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- Note -

Lethal (2) Essential for Life [*l(2)efl*] Gene in the Two-spotted Cricket, *Gryllus bimaculatus* (Orthoptera: Gryllidae)

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A cDNA encoding the protein lethal (2) essential for life [*l(2)efl*] was cloned from *Gryllus bimaculatus* and named *GBl(2)efl*. This protein is composed of 189 amino acids, including an N-glycosylation site and 15 phosphorylation sites. Its predicted molecular mass is 21.19 kDa, with a theoretical isoelectric point of 6.2. The secondary structure of GBl(2)efl was predicted from the identification of random coils (56.08%), alpha helices (22.22%), extended strands (17.99%), and beta turns (3.7%) through sequence analyses. A homology analysis revealed that GBl(2)efl exhibited a high similarity with other species at the amino acid level, ranging from 52% to 69%. While *GBl(2)efl* mRNA expression was higher in the dorsal longitudinal flight muscle following a three-day starvation and in the Malpighian tubules following a one-day starvation, no changes in expression were detected in other tissues. Furthermore, tunicamycin-induced endoplasmic reticulum (ER) stress resulted in an approximately 1.8-fold higher expression in the fat body compared with the wild type.

Key words : Endoplasmic reticulum (ER) stress, *G. bimaculatus l(2)efl* [*GBl(2)efl*], lethal (2) essential for life (*l(2)efl*), two-spotted cricket *Gryllus bimaculatus*

Introduction

Insects are the most diverse and abundant animals on Earth, greatly impacting natural ecosystems and human socioeconomics. These creatures have developed adaptability to almost all types of food over millions of years; in other words, various mouth-shaped and digestive organ adaptations have led to the intake of all kinds of fluids or solid food that are of plant or animal origin. However, much is unknown regarding their physiology and evolution of rapid adaptive mechanisms to environmental changes. In insects, starvation is one of the most common and difficult stressors, as a stable food supply is the most important factor for insect survival. If insects do not consume enough food within a certain period, growth and breeding are greatly reduced. However, insects can overcome disadvantageous external food shortages through mechanisms such as improving the availability of new foods, locomotion, and limiting metabolism [16]. Furthermore, there are reports of active gene regulation in some insects (*Mythimna separate, Aedes aegypti,* and *Formica exsecta*) in response to starvation [7, 13, 17]. It has been reported that aphids also break down their muscles and use them as an energy source to overcome serious external environments.

The two-spotted cricket, *Gryllus bimaculatus* (Orthoptera: Gryllidae), is an economically-significant cricket species and is of great interest as animal feed for pet reptiles, chickens, fish, as well as a food ingredient. Nutritionally, *G. bimaculatus* is rich in proteins, ranging from 57.49-70.10, lipids 14.93-33.44, and fiber 9.53±0.46 (g/100 g dry weight) [1, 2]. The chromosome structure of *G. bimaculatus* involves 2n = 28 + XX (F)/XO (M), and its genome size was estimated at 1.8 Gb [15]. Recently, genetic transformation techniques, such as RNA interference and transcription activator-like effector nucleases amenable to *G. bimaculatus*, have been established, and functional analyses of its genes have been performed in the field of biomedical science [9, 10, 14].

Kurzik-Dumke and Lohmann first reported the lethal (2)-essential-for-life [*l(2)efl*] gene on the right arm of the second chromosome at locus 59F4,5 in *Drosophila melanogaster*, which is constitutively expressed during normal development [4]. The l(2)efl protein shares high homology with heat shock protein 20 and alpha-crystallin. So, far, studies of *l(2)efl* in insects focused on the sweet potato whitefly (*Bemisia*

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tabaci), which, under thermal stress, demonstrated differential expression, as well as in the mosquito (*Aedes aegypti*) to reduce the replication of dengue virus type 2 (DENV-2) through overexpression of the *l(2)efl* gene [3, 8]. However, the exact biological function of *l(2)efl* remains unclear. To understand how insects can survive and overcome starvation, we studied the gene expression patterns of *GBl(2)efl*.

Materials and Methods

The fifth-instar larvae of the two-spotted cricket (*G. bimaculatus*) used in this study were obtained from the Rural Development Administration of Korea (Jeonju, Korea). The adults were reared at 28-30℃, with a humidity of 70% under a 10 hr/14 hr light/dark photoperiod in transparent plastic cylinders (Ø 9cm; h 10cm). These crickets were fed commercial feed that were suited for rats and rabbits (1:6), with unlimited water supply (Hello Super Premium Rabbit Food, Petco Co. Incheon, Korea). Crickets with synchronous growth from the fifth-instar stage to adult emergence were selected. Only males were used in the experiments. Due to cannibalism, each vial contained only a single male cricket. During a 6-day starvation period, the crickets were provided free access to water [6, 12].

G. bimaculatus was anesthetized by CO_2 gas exposure for dissection. Its body was cut open 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 further treated with RNase free DNase-I to remove any potential genomic DNA contamination. A marathon cDNA Amplification Kit (Clontech, Palo Alto, CA, USA) was then used to construct a cDNA library using 1.5 μg of mRNA as a template [11]. 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 the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) and Motif Databases (GenomeNet, Institute for Chemical Research, Kyoto University, Japan). *GBl(2)efl* forward and reverse primers are as follows: F (5′-CTT CGC CAA GAT GTC TCT GGT-3′) and R (5′-AGC CGAACC ATC TCT TCA TTT GG-3′). The open reading frame, molecular weight, theoretical isoelectric point, and prediction of protein secondary structure were estimated using bioinformatics tools available at the ExPASy server (http://www.expasy.org/). Multiple protein sequence alignment was performed using the NCBI server (http://www.ncbi.nlm.nih.gov/).

Each tissue section was removed from *G. bimaculatus* under a dissecting microscope (Olympus SZ51) and placed into a 1.5 ml tube with a 100 μl TRI^{zol} reagent (Invitrogen). Total RNA was extracted using the TRI^{zol} reagent in accordance with the company's instructions [5]. mRNA in the samples were reverse transcribed using the SuperscriptIITM First Strand Kit (Invitrogen). The resulting cDNA was PCR-amplified using primer pairs for the *G. bimaculats* actin: F (5′-ATC ACT GCC CTT GCT CCT TC-3′) and R (5′-TTCCTGTGGAC AATGGATGG-3′) and *GBl(2)efl*: F (5′-AGG ACT CTG GCT CCT CCA AT-3′) and R (5′-TGA CAT CAG CAG CAT TGA CA-3′). RT-PCR conditions (30 cycles) were as follows: 94℃ for 30 sec; 58℃ for 30 sec; and 72℃ for 1 min. A single band of *GBl(2)efl* amplicon (591 bp) was detected by RT-PCR. The resulting PCR product was subcloned into pGEM-T plasmid (Invitrogen) and transformed into competent *E. coli* DH5α for sequencing. The GenBank accession number for the *GBl(2)efl* cDNA is MN205433. Induction of endoplasmic reticulum (ER) stress was performed using tunicamycin treatment: injection at the #11 abdominal cavity. Using a syringe (10 μl), 5 μl of tunicamycin was intraperitoneally administered three times over 2 days.

Results and Discussion

We isolated a cDNA encoding lethal (2) essential for life [*l(2)efl*] from *G. bimaculatus,* coined by *GBl(2)efl* (GenBank accession number: MN205433). It contains 189 amino acids, including an N-glycosylation site and 15 phosphorylation sites with a theoretical pI of 6.2 and MW of 21.19 kDa. Phosphorylation of this protein is highly associated with its function and cell signaling (Proud, 2018), which occurs on 15 sites: 8 serines (S), 5 threonines (T), and 2 tyrosines (Y) (Fig. 1). On the other hand, N-glycosylation is important for both the structure and function of many eukaryotic proteins. *GBl(2)efl* only has one N-glycosylation at amino acid position 133. Furthermore, sites for N-terminal acetylation, C-terminal mannosylation, and GalNAc O-glycosylation were not detected in *GBl(2)efl*. Information on these protein structures provide valuable insights into the unique functions of *GBl(2)efl* and should be explored in future studies. The secondary structure of *GBl(2)efl* was predicted from sequence analyses showing the following domains: random coils

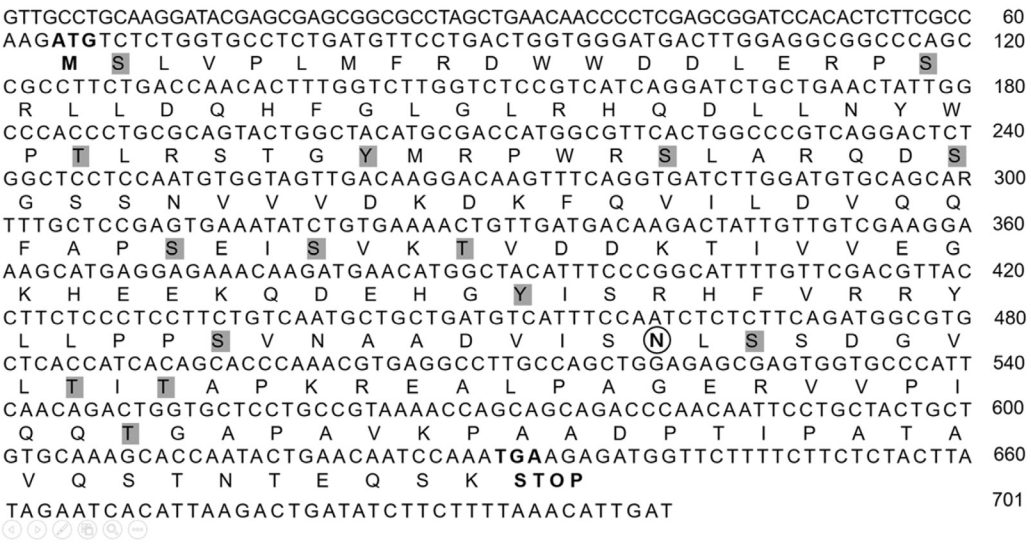

Fig. 1. The nucleotide and deduced amino acid sequences of *GBl(2)efl*. *GBl(2)efl* cDNA encodes a protein of 189 amino acids. The predicted amino acid sequence (single-letter abbreviation) is shown below the nucleotide sequence within the open reading frame. Fourteen phosphorylation sites are indicated by shading for the 8 serines (S), 5 threonines (T), and 2 tyrosines (Y), while an N-glycosylation site is circled (N). The amino acid sequence of the *GBl(2)efl* protein has been submitted to GenBank, and the accession number is MN205433.

(56.08%), alpha-helices (22.22%), extended strands (17.99%), and beta turns (3.7%).

The protein sequence of *GBl(2)efl* was compared with those of other known l(2)efl homologs in the GenBank database, which showed a 52% homology with that from *D. melanogaster* (NP_523827.1) and 69% with that from *Cryptotermes secundus* (XP_023705725.1) (Fig. 2A). Compared to the other regions of *GBl(2)efl*, homology at the N- and C-terminals were relatively lower. The evolutionary distances were computed using various difference methods and are expressed as the number of amino acid differences per sequence (Fig. 2A). *GBl(2)efl* exhibits an independent evolution pattern

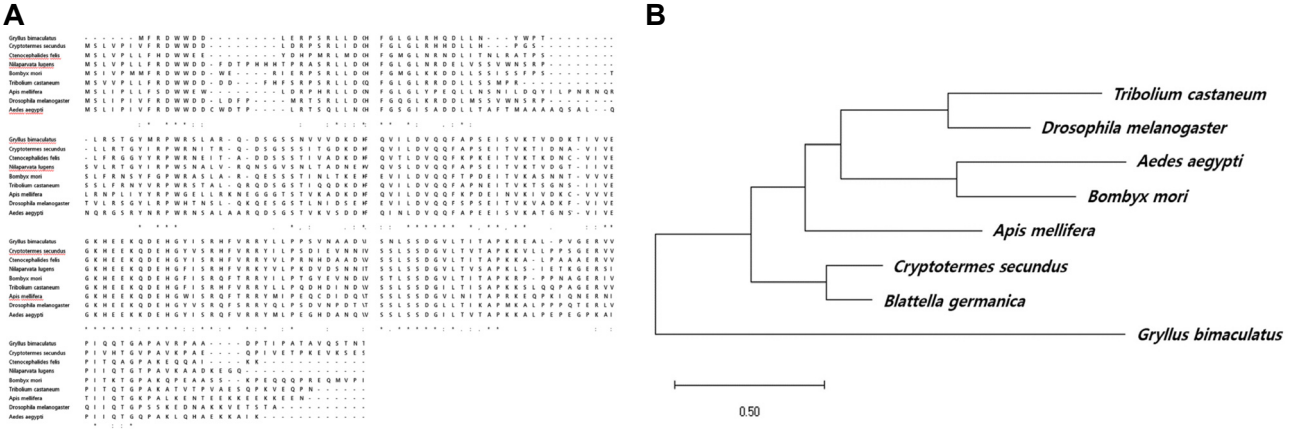

Fig. 2. Comparison of *GBl(2)efl* sequences. (A) Alignment of the *GBl(2)efl* with homologs from eight others species. Identical amino acid residues in this alignment are indicated by dark gray stars (*). Highly-conserved regions of amino acid residues are indicated by gray colons (:), and weakly-conserved regions of amino acid residues are indicated by light gray points (.). Deleted positions in the amino acid residues are indicated by dashes. (B) A molecular phylogenetic tree of *GBl(2)efl*. Phylogenetic tree based on homologs of *GBl(2)efl* from seven species constructed by the NCBI program. The length of the section indicates relative distances between the sequences. The species and gene accession numbers are as follows: *Tribolium castaneum* (XP_968760.1), *Drosophila melanogaster* (NP_523827.1), *Aedes aegypti* (XP_001663494.1), *Bombyx mori* (NP_001091767.1), *Apis mellifera* (XP_001120194.1), *Cryptotermes secundus* (XP_023705725.1), *Blattella germanica* (PSN45237.1.), *Nilaparvata lugens* (XP_022188126.1), and *Gryllus bimaculatus* (MN205433).

from the early stages of evolution with other l(2)efl homologs (Fig. 2B). However, considering that the homology between *GBl(2)efl* and other identified l(2)efl's were relatively high (52%-69%), the unique biological function of *GBl(2)efl* may be conserved across different species.

The mRNA expression of *GBl(2)efl* in various tissues of *G. bimaculatus* was analyzed using RT-PCR. As shown Fig. 3A, although *GBl(2)efl* expression is unremarkable in eight different tissues, it was found to be slightly higher (~1.25 fold) in the foregut. ER stress is activated by posttranslational modification to maintain cellular homeostasis through a sophisticated signaling system. Activation of ER stress is also required to survive against rapid environmental changes. Here, we induced ER stress in *G. bimaculatus* by tunicamycin injection. This induction resulted in *GBl(2)efl* mRNA expression being highly elevated in the dorsal longitudinal flight muscle (DL) and dorsal ventral flight muscle (DV) (~1.5 fold) as well as the fat body (FB) (~2 fold). Starvation is one of the most common and forcible stresses for insects. Therefore, it is essential for insects to adapt against starvation through various mechanisms, including gene regulation and protein modification. Next, we tested whether *GBl(2)efl* mRNA expression is regulated by starvation (1, 3, or 6 day) and refeeding (1 or 2 day) following a 6-day starvation. Although an increase in *GBl(2)efl* mRNA expression (~1.25 fold) was observed in Malpighian tubules (MP) following starvation, no other tissues demonstrated remarkable changes in expression in both starvation and refeeding experiments.

Kurzik-Dumke and Lohmann reported that the *l(2)efl* gene in *D. melanogaster* exhibited high homology with small HSP and alpha crystallin, both of which are constantly expressed throughout development [4]. Although the mechanism remains unclear, this *l(2)efl* homolog is capable of inducing the phosphorylation of the eukaryotic initiation factor 2α (eIF2α) in *Drosophila*, therefore, inhibiting protein biosynthesis. In the sweet potato whitefly, *Bemisia tabaci*, the *l(2)efl* homolog (*BtsHSP*) functions in thermal stress response as its mRNA expression is significantly upregulated by a high temperature stress (39-45℃) [3]. It has been reported that the upregulation of *l(2)efl* inhibits DENV-2 replication (*A. aegypti*) [8]. Thus far, many questions remain regarding the

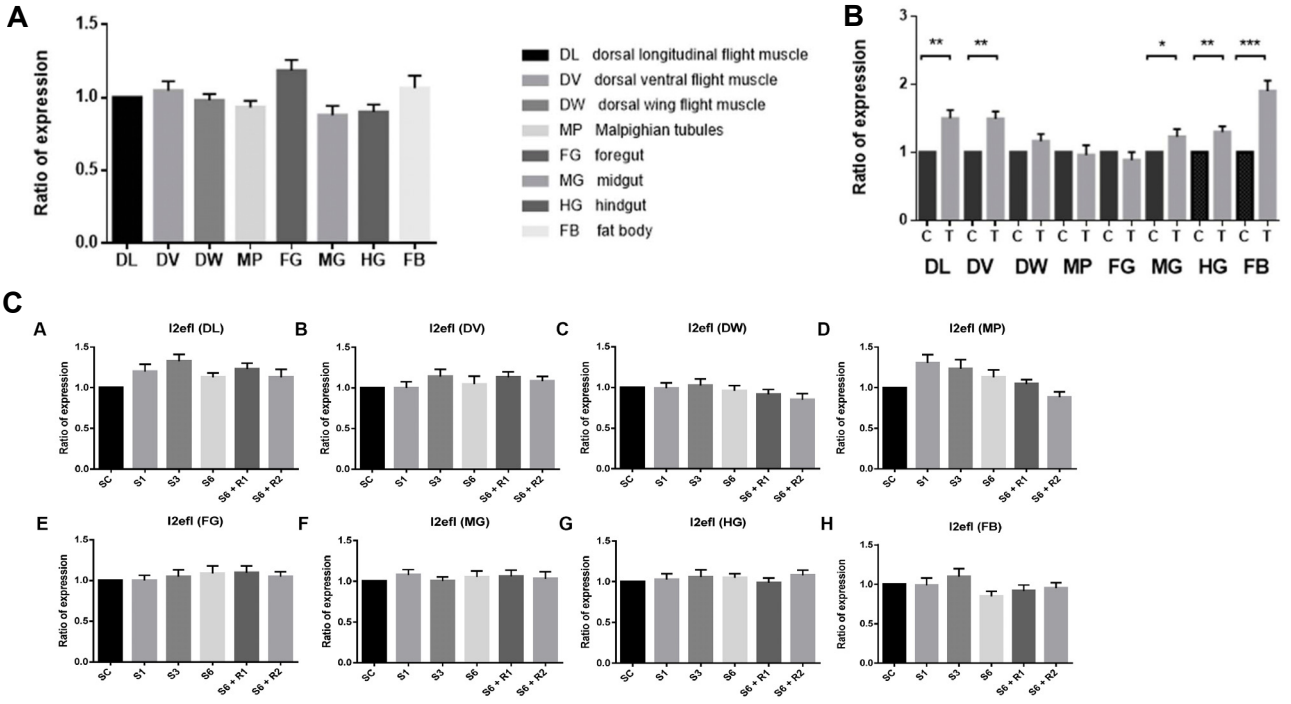

Fig. 3. *GBl(2)efl* gene expression. (A) In the various tissues. (B) Under ER stress. Tunicamycin (5 μl) was injected intraperitoneally three times across 2 days. (C) Under starvation or refeeding conditions. S1–6, starvation for 1–6 days; S6+R1, a day of refeeding after 6 days of starvation; S6+R2; 2 days of refeeding after 6 days of starvation. DL, dorsal longitudinal flight muscle; DV, dorsal ventral flight muscle; DW, dorsal wing flight muscle; FB, fat body; FG, foregut; MG, midgut; HG, hindgut; MP, Malpighian tubules. Values are presented as mean ± SEM, n = 3, *p<0.05 **p<0.005 ***p<0.001. Statistical significance between multiple groups: one-way analysis of variance test. Graph Pad Prism 6 software (Graph Pad Software Inc.).

biological function of *l(2)efl*; however, considering its ability to phosphorylate eIF2α, adapt to external temperature changes and starvation, we may be close to understanding the physiological mechanisms that contribute to insect survival. Further studies on this front will also help improve our capability to conduct more effective pest management control.

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The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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초록 : 쌍별귀뚜라미(*Gryllus bimaculatus*)의 *l(2)efl* cDNA 클로닝과 발현분석

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쌍별 귀뚜라미(*Gryllus bimaculatus*)에서 lethal (2) essential for life [l(2)efl]을 코드한 cDNA를 분리하여 *GBl(2)efl* 이라 하였다. GBl(2)efl는 N-glycosylation 한곳과 phosphorylation site를 15곳 가진 189 aa로 구성되며6.2등전점과 21.19 kDa 분자량을 가진다. GBl(2)efl 단백질의 이차구조는 random coils (56.08%), alpha-helix (22.22%), extended strands (17.99%), beta turns (3.7%)로 이루어진다. GBl(2)efl 는 지금까지 보고된 l(2)efl들과는48-69%의 상동성을 보인다. *GBl(2)efl*은1일, 3일 starvation일때에 각각 dorsal longitudinal flight muscle과 Malpighian tubules에서 mRNA발현이 증가하였다. 한편, ER stress 조건에서는*GBl(2)efl* 발현은 fat body에서 증가하였다. 본 연구는 곤충의 생존에 기여하는 생리학적 메커니즘을 이해와 효과적인 해충 관리 통제를 수행할 수 있는 능력을 향상에 많은 힌트를 줄 수 있는 실마리를 제공할 수 있을 것이다.