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Cloning, Characterization and Expression Analysis of a Cyclophilin Gene from the Entomopathogenic Nematode *Steinernema carpocapsae* *

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Abstract: Cyclophilins (CYN) are ubiquitously present in divergent species from prokaryotes and eukaryotes. They are structurally conserved throughout evolution and most of them, if not all, possess PPIase activity. A cDNA encoding cyclophilin (Sca-CYN) was cloned based on *Steinernema carpocapsae* suppression subtractive hybridization analysis. The predicted protein contained 171 amino acid residues with a predicted molecular mass of 16.2 kD and a theoretical pI of 6.89. Subcellular localization was localized in cytoplasm. BLAST analysis showed 75%~89% identity to cyclophilin from nematodes, insects, and other organisms. Expression analysis revealed that the *Sca-CYN* was up-regulated during the initial parasitic stage. Sequence comparison and evolutionary marker analysis showed it was an orthologue of *Caenorhabditis elegans* CYN3. Homology modeling revealed that Sca-CYN adopts the typical structure of cyclophilin proteins and phylogenetic analysis indicates that Sca-CYN branched off early during the evolution. These data provide a basis for future research into the function of *Sca-CYN*, and offer further insight into the molecular evolution of nematode cyclophilin gene family.

Key words: entomopathogenic nematode; cyclophilin; bioinformatic; *Steinernema carpocapsae*
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The peptidyl-prolyl cis/trans isomerase (PPIase) (EC5.2.1.8) of protein is comprised of three known protein families, the cyclophilins (cyclosporin A binding proteins), FKBPs (FK506-binding proteins) and parvulins. They are found widely distributed and highly conserved in diverse prokaryotic and eukaryotic major compartments of the cell[4-6]. The diverse roles and still emerging new functions suggested that organisms[1-3], implying that their function is required in cellular processes from bacteria to man, and in the cyclophilins processing more functions than already defined roles in protein folding. However, the mechanisms by which they fulfill these cellular events have been difficult to establish and are still largely unclear yet.

The cloning and characterization of cyclophilin genes from nematode *Haemonchus contortus*, *Caenorhabditis elegans* and other nematode species provides an excellent opportunity to study the various biological roles played by representatives of all the cyclophilin subfamilies within this system. The immunosuppressant drug cyclosporin A has been shown to have an effect on parasitic helminth worms, causing structural tegumental damages. A possible mechanism for this anti-parasitic effect is the inhibition of proper cyclophilin activity[7-9] resulting in production and accumulation of misfolded protein. Detailed structural information on the nematode cyclophilins may therefore provide useful targets for the design of new families of anti-parasitic drugs.

In this study, we described the characterization of a gene encoding a cyclophilin protein that was identified during the analysis of a suppression subtractive hybridization (SSH) library of entomopathogenic nematode

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Steinernema carpocapsae. The expression patterns in different developmental stage and insect hemolymph induced nematode were also investigated. The expression pattern, when considered together with emerging structural and sequence data, provides an insight into the possible roles played by cyclophilins in multicellular organisms.

1 Materials and methods

1.1 Nematode collection and induction

Infective juveniles (IJs) were multiplied in *Galleria mellonella* larvae and harvested in a white trap[10]. To induce recovery to the parasitic stage, IJs were surface sterilized with 0.5% sodium hypochloride, induced by 1% insect hemolymph plus 1% antibiotic (penicillin-streptomycin-neomycin, Sigma). Induced nematodes were collected at 0, 6, 12, 24, 36, 48 and 72 h, immediately frozen in liquid nitrogen and stored at −80 ℃ for further analysis.

1.2 Suppression subtractive hybridization (SSH) screening and gene identification

Total RNA was isolated using the Trizol reagent (Invitrogen, USA). Suppression subtractive hybridization (SSH) was performed using the PCR-Selected cDNA Subtraction Kit (Clontech, USA) and differentially expressed gene screening was done by reverse Northern blot[11]. Homology searches were carried out using the Blast against GenBank database.

1.3 Full-length cDNA cloning

Rapid amplification of cDNA ends (5' RACE) was conducted to obtain full-length cDNA of cyclophilin using the SMARTTM RACE cDNA Amplification Kit (Clontech-Takara, UK) under the manufacturer's instruction. Gene specific primer ScCYN (5'-GGCGTTCCTTGAAGTTCTCGTCGTTG-3') was designed based on the EST fragment in SSH library. The PCR condition was as follows: 30 cycles of 94 ℃ for 30 s, 68 ℃ for 30 s, and 72 ℃ for 3 min, and a final extension at 72 ℃ for 5 min. The amplified fragment was cloned into the pCR4-TOPO vector, and then transformed into *E. coli* DH5α cells. Positive clones were screened and sequenced. The full-length cDNA was obtained by joining the two fragments.

1.4 Sequence analysis and subcellular localization

Sequences similarity analysis was performed using BLAST program. Domain was identified using the Conserved Domain Database sever in NCBI with the Conserved Domain Architectural Retrieval Tool (CDART), SMART (http://smart.embl-heidelberg.de/) and MotifScan (http://motifscan.stanford.edu/). The theoretical isoelectric point and molecular weight were predicted using Compute pI/Mw (http://web.expasy.org/compute_pi/); Sub-cellular localization was predicted by WOLFPSORT (http://wolfpsort.org/).

1.5 Gene expression analysis

Total RNAs from the different development stages or induced nematodes were isolated and reverse-transcribed into cDNAs. Relative gene expression of Sca-CYN was detected by real-time RT-PCR using 18S rRNA as an endogenous control. Primers for 18S rRNA were 18SF (5-GCTAATCGGAAACGAAAGTC-3) and 18SR (5-CATCCACCGAATCAAGAAAG-3). Primers for cyclophilin forward and reverse were (5-TACAAGGGCTCCAAGTTCC-3) and (5-CTTGACGATGTCCAGTCC-3). Real-time RT-PCR was performed using SYBR Green Mix and the thermal cycle conditions were as follows: 95℃ for 10 min, and 40 cycles at 95 ℃for 15 s and 60 ℃ for 60 s. qRT-PCR data from three replicate samples were analyzed with the Relative Manager Software (Applied Biosystems, USA) to estimate transcript levels of each sample using the $2^{\Delta\Delta Ct}$ method[12].

1.6 Phylogenetic analysis

Multiple sequence alignments were generated using Muscle in MEGA5.0[13] and were refined manually using BioEdit7.0. Unambiguously alignment positions were used for subsequent phylogentic analysis. A Bayesian inference tree was reconstructed using MrBayes v3.1.2[14] under the best evolution model (VAG+G) determined by the ProtTest3 program[15]. Four independent Markov Chain Monte Carlo (MCMC) chains were used with the default temperature of 0.01. The analysis was run for 200 000 generations with a sample frequency of 100 and burn-in was set to correspond to 25% of the sampled tree.

1.7 Homology modeling

Web-available ESyPred3D (http://www.fundp.ac.be/sciences/biologie/urbm/bioinfo/esypred/) and 3Djigsaw (http://www.bmm.icnet.uk/servers/3djigsaw/) server were used to find the best structural templates. Then SWISS-MODEL server (http://swissmodel.expasy.org/) was applied to create a 3D structural model.

2 Results

2.1 Cloning and characterization of cyclophilin

Suppression subtractive hybridization (SSH) was used to identify differentially expressed genes involved in the host-parasite interactions of entomopathogenic nematode S. *carpocapsae* and their insect hosts. Analysis of the SSH library revealed that a partial clone encoding for a *C. elegans* cyclophilin homolog [11]. The preliminary screening of selected gene by RNA blot analysis confirmed that it was up-regulated by insect hymolymph induction.

The final cDNA sequence (Genbank Accession DQ861405) was obtained by assembly of the sequence of the EST clone and a 5' end sequence from a 5' RACE product. The complete cDNA encoded 171 amino acid residues (designed as Sca-CYN). The predicted mature protein would have a molecular mass of 16.2 kD and an isoelectric point of 6.89. It was localized in the cytoplasma and contained two potential N-glycosylation sites (N^{57} and N^{94}). Motif analysis revealed that Sca-CYN had a conserved PPIase domain (2.8e−48) and its signature (YKGSKFHRVIPNFMIQGG). Sequence logo alignment of the conserved signature was created (see Fig. 1 on the inside back cover of the issue) and highly conserved in cyclophilin family.

BLAST analysis showed that Sca-CYN had 75%～89% identities at AA level to many nematode cyclophilin proteins. It showed 89% identity with *C. briggsae* CYN3 and 86% with *C. elegans* CYN3. Based on the size and sequence identity, Sca-CYN was identified as an orthologue of *C. elegans* CYN3 (see Fig. 2 on the inside back cover of the issue).

2.2 Gene expression analysis

Expression analysis throughout the life cycle in the parasitized insect *G. mellonella* larvae revealed that Sca-CYN was up-regulated in all larvae stages after infecting insect (Fig. 3A). Since the *Sca-CYN* gene was initial isolated from insect hymolymph induced nematodes, we want to investigate the kinetics of the induced expression of this gene. As shown in Fig. 3B, resistant stage nematodes quickly responded to insect homogenates *in vitro*, displayed a low level expression after 6 h induction, then significantly increased at 12 h and remained relatively constant until 48 h.

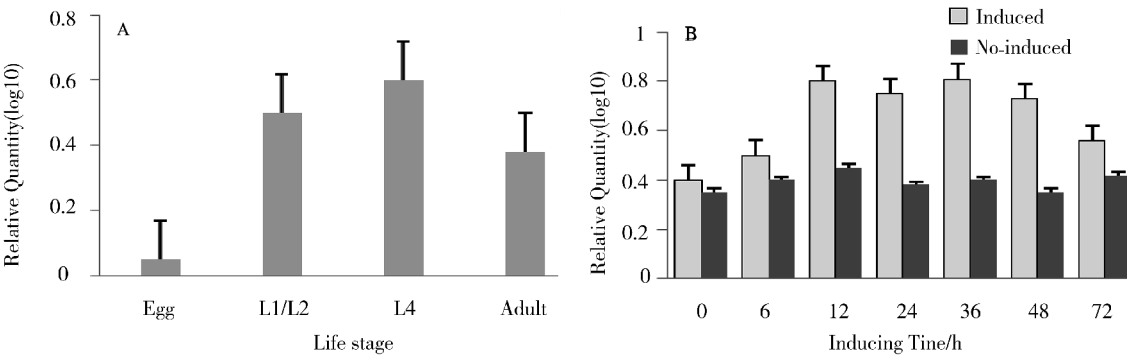


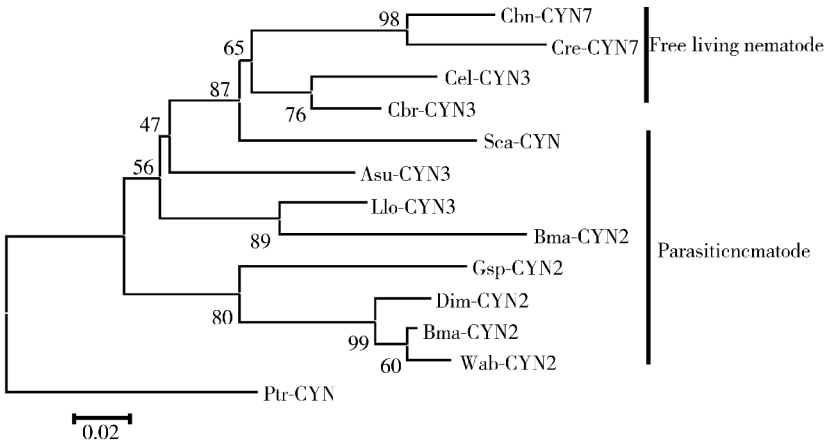
Fig. 3 Sca-CYN gene expression throughout the life cycles (A) and in response to insect homogenate induction (B). 18S rRNA was used as an endougenous control

2.3 Phylogenetic analysis

To shed light on the evolutionary position of Sca-CYN, a phylogenetic tree was constructed based on the deduced amino acid sequences from other nematode species (Fig. 4). This analysis indicated that Sca-CYN was clustered with cyclophilin from free-living nematode *C. remanel* and *C. brenneri* and animal parasite *C. briggsae*. In this group, Sca-CYN formed an ancestral branch, suggesting that Sca-CYN branched off at an early stage of the cluster divergence.

2.4 Homology modeling

The structural homology of Sca-CYN was obtained with SWISS-MODEL server based on the cyclophilin structure of *C. elegans* (PDB ID: 1dywA). Both Sca-CYN and template have eight β-sheets and two helixes (see Fig. 5 on the inside back cover of the issue).



Notes: Numbers after internal branches indicated the posterior probability (%) for each branch. GenBank accession number (upside down) was: Cbn-CYN7 (*Caenorhabditis brenneri*, EGT45232), Cre-CYN7 protein (*Caenorhabditis remanei*, EFO99944), Cel-CYN3 (*C. elegans*, NP_506751), Cbr-CYN3 (*C. briggsae*, CAP35999), Sca-CYN (*S. carpocapsae*, ABI18377), Asu-CYN3 (*Ascaris suum*, ERG84468), Llo-CYN3 (*Loa loa*, EJD75189), Bma-CYN3 (*Brugia malayi*, EDP30282), Gsp-CYN2 (*Gnathostoma spinigerum*, ACX47902), Dim-CYN2 (*Dirofilaria immitis*, AAC47232), Bma-CYN2 (*B. malayi*, EDP28522), Wba-CYN2 (*Wuchereria bancrofti*, EJW85904) and Ptr-CYN (*Populus trichocarpa*, EEE87691).

Fig. 4 Phylogenetic tree of Sca-CYN with the other nematode cyclophilins inferred by Bayesian method using Populus trichocarpa cyclophilin as the outgroup

3 Discussion

In this study, the full length cDNA encoding cyclophilin was cloned based on the EST sequences in SSH library of *S. carpocapsae*. Sequence analysis showed that it was a single PPIase domain with the typical signature of cyclophilin family. BLAST analysis revealed that Sca-CYN has high identity with many cyclophilins from nematodes, insect, plant and other organisms.

Bioinformatic analysis showed that *Sca-CYN* gene was an orthologues of *C. elegans CYN3*. The clustering of Sca-CYN with orthologues in other nematode species provided clues to potential roles of these proteins. The result showed that Sca-CYN was grouped with CYN3 of animal parasite nematode *C. briggsae*, *C. remanel* and free living nematode *C. elegans*, and formed an ancestral branch, suggesting that Sca-CYN branched off at an early stage of the cluster divergence. *C. elegans Cyn-3* encodes a cyclophilin A isoform and is not detected for peptidyl-prolyl isomerase activity *in vitro*[16]. Sequence analysis showed that it is very similar to CYN-3 and closely resembles the prototypical hCypA, and is therefore likely to be a member of the cytosolic CsA-binding cyclophilin subfamily. CYN-3 was required for embryonic viability in one set of mass RNAi assays[17]. There was growing evidence that pathogene cyclophilins, in particular homologs of hCypA, play a role in the infection of both plant and animal hosts[18-19]. The expression pattern of *Sca-CYN* during the development and in the induced nematodes revealed its important role during the whole life cycles or in the host-parasite interaction processes. In addition, the cyctosol localization suggested that Sca-CYN is likely to chaperone or help the folding of cytoplasmic proteins. Our results provided a framework for future studies of the function of cyclophilins in the entomopathogenic nematodes.

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动物科学

昆虫致病性斯氏线虫亲环素基因的克隆、特征及表达分析

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摘要:亲环蛋白(Cyclophilin)广泛存在于原核和真核生物中,在进化过程中结构保守,多数具有肽基脯氨酰基顺反异构酶活性。本文在斯氏线虫(*Steinernema carpocapsae*)差减杂交分析的基础上,克隆一个亲环蛋白基因(*Sca-CYN*)。该胞质蛋白有 171 个氨基酸组成,分子量约为 16.2 kD,等电点约为 6.89。BLAST 分析表明,Sca-CYN 蛋白与线虫、昆虫及其它生物亲环蛋白的序列一致性达 75%～89%。基因表达分析显示该基因在寄生初期就上调表达。序列比较及进化标记分析发现 Sca-CYN 为秀丽隐杆线虫亲环蛋白 3 的直系同源蛋白。同源建模分析显示该蛋白具有亲环蛋白的经典三维结构。系统发育分析结果表明该蛋白在进化早期与其同源基因分离。研究结果为进一步研究该基因功能及探讨线虫亲环蛋白基因家族的分子进化提供基础。

关键词:昆虫致病性线虫;亲环蛋白;生物信息学;斯氏线虫

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