

Sociobiology

An international journal on social insects

RESEARCH ARTICLE - ANTS

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

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Article History

Edited by:

Kleber Del-Claro - UFU, BrazilReceived07 January 2013Initial acceptance04 April 2013Final acceptance26 July 2013

Keywords

Calreticulin homologous gene, Molecular cloning, *Polyrhachis vicina* real-time quantitative RT-PCR

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Introduction

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.

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.



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 28°C, 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.giagen.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 (<u>http://www.ncbi.nlm.nih.gov/gorf/gorf.html</u>). Signal peptide prediction was completed using the SingalP program (<u>http://www.cbs.dtu.dk/services/SignalP/</u> Bendtsen J.D. et al. 2004; Center for Biological Sequence Analysis;). The secondary structure of the PvCRT protein was predicted usingSOPM (<u>http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_hnn.html</u>).

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

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 (<u>http://www.sbg.bio.ic.ac.uk/phyre2/</u><u>html/page.cgi?id=index</u>). Potential functional motifs were analyzed using PROSITE database (Expert Protein Analysis System, Swiss Institute of Bioinformatics, Basel; <u>http://</u><u>myhits.isb-sib.ch/cgi-bin/motif_scan</u>). 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 60°C, 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: 95°C for 10min, 40 cycles of 95°C for 15s and 60°C for 1min, followed by a melting-curve program from 59 to 95°C with a heating rate of 0.3°C 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 $F=10\Delta Ct$,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

GAAAATTATCGGCCAGTTCGTCGTGGTGGCAAGTGCCTCCTT CGTTGTCGTCTCTTCGGGAACACGTCGTTTTGTCGGCGTCCGAAA 88 ATGCGGTCTCTCTGCACGTTTGTCTTACTCGTAGTACTCGCTATT 1 MRSLCTFVLLVVLAI 133 GTCACAGCGGACATCTACCTGGACGAGAAATTCAACGATGATTCA 16 V T A D I Y L D E K F N D D S 178 TGGGAAAAAACTTGGGTCTACTCTGAACATCCGGATAAAGAATTT 31 W E K T W V Y S E H P D K E F 223 GGAAAGTTTGTTTTAAGTCATGGGAAATTTTGGAATGACCCAGA 46 G K F V L S H G K F W N D P E 268 AATGATAAAGGTATTCAGACATCACAAGATGCAAGGTTTTATGCT 61 N D K G I Q T S Q D A R F Y A 313 CTCAGTAAAAAATTCCCTCCGTTCAGCAATAAGGACAAGACTCTT 76 L SKKFPPFSNKDKTL 358 GTCATCCAATTCACGGTCAAGCATGAACAAAACATCGATTGTGGT 91 VIQF<u>TVK</u>HEQNIDCG 403 GGTGGATATCTCAAAGTGTTCGATTGCTCGCTCGACCAGAAAAAT 106 G G Y L K V F D C S L D O K N 448 ATGTATGGAGAAAGCCCATACCTAATTATGTTTGGACCTGACATT 121 MYGESPYLIMFGPDI 493 TGTGGCCCAGGAATTAGGAAAGTGCACGTTATCTTTAATTATAAA 136 C G P G I R K V H V I F N Y K 538 GGCAAGAACCATTTGGTTAATAAGGACATTCGCTGCAAGGATGAT 151 GKNHLVNKDIRCKDD 583 ATCTATACACATTTATATACGTTGATTGTTAAACCTGATAATACA 166 I Y T H L Y T L I V K P D N T 628 T ACAAGGTACTCATCGACAACGAGGAAGTAGAGTCTGGTGAGTTG 181 YKVLIDNEEVESGEL 673 GAAGCAGACTGGGGTTTCCTCCCTCCAAAGAAAATCAAGGATCCA 196 E A D W G F L P P K K I K D P 718 TCTCAAACCAAGCCTGCAGATTGGGATGACAAACCTAGCATCGAT 211 S Q T K P A D W D D K P S ID 763 GATCCTGAGGATAAAAAACCAGAGGACTGGGACAAACCCGAGCAT 226 D P E D K K P E D W D K P E H 808 ATCCCCGATCCCGAAGCTACCGAACCTGAGGACTGGGATGACGAA 241 I P D P E A T E P E D W D D E 853 ATGGATGGCGAATGGGAAGCACCCATGATCGATAATCCCGATTAC 256 M D G E W E A P M I D N P D Y 898 AAAGGTGAATGGAAACCGAAACAAATCGACAATCCTAATTATAAG 271 K G E W K P K Q I D N P N Y K 943 GGACCATGGATACATCCCGAGATCGATAATCCTGAATATACACCT 286 G P W I H P E I D N P E Y T P 988 GATCCCGAGCTCTATAAACGTGATGAAATCTGCGCTGTCGGTTTT 301 D P E L Y K R D E I C A V G F 1033 GATTTATGGCAGGTTAAATCTGGCACCATTTTTGATAACGTTCTA 316 DLWQVKSGTIFDNVL 1078 ATCACCGATGATGTGGAAGTCGCGCGCAAATTTGGAGAAGATGTC 331 I T D D V E V A R K F G E D V 1123 TGGAAACCTACATTGGAAGGTGAAAAGAAAATGAAAGATGCGCAA 346 W K P T L E G E K K M K D A Q 1168 GACGAAGAGGAGAGAAAGCAACGAGATAAAGAGGTAAAAGAAAAT 361 DEEERKQRDKEVKEN 1213 GAAAACAAAGACGATGAAGATGACGATGAGGAAGAACAACATCGTA 376 E N K D D E D D D E E D N I V 1258 CCAGAAAATGAAGAAAACAATATACCCGATAGCGACGATGATAAT 391 PENEENNIPDSDDDN 1303 AATATACCTGAAATTGAAGAGCACGATGAATTGTAATAAATG 406 NIPEIEE HDEL ER retention signal TGTCGCGCGTGGACATAAACACACAGACTGTATTCCAGAAGCTAC GTGATCGCGACGCGTACTAATTCTCCCACAGGCACAAAATTATAA TCTAAAGATATGCAATAGCAAAAGAACAGGATAATAGCCTCGTTG ATTATGTCGCCAGCCATATTTTCTACGAAGATGTTACGTTGAGTT CGGTTTTAAATTTCAAAAAAAGTCTCGCCCAATTTTTCCAAAAA

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).

AAAAAAAAAAAAA

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 (ID-DPEDKKPEDWD); 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

10	20	30	40	50	60	70
	1	1	1	1	1	- E
MRSLCTFVLLVVLA	IVTADIYLDE	KFNDDSWEKTV	VYSEHPDKE	FGKFVLSHGK	FWNDPENDKG	IQTSQD
chhhhhhhhhhhh	hhhheehcc	cccccccee	eeeecccccc	cceeeeeccc		cccchh
ARFYALSKKFPPFS	NKDKTLVIQF	TVKHEQNIDCO	GGYLKVFDC	SLDQKNMYGE:	SPYLIMFGPD	ICGPGI
hhhhhhhcccccc	cccceeeeee	eeeccccccc	cccceeeec		cceeeeeccc	cccccc
RKVHVIFNYKGKNH	LVNKDIRCKD	DIYTHLYTLIV	VKPDNTYKVL	IDNEEVESGE	LEADWGFLPP	KKIKDP
ceeeeeeecccccc	eeccocccc	hceeeeee	eccccceeee	ecccecccc		cccccc
SQTKPADWDDKPSI	DDPEDKKPED	WDKPEHIPDP	EATEPEDWDD	EMDGEWEAPM	IDNPDYKGEW	KPKQID
cccccccccccc			cccccccch			cccccc
NPNYKGPWIHPEID	NPEYTPDPEL	YKRDEICAVGI	FDLWQVKSGT	IFDNVLITDD	VEVARKFGED	VWKPTL
cccccccccccc		cchhhhhhhc	cc <mark>hhee</mark> cccc	ecceeeecccl	hhhhhhhhh	cccccc
EGEKKMKDAQDEEE	RKQRDKEVKE	NENKDDEDDD	EEDNIVPENE	ENNIPDSDDD	NNIPEIEEHD	EL
ccccccccchhhh	hheeceecee					cc

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.

shares high identity with *P. puparum* and *N. vitripennis* calreticulin (Fig.5).

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-

Nvi	MIHFYL.CYTILLLYTVRAEVFF22REIDDINEHNKIYSCHCS2PCYFTLSGGEWND2DND.GIOTSODARFYAVSRA MIHFCL.CYTILLLYTVRAEVFF22REIDDINENHKIYSCHCS2PCYFALSCGF3ND2DND.GIOTSODARFYAVSRA	79
Ppu	MIHFCL.CYTILLLYTVRAEVFFEERFIDDSKENHKIYSEH;GOEFGWFALSOCWFADPDND GIOTSODARFYAVSRK	79
Pvi	MRSLCT, FVLLVVLAINTALTYLDHONDDBAEKTKVVSEHEDROHERRANDSHERANDFENDHEIONSODARNALSEK	79
Bmo	MRSLCT.FVLLUVLAIVIAIIYLDE (PNDDIWEXIVVSEHFDKEFGYFVLSHGYFNDDENDGIGTS)DARFYALSXX MKAVVLVVVSLLALSSINCIVFFEF <mark>EPDIWESNVVYS</mark> EHFGKEF <mark>GY</mark> FKLINGYFS <mark>DDFGLA</mark> GL <mark>AT</mark> SEDARFYALSXX	80
Pra	MKSPLLGVVSLLAIYSVNCEVFYE <mark>PPLDDSWE SKVVYS</mark> EHEGK <mark>EFGVFKLTAGKFVNDPREDKGLQTSEDARFVALST</mark> K	80
Ржу	MKLLAFTLVSLLSIVSINCEVFFE ² ve ² dds ^v e bd ^{3ve} se ² vys <mark>2</mark> ffgk <mark>Pf</mark> erkltxGxPrndaeA <mark>D</mark> ;Glo <mark>TsedARFY</mark> AISKK	80
Cru	MRALVA, VLAIAAIATASAEVFFE <mark>BRESDDSVE</mark> KNOVYS <mark>EHFGKEFE</mark> KEKRTA <mark>GKEYHE</mark> ELQDIGIQTIEDAREVAISNK	79
Dme	MMCKIVIVLLATVGFTSAFVYLKENEDNENCEDIGIYSZHFGKEVEVLTEGTEVNDAEADHGIOTSCDAREYAASZK	80
Ast	MATLAVLLAALLAVNAEVYFOERKDDANOKTVOSEHKGVEYENEVHTAGHENDADAD.GLOTSODARFAISNK	77
Cqu	MKSFLLGVVSLLAIVSVNCIVFYEZRELDDIWESWVVSCHFGWERKVEYGERKLING FYNDERED GLOT SEDARFYALSTN MKLLAFTLVSLLSIYSINCIVFYERREDDIWERWVYSCHFGWERGYFKLING FYNDAEAD GLOT SEDARFYALSN MRALVA.VLAFAAIATASAFVFFERREDDIWERWVYSCHFGWERGYFKRING FYNDAEAD GLOT SEDARFYALSN MMCKTVIVLATVOFTSREVYLWERD NEW DIWIYSCHFGWERGYFWITG FYNDAEAD GLOT SEDARFYALSN MRTLAVLLANL.LAVNAFVYFERREDDIWERWVYSCHFGWERGYFWITG FYNDAEAD GLOT SEDARFYALSN MRTFGTALAVLSVALVAREIYFERFERDIWERWVSCHFGWERGYFYITG FYNDAEAD GLOT SEDARFYALSN MRTFGTALAVLSVALVAREIYFERFERDIWERWVSCHFGWERGYFYLTG FYNDAEAD GLOT SEDARFYALSN MRTFGTALAVLSVALVAREIYFERFERDIWERWVSCHFGWERGERFERFERFERFERFERFERFERFERFERFERFERFERFE	80
Lva	MKTW. VPLALEG. VVIVESFVEFEREDSPINEKNVVSAHKGNPGERLTECHEFGDAEKDRGIOTGODARFYGISTK	78
Nvi	FF <mark>FFSNENKS<mark>U VICFTVKHECNIDCGGGYIF</mark>VFDCSLNOKDNEGDSEYLIMFGPDICGFGTHEVHATFSYKGKNILIKKD</mark>	159
Ppu	FERSMENKS <mark>U VIÇETVKHEÇMIDCGGGYIEVFDCSLNOKDHEGDSEYLIMFGPDICGFGTHEVHALFSYKGKNILIKO</mark> FERSMENKS <mark>U VIÇETVKHEÇMIDCGGGYIEVFDCSLNOKDHEGDSEYLIMFGPDICGFGTHEVHALFSYKGKNILIKO</mark>	159
Pvi		159
Bmo	FEIFEN KOKTUVICE IVKHEONIDCGGGYLIVEDCELDOKNNIGE SEYLIMFGEDICGEGIRFVHVIFSYKCKNEIVKC FKIFSNECKUVVCETVKHEOTIDCGGGYLVEDCELEOKONEGETEYEIVEGEDICGEGIRFVHVIFSYKCKNEIIKC	160
Pra	FRIESNEGKILVICF VKHEQIIDCGGGY HVFDCHLESKDNHGETFY IMFGPDICGPGTHEVHVIF YKGANHLINKD	160
Pxy	FRIFSNEGKULIVCFTVKHEQTIDCGGGYLVFDCILDCKDNEGETFYEIMFGPDICGFGTHEVHVIFSYKCKNELINKO	160
Cru	FRIETNKOKSTUNGET VKHEQ IDCGGGYDEVEDCELDQKDKEGETEYLLMEGEDICGEGTKKVEVVETYKGKNLIKKO	159
Dme	FKEFSNEGKO <mark>U VICFEVKHEODIDCGGGYAE</mark> VFDCSLESKDNEGETEYEIMFGPDICCFGTHWUHVIFSYKGMNEDIKKD FKEFSNEGKODIVCFIVKHEODIDCGGGYLEVFDCSLDOKDNEGETEYEIMFGPDICCFGTHWUHVIFSYKGMNEDIKKD FKEFENKDKSUVVCFIVKHEOXIDCGGGYVEVFDCSLDOKDNEGETEYLLMFGPDICCFGTHWUVVFYKGKNEDIKKD FDGFSNEDKSLVVCFIVKHEOXIDCGGGYVEIFDCSLDOXDNEGETEYLLMFGPDICCFGTHWUVVFYKGKNEDIKKD	160
Ast	PD <mark>GFSMEDKS^I VVCPSVKHEQMIDCGGGYVEIPDCSLDOIDHEGESFYEIMFGPDICGFGTHEVHVI<mark>F</mark>SYKGMNEDICGFGTHEVHVI<mark>F</mark>SYKGMNEDISKD Fr<mark>ess</mark>NEDDT<mark>UVICFS</mark>VKHEQMIDCGGGYLE<mark>VFDCSVDOKDIEGETSYLVM</mark>FGPDICGFGTHEVHVI<mark>F</mark>SYKGMNEDING</mark>	157
Cqu	FRESNEDINIVIÇESVEHEQNIDCGGGYDEVEDCSVDQEDIHGDTEYLVMEGEDICGEGTERVEVIESVEGENELINED	160
Lva	FERENKDSELVICETVKHECHIDCGGGYLEVEDCELDOKDNHGE SEYLIMEGEDICGPGTREVHVIFLYKGENHEIRKE	158
Nvi	IRCKDDIYTHIYT FUYF EVIKEDNTYE ILIDNE FVESCUE EDWDFF PAKNIKDPTOSKPAUNDDRPTIPDE DRKE EWDWPE	239
Ppu	IRCKDD VYTH LYTIVIKEDNTYSILIDNERVESG LEEDWDED PAKHIKDELOSKPAIN DDKETIEDEND WERELWDRPE IRCKDD LYTH LYTIIVKEDNTYSULIDNEEVESG 212 ADWGED FFKHIKDESOTKPAIN DDKE ST DDF 20 %KPEIWDRPE	239 239
Pvi	IRCKDD II DI WILLVKEDATES VLIDAES VESGUEEDAGED PRAIKDES VIKERIAD DRECH DESDRAFSIA DREE IRCKDD VYTELYTLIVKEDATES VLIDAERVESG VLEDAGED PERSIKDES ARKEE IN DRECH DESDRAFSIA DREE	
Bmo	IRCKDIVITE FTIIVEDATE OIIDAEVESGIEDDWEEDPEKTROPARKEELWOORFIEDPEORKEELWORPE	240
Pra	IRCKDI VYTHIYTIIVN FONTYEVIIDNEFVESGELE DOWDED PAKFIKDESAKKEEINDERATIFOFID AKFELMEKPE IRCKDI VYTHIYTIVVKFONTYEVIIDNEFVESGELEADWDED FKKIKDE AKKPEINDDRATIFOFID AKFELMOKPE	240 240
Pxy	IRCKDD YYTHI YYTIIVKFDATHCYTDADAYUSGDEDDAUDE FARIADE AAAFELT DDAAIHDDHDAAPAA DAFE IRCKDD YYTHI YTIIVKFDATYEVIIDAE YESGDEDDAUDE PEKKIKDE AAAFELT DDAAIFEDHDAAPAA DAFE	240
Cru Dme	IRCKDD Y THE WILLYR DATHS VEIDADAY USGOLEDWALFPERKIKDEN SAKELED DRAITEDE DRAFFUNDRES IRCKDD YYTE WILLYR DATHS VEIDADAY USGOLEDWALFPERKIKDETA IRESER DRAFIEDE DRAFFUNDRES	239
Ast	IRCKDIVETHEVIIVERADITYEVIIDDEVECCULUURAANDEDEVECULUURAANDEN ANDELADEN ANDELEDEVECULUURAANDEDEVECULUURAANDEDEVEC	237
	IRCKDIVTHEYTIIVKADA HUVIDAG VUSGAD DA DE PRATKDE AKADIM DRADI DE DA KOMANA DA DE DA KADA DA DE DA KADA DA DE D IRCKDIVTHEYTIIVKADA HUVIDAG VUSGAD DA DE DE PRATKDE AKADIM DRADI DE DA KADA DA DE DA KADA DA DE DA KADA DA DE D	240
Cqu Lva	IRCKDFVF3ELYTLIVNFDNTYEVLIDNSK 1086312 DA DEP FRAIRDEZANKEDIWD DRPTIADELDUKEZDA DASF	238
		200
Nvi	HIEDE BATHETDWIDEMDGEWEAPMIDNEE EKGIWEEKÇIDNENYKGEWVHEEIINEEYLEDFIIYKHNEICIVGIDIWQ	319
Ppu	hiedeeaaheddwidemdgeweapwidnes xkgiwerkoidnexykeewvheeidneeyiedsilyrofficiveldlwo	319
Pvi	HIEDBOAT 3PS DWIDEMDGEWELPWIDNPD'KGOW, PKOIDNP <mark>Y</mark> KGOWIHPETINPEYTPD 92LYKKIE ICAVGODIWO HIEDPO <mark>ATHE</mark> DWIDEMDGEWELPWIDNPD'KGOWEPKOIDNPAFKGOWIHPEIINPEYTPD 3NLYKKIE ICAVGIDIWO	319
Bmo	HIEDEDATHERDWIDEMDGEWERPHIDHEDYKG WARKOIDNEAFKGEWUHEETUNEEYTEDENLYKKIETCAVGUDIWO HIEDEDATHERDWIDEMDGEWERPHIDHEYKG WARKOIDNEAFKGEWUHEETUNEEYTADETLYKKIE CAVGUDIWO	320
Pra	HIEDEDATHEEDWIDEMDGEWEEPHIDNEENKGJWEEKQIDNEATKGEWVHEEIDNEETADETLYKRIEECAVGIDLWQ	320
Pxy	HIEDELA AFRIDWIDEMDEENE PMIDNPDIKGSW ² PKCIDNPAYKCEWUHPEINPEYTEDASLYKK EKGAVGIDIWQ Tiedea <mark>th</mark> e dwidemdeene pwidnpdikGew ² PKCidnpa <mark>y</mark> kcewihpei <mark>npeyykdeelykkiejCaiced</mark> wQ	320
Cru	TIEDEPATEEDWIDEMDGEWE PNIDNEDEKGEWEYKGIDNEAKKEWIHEETNEEYKDEELYKKIE JCAIGEDIWQ HIEDEPATEEDWIDEMDGEWE PNIDNEEKGEW <mark>EYKGIDNENYKGWE</mark> HEEINEEYKEDHEYZEDEUGTIGEDIWQ	319
Dme	HIEDPLATE PEDWEDEMDGEWES PHIDNEPEKGEW, PKQLDNP NYKGWEHPEIEN PEY VED DELYERSE UCTIGED WQ	320
Ast	HIEDEN AT FELDWIDENDEND FRADREF KÖMFKÖNDEN KÖMFELT HELT HELT HELT HELT HELT HELT HELT H	317
Cqu	HIEDELATPELDWIDEMDGEWE EMIDNEENKGEN, PKOTDNES <mark>y</mark> kgiwiheeiinpeytedkiiyireekoweidiwq	320
Lva	<mark>hv</mark> eded <mark>a the</mark> edwidemdgewe pridnedikgen pro <mark>i</mark> dned <mark>n</mark> kgewiheet inpeytedeet takyte iChtoino	318
Nvi	VKSGTIPDNILLTIDPEYAKQFGQDVWKPTIEGEKEMKSSQINEEKKIKEQPAKDSSEDKEIDDEEDDVK	390
Ppu	VKE <mark>gtipdnidltidpe yak@pg@diwkptiegekkwksl@ineekklkeebakdnledve id</mark> dedd <mark>d</mark> dyk	390
Pvi	VKEGTIFDN, DITIDVE VARKEGEDVNHETLEGEKKNKDAGIEEERKORDRENKENENKIDEDDIEEDNIVPENEE	395
Bmo	VK GTIED IIID PAARKERGEVIKK.RCEGEKKMKSECIEAEREKAEKPDIEED DEDLDDET VK GTIED NED FIID PAARKERGEEIKK.KIEGEKKMKNECIEAEREKEKSEKPEIE. <mark>D</mark> DEDL <mark>D</mark> DEA	38.6
Pra	VKNGTIED HELETIDPAAAKCRGEEIKK. KIEGEKMENENENENENENENENEKSEKPEIE.DDEDLDDEA	385
Ржу	VRSGTTED NED TTID LE AARAVGE EVRR. TIE GERMANSQCIE AERERERES PROF DEEED DED LD D VE	38.6
Cru	VK GTIFD NEVITID LEARAVGE EVKN. TIEGEKKMKSQOIEAEREKSERP	391
Dme	VRAGTER HUDITED VELAAMAAA EV KN. TOAGEKKEKE AOTE VORHKDE EMAKKA SOKDDE DEDIDDEE KODE S	394
Ast	VK GTIED NE ITNDLE RAMA AATVKDIE GERKYAO OLA ERKAAE RAAR. AAR AAR DE DE DE ALM. VK GTIED NE ITNDLE RAMA AATVKDIE GERKYAE GERKAAE RAAR. AAR DE	389
Cqu	VROGTIFDING INDIE AANKA, AASFRET, EGERETIKEAOEAEERRAAEDPAKAAEDARDEDEIDEDDED.	391
Lva	VK <mark>EGTIEDNEDISNDPE SA</mark> BKIGE ETWGATI <mark>E AAKKM</mark> KDE <mark>CIEEERPRAESE</mark> AKAAADAEIDE <mark>D</mark> DIDEDLG	390
Nvi	DNNIPEIBEHDEL	403
Ppu	DNAIPEIEEHDEL	403
Pvi	NNIPDSDDDNNIPEIEE HDEL	416
Bmo	GDAAPVEEHDEL	398
Pra	AEGAPVEEHDEL	397
Pxy	GDAAFVDEHDEL	398
Cru	NTVPEVE 2 HDEL	403
Dme	KQDKDQS <mark>BHDEL</mark>	406
Ast	ALPGEATEVEDE. CHDEL SHAGQATEIDEE CHDEL	406
Cqu		409
Lva	DEDEDDLDNEL <mark>EHDEL</mark>	40.6

Fig.3. Amino acid sequence alignments of PvCRT with the homologues sequences of other insects and one crustacean. Species and Gen-Bank 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.

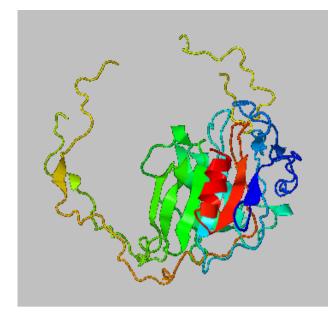


Fig.4. The tertiary structure prediction of PvCRT.

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.

Acknowledgment

This work was supported by the National Natural Science Foundation of China, NSFC (31171195). We thank two anonymous reviewers for contributions that helped to improve this article.

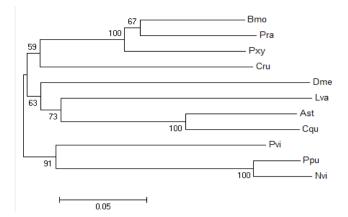


Fig.5. The phylogenetic tree shows the envolutionary relationship of PvCRT with CRTs of other insects on the basis of NJ method, confidence values based on 500 repeats are shown in the nodes; the abbreviation of species is given in Fig.3 and text.

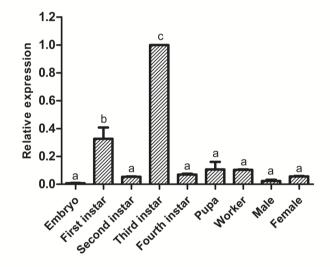


Fig.6. The relative expression profiles of PvCRT mRNA at different development stages and in different castes, the same letter is indicated that there are no significant differences between them (p<0.05).

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