Identification and Expression Analysis of a cDNA Encoding Cyclophilin A from *Gryllus bimaculatus* (Orthoptera: Gryllidae)

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Abstract: - Cyclophilin A (CypA), a cytosolic binding protein of cyclosporine A, is an immunosuppressive drug. In this study, CypA cDNA was cloned from the two-spotted cricket *Gryllus bimaculatus* (*gCypA*). The protein encoded by gCypA comprises 165 amino acids with a molecular mass of 19.23 kDa and an isoelectric point of 9.38 and possesses three N-glycosylation sites and 17 phosphorylation sites. The secondary and tertiary structures of gCypA were identified, and homology analysis revealed that it shares around 73%-81% sequence identities with other CypA proteins. When the researchers analyzed the expression levels of *gCypA* mRNA in various tissues, they found that the foregut exhibited nearly the same expression level as that of the dorsal longitudinal flight muscle (the control). However, *gCypA* mRNA expression in the fat body, Malpighian tubes, and midgut was less than half of that in the dorsal longitudinal flight muscle. Under endoplasmic reticulum stress conditions, *gCypA* mRNA expression was highest in Malpighian tubules (about two times higher than the expression in the control). Under starvation conditions, gCypA mRNA expression increased to three times that of the dorsal longitudinal flight muscle 6 days after starvation. Nonetheless, its expression levels decreased in Malpighian tubules under all starvation conditions. This study provides insights into the physiological role of gCypA in *G. bimaculatus*.

Keywords - *Gryllus bimaculatus*, Cyclophilin A of *G. bimaculatus* (gCypA), endoplasmic reticulum (ER) stress, starvation

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1 Introduction

Cyclosporin A is a metabolite from telluric fungi of *Tolypocladium inflatum gams*. It has been developed as an immunosuppressant drug, [1]. After organ transplantation, cyclosporin A can be used to inhibit graft rejection, [2]. At a certain period after administration, cyclophyllin proteins can bind to cyclosporin A, [3]. Among cyclophilines, cyclophilin A (CypA), an evolutionarily and three-dimensionally preserved protein from bacteria to mammalian, is a ubiquitously distributed intracellular protein that plays a crucial role in several cellular functions, such as protein folding for chaperone signal pathway, apoptosis, and transcriptional regulation, [4], [5]. CypA can form a ternary complex with cyclosporin A to inhibit the transcription of genes involved in immune responses of mammals, [6]. Recent studies have demonstrated that CypA has critical functions in various human diseases such as cardiovascular diseases, type 2 diabetes, viral infections, neurodegenerative diseases, aging, periodontitis, sepsis, asthma, rheumatoid arthritis, and cancer, [7], [8]. CypA has also been suggested to play a crucial role in the innate immune system of insects. For instance, at least nine cyclophilins are known in *Drosophila melanogaster*, [9]. One CypA cDNA from *Blattella germanica* and one CypA cDNA from *Bombyx mori* have also been reported, [10], [11]. However, the function of CypA protein and its detailed mechanism in insects remain unclear.

The two-spotted cricket, *Gryllus bimaculatus* (Orthoptera: Gryllidae), is the most commercially noteworthy cricket species. It is used as an experimental model insect and feed for poultry, [12], [13]. *G. bimaculatus* has chromosomes of 2n = 28 + XX (F)/XO (M). Its estimated genome size is 1.8 Gb, [14], [15]. Several types of gene manipulation systems, RNA interference (RNAi) and transcription activator-like effector nucleases (TALEN) have been applied directly and specifically to *G. bimaculatus* individuals, [16], [17]. *G. bimaculatus* has recently been considered an experimental animal. It has attracted attention in the field of biomedical science, [18]. Identifying the biological function in an individual simpler than mammalian is suitable for determining new molecular mechanisms of CypA. It will help us develop novel pharmacological therapies. The main goals of this research were: (I) to clone a cDNA of *CypA* from *G. bimaculatus* (*gCypA*), (II) to investigate gCypA molecular characteristics including tissue distribution of *gCypA* mRNA expression, homology analysis, and structure prediction, and (III) to determine *gCypA* mRNA expression levels in response to starvation and endoplasmic reticulum stress.

2 Materials and Methods

Fifth-instar larvae of *G. bimaculatus* were obtained from the Rural Development Administration of Korea (RDAK). They were reared at 28°C to 30°C with a humidity of 70% under a 10 h/14 h light/dark photoperiod in plastic cylinders. Crickets were provided with food (rat and rabbit food at 1:6) and water. Synchronously grown male cricket adults (5 instars) were used for the experiments. During a six-day starvation period, only water was provided for crickets. During re-feeding, 30% dextrose (carbohydrate), 30% casein (protein), and 40% cellulose containing a drop of commercial soybean oil (lipid) were supplied. An endoplasmic reticulum (ER) stress cricket model was prepared using tunicamycin (Sigma Chemical, St. Louis, MO, USA). The injection site was the 11th abdominal cavity of the insect. Tunicamycin (5 μL) was intraperitoneally administered to the abdominal segment using a syringe (10 μL).

G. bimaculatus was anesthetized by exposure to CO2 gas. The body was incised ventrally from the last abdominal segment to the neck. Each tissue was obtained under a virtual microscope (Nikon Eclipse E600). Total RNA was extracted from each tissue using a TRI^{zol} reagent (Invitrogen, Carlsbad, CA, USA) and treated further with Rnase free Dnase-I. A marathon cDNA amplification Kit (Clontech, Palo Alto, CA, USA) was used to construct a cDNA library using 1.5 μg of mRNA as a template. BLAST search was used for gene identification. PCR was performed using primers designed with Primer3 (http://simgene.com/Primer3) based on Conserved Domain Databases from National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) and Motif Databases (GenomeNet, Institute for Chemical Research, Kyoto University, Japan). The number of PCR cycles was optimized to obtain a linear range of amplification. RT-PCR was performed using primers *gCypA*-F 5'-GTCGCGTCAATCTAGTGTATG-3', *gCypA*-R 5'-TCAAGAAAGTTGGCCGCAATT-3'. The PCR program was: 30 cycles of 94℃ for 30 sec, 58℃ for 30 sec, and 72℃ for 1 min; followed by a final extension step at 72℃ for 10 min. DNA fragments of the PCR product were subcloned into TOPO^{TM} TA Cloning plasmid (Invitrogen) and confirmed by sequencing. Other chemicals and drugs not described were purchased from Sigma Chemical. Open reading frame, molecular weight, and

theoretical isoelectric point were determined with the ExPASy server (http://www.expasy.org/). Multiple protein sequence alignment was performed with the NCBI server (http://www.ncbi.nlm.nih.gov/). Protein structures (secondary and tertiary) of gCypA were constructed using both DTU Health Tech of NetSurfP-3.0 (https://services.healthtech.dtu.dk/service.php?NetSurfP-3.0) and SWISS-MODEL (https://swissmodel.expasy.org/interactive/UVytRb/models).

Each tissue was selected from the insect under a dissecting microscope (Olympus SZ51) and placed into a 1.5 mL Eppendorf Tubes® with 100 µL TRI^{zol} reagent (Invitrogen). It was then homogenized with a plastic pestle (SP Scienceware, Wayne, NJ, USA). Total RNA was extracted with TRI^{zol} reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the company's instructions. After quantifying the purified total RNA with a NanoDrop Lite UV-spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), a cDNA library was constructed with a Superscript II™ First Strand Kit (Invitrogen). The cDNA was PCR-amplified with primers of *gCypA*-F (5'-CGTGCTTTATGCACTGGAGA-3′) and *gCypA*-R (5'-GAAAAACTGGCTGCCGTTAG-3′). RT-PCR conditions were: 30 cycles of 94° C for 30 sec, 58° C for 30 sec, and 72°C for 1 min; one extension step at 72°C for 10 min using both primers *gCypA*-F and *gCypA*-R. Data are presented as mean ± SDM (one-way ANOVA: **, $P < 0.005$; ***, $P < 0.0005$) (Fig. 4).

3 Results and Discussion

A cDNA encoding cyclophilin A (*CypA*) was isolated from a *G. bimaculatus* (*gCypA*). It was deposited into GenBank with an accession number of MN205431.

Fig. 1: Nucleotide and deduced amino acid sequences of gCypA. Three N-glycosylation sites are indicated by circles and seventeen phosphorylation sites for serine and threonine are indicated by shadow boxes. Two regions for peptidyl-prolyl cis-trans isomerase (PPIase) activity are indicated by two boxes.

It encoded a protein of 165 amino acids in length with a theoretical pI of 9.38 and an Mw of 19.23 kDa (Fig. 1). Although the genome size of *G. bimaculatus* has been determined to be 1.8 Gb, information on its 2n = 28 + XX chromosomes is insufficient, [14], [15]. Information about its chromosomal location and genomic exon & intron of *gCypA* has not been reported yet. Limited *G. bimaculatus* genes and BLAST information can be obtained at https://gbimaculatusgenome.rc.fas.harvard.edu/.
Protein N-glycosylation is one important metabolic process in the ER lumen in which a newly synthesized secretory protein undergoes a post-translational modification to provide a diversity of both structures and functions highly conserved in evolution, [19], [20]. Phosphorylation of protein is distinctly associated with protein activity or inactivity, which is one of the important regulations for protein function and cell signaling, [21]. Aberrant N-glycosylation and/or phosphorylation is strongly linked to several types of diseases, [22], [23]. Results of sequence analysis of gCypA protein (Fig. 1) revealed that it had three N-glycosylation sites (circle) and 17 phosphorylation sites (grey shadow) (10 serines, 5 threonines, and 2 tyrosines). A typical CypA protein exhibits PPIase activity important for several signaling pathways of eukaryotic cells, [24], [25]. A gCypA protein has two highly conserved signature sequences of PPIase (29RITMELRSDVVPKTAENF46 and 58YKGSTFHRVIPHFMCQGG75) indicated by two boxes in Fig. 1.

The sequence of gCypA protein was compared with those of 10 other CypA proteins (Fig. 2). It was found gCypA shared 85% sequence similarities with *Cryptotermes secundus* (drywood termite) CypA. It shared 81% sequence similarities with *D. melanogaster* (fruit fly) and *Galleria mellonella* (the greater wax moth) CypAs. It also shared 76% and 73% sequence similarities with *Danio rerio* (zebrafish) CypA and *B. mori* (silkworm) CypA, respectively. It is a protein that is well preserved among species, showing 76% homology with *Homo sapiens* CypA. Its N-terminal sequence showed more variations than its C-terminal sequence. Its two functionally important regions (boxed 29R-F46 and 58Y-G75 in Fig. 1) for PPIase activity are highly conserved. It has been suggested that gCypA is highly conserved among various species for

several cellular functions. Information about protein structures can provide valuable insights into binding proteins, carry atoms, and small molecules for regulating their functions in cells. Thus, we predicted gCypA secondary structure using DTU Health Tech of NetSurfP-3.0 (https://services.healthtech.dtu.dk/service.php?NetSurfP-3.0).

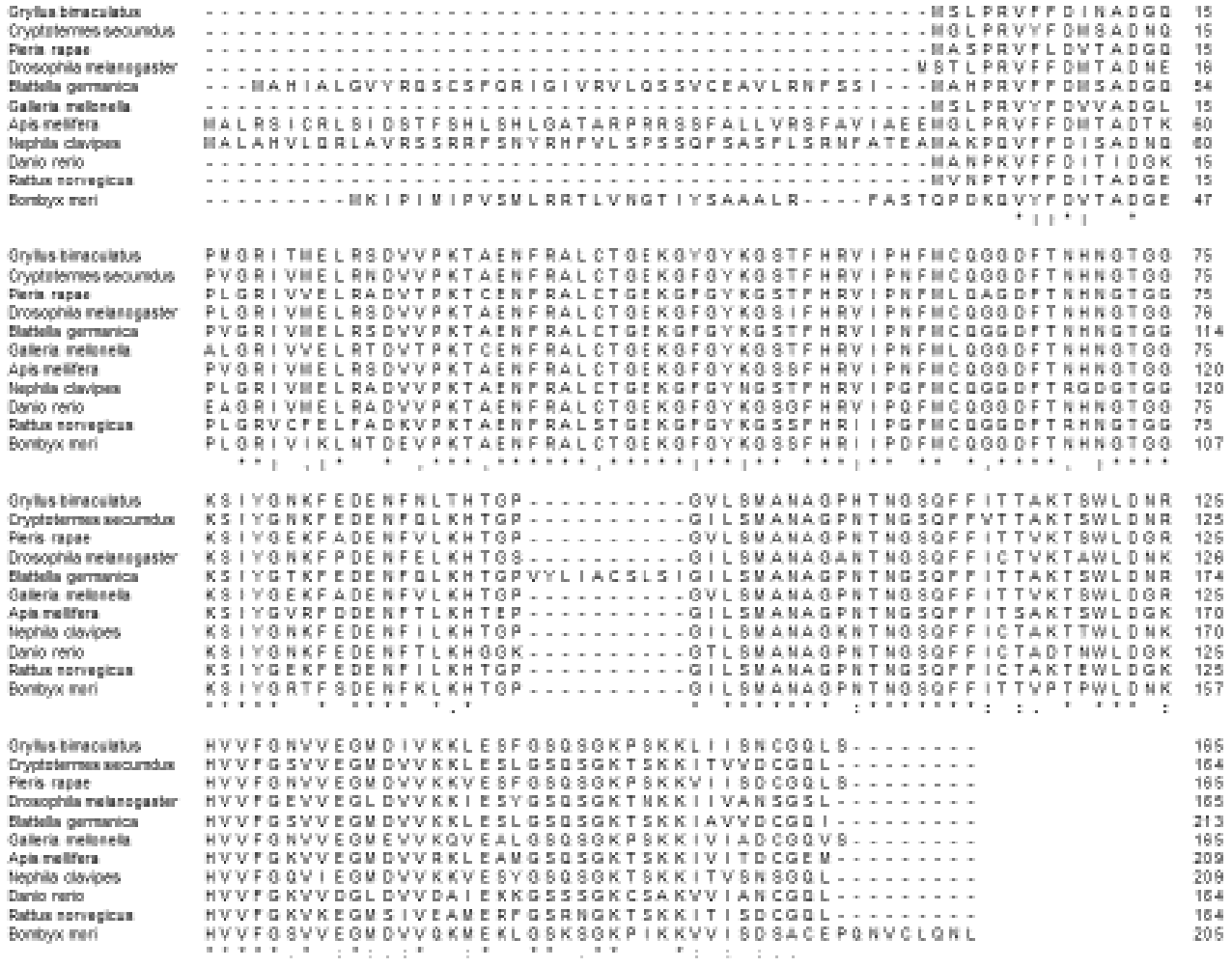

Fig. 2: Comparison of CypA sequences. Identical amino acid residues in this alignment are indicated by stars (*). Highly conserved regions of amino acid residues are indicated by colons (:). Weakly conserved regions of amino acid residues are indicated by points (.). Deleted positions in the amino acid residues are indicated by dashes. Species and gene accession numbers are as follows: *Cryptotermes secundus* (PNF42238), *Pieris rapae* (XP_022118540), *Drosophila melanogaster* (NP_523366), *Blattella germanica* (PSN50434), *Galleria mellonella* (XP_026755402), *Apis mellifera* (XP_006620611), *Nephila clavipes* (GFS56219), *Danio rerio* (NP_001315353), *Rattus norvegicus* (NP_058797), and *Bombyx mori* (XP_021206182).

The secondary structure of gCypA was predicted based on its 165 amino acids of one strand. It has three alpha helices (K31-C40, S120-L122, D137-H144), 14 random coils (M1-P4, D13-G14, R25-P30, T41-S51, I57-F60, G65-S77, Y79-G96, N102-Q111, A117-T119, D123-H126, G135-M136, F145-K151, S153-K155, L164-S165), and 9 extended strands (R5-A12, Q12-L24, T52-V56, I78, V97-A101, F112-T116, V127-E134, P152, L156-Q163). As shown in Fig. 3, a tertiary structure of gCypA was derived from its secondary structure using SWISS-MODEL. The predicted structure with no signal peptide was foundbased on SignalP analysis, suggesting that the gCypA protein is not secreted. It is located in the cytoplasm. The evolutionary position of gCypA was determined by comparing it with other CypAs by NJ analysis. A total of 11 amino acid sequences including gCypA were used for the analysis (Fig. 3). The overall tree topology showed that *Galleria mellonella* (the greater wax moth) and *Pieris rapae* (cabbage butterfly) CypAs were evolutionarily closer to gCypA among insect

CypAs. Among insect CypAs, *Apis mellifera* (western honeybee) CypA and *B. mori* (silkworm) CypA had the most distant evolutionary relationships with gCypA.

Using dorsal longitudinal flight muscle (DL) *gCypA* (Fig. 4) expression level as a control, relative *gCypA* expression levels in *G. bimaculatus* tissues were determined by RT-PCR (Fig. 4A). Results showed that *gCypA* mRNA expression level in the foregut (FG) was similar to that in the DL. However, its expression levels in most tissues were less than that in the DL. Its expression levels in Malpighian tubules (MP), midgut (MG), and fat body (FB) were 0.5 times or less than that in the DL. Expression levels of *gCypA* in three types of muscles tested showed the following order: DL > dorsal-ventral flight muscle (DV) > dorsal wing flight muscle (DW). It was found that *gCypA* was expressed in all tissues tested, although there was a difference in its expression level.

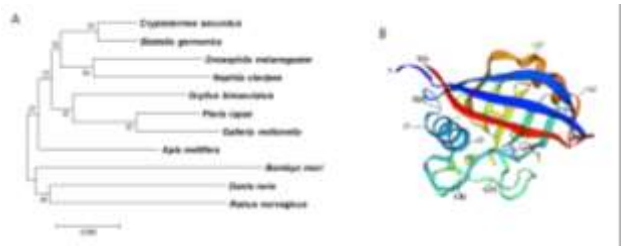

Fig. 3: Evolutionary relationships and predicted tertiary structure of gCypA protein. (A) A phylogenetic tree is drawn to scale, with branch lengths of the same units as those of the evolutionary distances used to infer the phylogenetic tree. (B) Three-dimensional structure of gCypA protein predicted by SWISS-MODEL. Two regions of PPIase activity (29R-F46 and 58Y-G75) and two alpha helices (31-40 and 137-144) are shown.

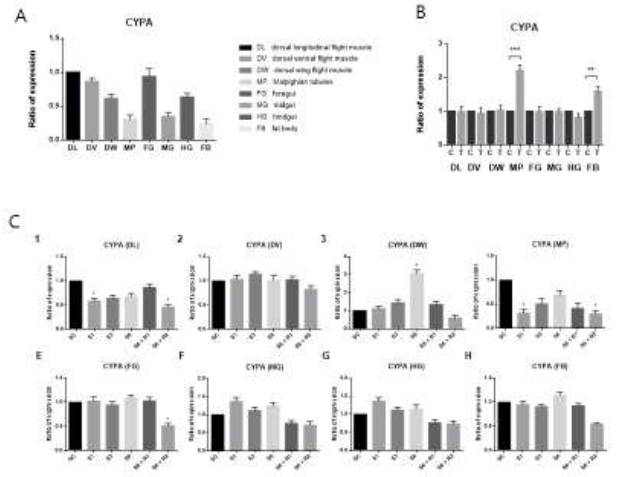

Fig. 4: Gene expression of *gCypA* under ER stress, starvation, and/or re-feeding conditions. (A) *gCypA* mRNA expression pattern under normal condition. (B) *gCypA* mRNA expression pattern under ER stress condition. (C) *gCypA* mRNA expression pattern during starvation for 1 day, 3 days, or 6 days and during 1 day or 2 days of re-feeding after a 6-day starvation.

The unfold protein response (UPR) is a mechanism for maintaining ER homeostasis, [26]. It is associated with several human diseases such as high blood pressure, diabetes mellitus, Parkinson's disease, dementia, cancer, and autoimmune & inflammatory disease, [27], [28]. Although the role of CypA in UPR remains unclear, it has been recently demonstrated that CypA can induce ER stress in human tubular cells, [29]. As described in *MATERIALS & METHODS*, tunicamycin was injected into *G. bimaculatus* to induce ER stress. Gene expression of *CypA* in each tissue was then investigated. We observed no notable *CypA* gene expression in tested issues of *G. bimaculatus* except that its expression was upregulated two-fold in the MP and FB (Fig. 4B). It has already been reported that insect FB is an organ analogue to the vertebrate liver and that *D. melanogaster* MP encounters elevated levels of ER stress by performing normal secretory functions, [29]. Although why *gCypA* gene expression is enhanced in MP and FB under ER stress remains unclear, the same results have already been reported in other insects, [30]. *CypA* has some active functions against ER stress. For example, it can act as an antioxidant to exhibit protective effects on virus-induced liver sizes, acetaminophen-induced liver toxicity, liver inflammation, and fibrosis, [31], [32].

Starvation is the most commonplace and influential stress for insects that are relatively environmentally sensitive and dependent. Therefore, insects must adapt to starvation to maintain environmental homeostasis through various mechanisms, including rapid gene expression and physical homeostasis. It has been reported that starvation can regulate some gene expression, such as allatotropin in *Mythimna separata* (northern armyworm) and tissue-peculiar genes in *Formica exsecta* (narrow-headed ant), [33], [34], [35]. We have reported starvation-associated troponin complex (TnT, TnI, and TnC), tropomyosin, ER stress-associated proteins (ATF6, BiP, PDI, and calreticulin), digestive enzyme genes (amylase, trypsin, and lipase), lethal (2) essential for life gene, and autophagy induction in Malpighian tubules of *G. bimaculatus*, [36], [37], [38], [39], [40], [41]. Fig.

4C shows *gCypA* mRNA expression after starvation for 1 to 6 days and re-feed for 1 to 2 days following 6-day starvation in tested tissues of *G. bimaculatus*. During starvation for 1 to 6 days, remarkable *gCypA* mRNA expression changes were observed in both the DW and MP. *gCypA* mRNA expression was increased by about three times in DW. The highest was found at 6 days after starvation. Meanwhile, in MP, it was reduced to 0.3 times at 1 day after starvation. It was then gradually increased to 0.7 times at 6 days after starvation. However, it was expressed at 0.5 times or less after re-feeding. A very interesting result here was that *gCypA* mRNA expression was lower in all tissues upon re-feeding after starvation. Its expression was decreased further after two days of re-feeding than that after one day of re-feeding. Currently, data associated with this result have not been reported. To understand the new function of CypA, investigating why *gCypA* mRNA expression is downregulated in tissues upon re-feeding after starvation will provide an important clue.

4 Conclusion

Cyclophilin A (*gCypA*) was cloned from a two-spotted crick, *G. bimaculatus*. It encodes a protein of 165 amino acids in length with an isoelectric point of 9.38 and an MW of 19.23 kDa. It contains three N-glycosylation sites and 17 phosphorylation sites. It shares about 73%-81% of sequence identities with other known CypAs. Secondary and tertiary structures of gCypA were determined using bioinformatics techniques. Expression levels of *gCypA* mRNA in the fat body, MP, and MG were less than 50% of that in the dorsal longitudinal flight muscle as a control. ER, stress enhanced *gCypA* mRNA expression in both MP and FB (about 2 times higher than that in others). The highest *gCypA* mRNA expression of about 3 times was observed in DW after 6 days of starvation. All these expression levels were reduced by starvation and re-feeding in MP. This study on gCypA is expected to give a clue for understanding the molecular levels of CypA protein.

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