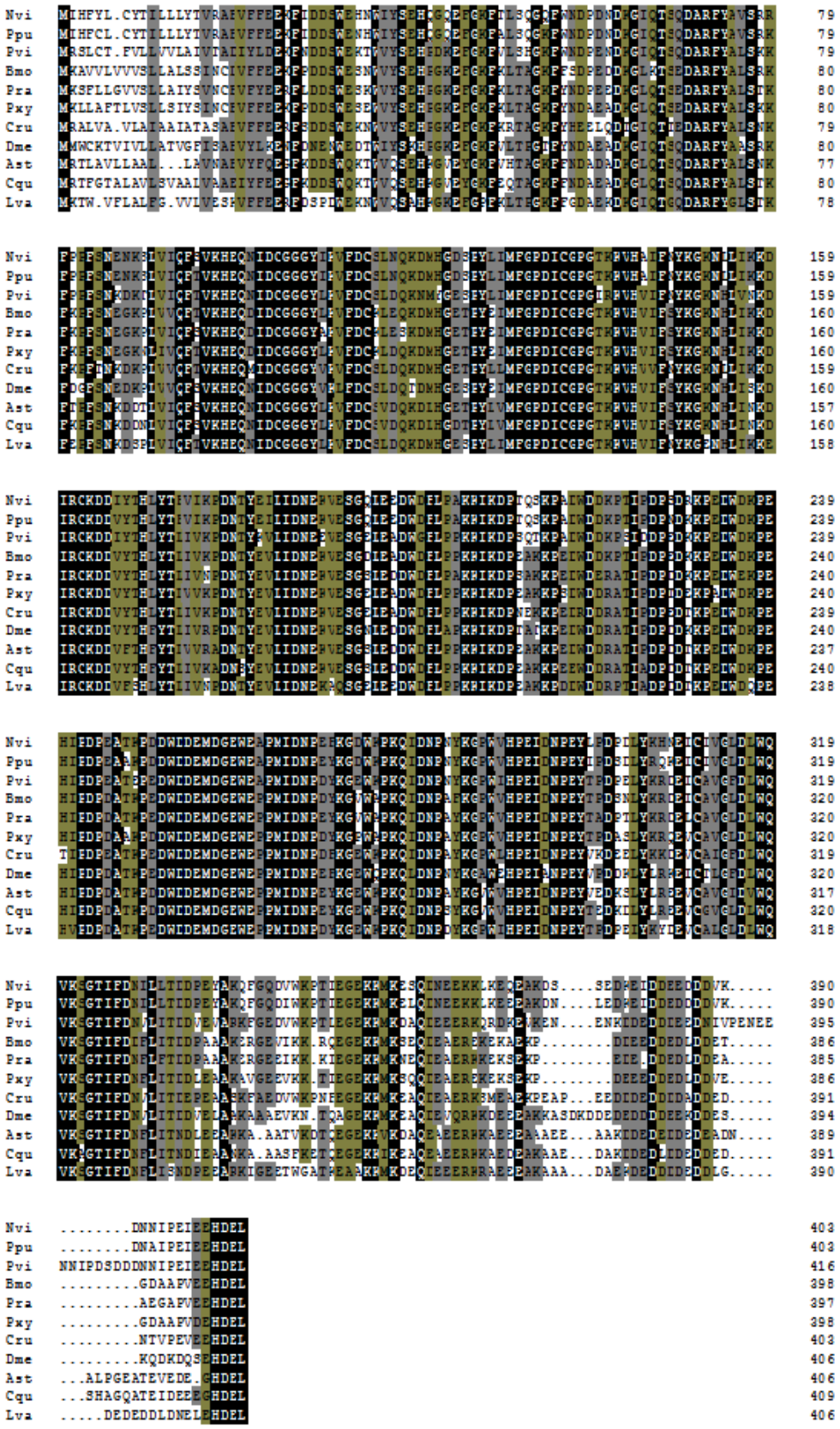

Fig.3. Amino acid sequence alignments of PvCRT with the homologues sequences of other insects and one crustacean. Species and GenBank accession numbers for alignmental and phylogenetic analysis are as follows: *N. vitripennis* (Nvi) NM_001161679; *P. puparum* (Ppu) FJ882064; *P. vicina* (Pvi) JQ783054; *B. mori* (Bmo) FJ360528; *Pieris rapae* L. (Pra) EU826537; *Plutela xylostella* L. (Pxy) HM240516; *Cotesia rubecula* (Marshall) (Cru) AY150370; *D. melanogaster* (Dme) NM_079569; *Anopheles stephensi* Liston (Ast) JN559458; *Culex quinquefasciatus* (Say) (Cqu) XM_001848772; *Litopenaeus vannamei* Boone (Lva) (Crustacea) JQ682618. Similarity of 100%, (>=75%), (>=50%) in all CRTs are marked in black, dark green and gray, respectively.

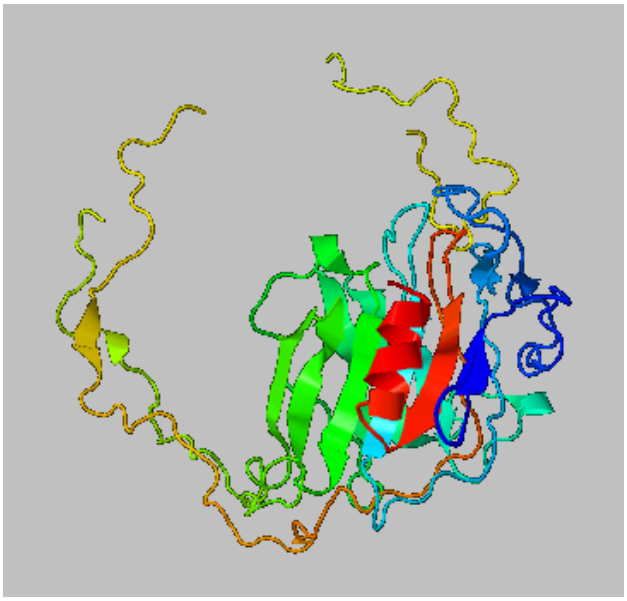

Fig.4. The tertiary structure prediction of PvCRT.

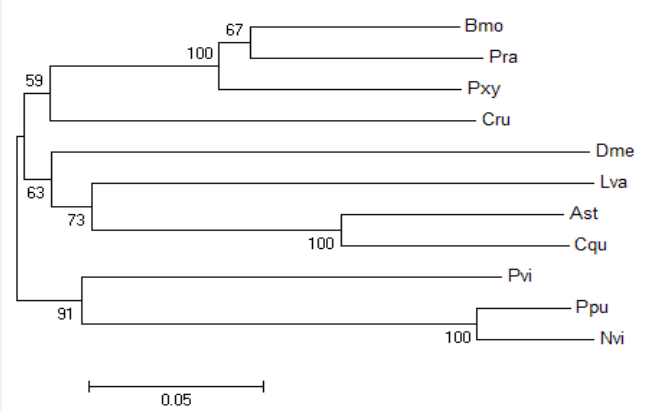

Fig.5. The phylogenetic tree shows the envolutionary relationship of PvCRT with CRTs of other insects on the basis of NJ method, confidence values based on 500 repeats are shown in the nodes; the abbreviation of species is given in Fig.3 and text.

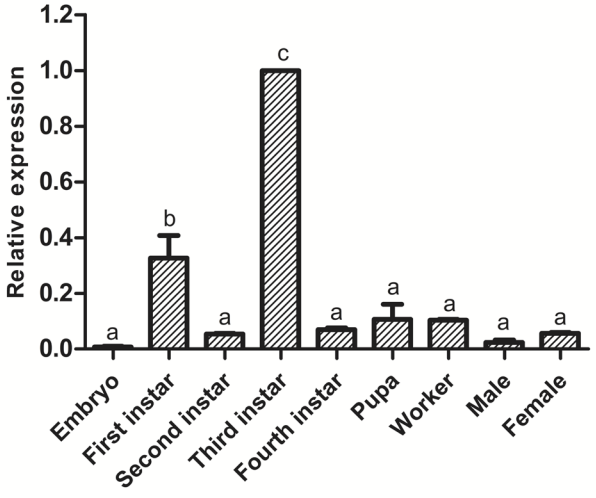

Fig.6. The relative expression profiles of PvCRT mRNA at different development stages and in different castes, the same letter is indicated that there are no significant differences between them ($p<0.05$).

sion levels are different. During the developmental stages, the highest expression level was found in the third instars and the lowest expression level was found in embryos. According to those results, we guess that third instars's nervous system and sense organs may reach an development peak due to calregulin can promote the development of nerve and Sense organs (Silerová et al., 2007; Gamo et al.,1998). In the three adult's castes, the PvCRT gene expression was highest in workers and lowest in males. This result coincide with Stoltzfus and Horton's conclusion (2003) "the calregulin plays a key role in olfactory system function, possibly by establishing its overall sensitivity to odorants". Among *P. civica* adult's castes, the worker olfactory system is the most developed especially in antenna and chest foot aspects because of the main responsibilities related to building and defending nests, feeding the young ants and queens. The main task of male ants is only to mate with the queen, so its olfactory system function may be the weakest among the castes.

From the above results we can deduce that PvCRT gene may play critical roles for the ant growth and development. More study of PvCRT functions will be further continueding.

In conclusion, the full-length PvCRT cDNA for the first time was cloned from *P. vicina*, and the characterization of PvCRT protein was analyzed. The differential PvCRT mRNA expression levels of PvCRT in different developmental stages and different castes, may indicate the physiological importance of PvCRT for this ant species.

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