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- Note -

Characterization of a cDNA Encoding Transmembrane Protein 258 from a Two-spotted Cricket *Gryllus bimaculatus*

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The cDNA that encodes transmembrane protein 258 (Tmem258) was cloned from *Gryllus bimaculatus* and named *GbTmem258*. This protein comprises 80 amino acids, has no N-glycosylation site, and contains five potential phosphorylation sites at two serines, two threonines, and one tyrosine. The predicted molecular mass of GbTmem258 is 9.06 kDa, and its theoretical isoelectric point is 5.5. The tertiary structure of GbTmem258 was predicted using the available secondary structure information, which suggests the presence of alpha helices (52.5%), random coils (22.5%), extended strands (16.25%), and beta turns (8.75%). Homology analysis revealed that GbTmem258 exhibits high similarity at the amino-acid level to Tmem258 found in other species. The effect of starvation and refeeding on *GbTmem258* mRNA expression was also examined in this study. It was found that *GbTmem258* mRNA expression in the hindgut progressively increased throughout the starvation period, peaking at almost 1.5 times the control level after six days of starvation. However, refeeding for one to two days after the six-day starvation period restored *GbTmem258* mRNA expression to the control level. In fat body, *GbTmem258* mRNA expression was almost 3-fold higher during starvation compared to the control level. Refeeding for one to two days after the six-day fast resulted in a decline in the expression to about a 2.5-fold increase over the control level. Throughout the starving and refeeding periods, no other tissues showed any discernible alterations in *GbTmem258* mRNA expression.

Key words : Starvation, two-spotted cricket *Gryllus bimaculatus,* transmembrane protein 258 (Tmem258), Tmem258 of *G. bimaculatus* (GbTmem258) cDNA

Introduction

The two-spotted cricket, *Gryllus bimaculatus* (Orthoptera: Gryllidae), is one of the most economically significant cricket species in the world, it is a hemimetabolous insect species [16]. This is due to the fact that it serves as an experimental model insect and poultry feed [17]. It goes through the first to eighth nymphal stages as it develops into an adult. As an adult, it lays eggs all year long. The *G. bimaculatus*' genome was estimated to be 1.8 Gb in size and has chromosomes that are 2n = 28 + XX (female)/XO (male) [18]. Studies on gene function demand the use of direct, focused gene alteration methods in experimental insect individuals. Through the development of genetic transformation techniques including RNA interference and transcription -like effector nucleases, functional assessments of the genes in the *G. bimaculatus* have recently been resolved [11]. As a result, the *G. bimaculatus* is a brand-new experimental animal model that has gained interest in the biomedical sciences [17]. Insects that are starving during development, especially those that live in deserts, can survive without food for extended periods of time by slowing down their metabolism until they come across a food source. Strong hunger resistance, often known as this survival tactic, is characterized by a decrease in body size. In reaction to famine, some insects, including *Aedes aegypti*, and *Formica exsecta*, actively control gene expression [12, 13]. In the absence of food, insects convert proteins, fats, and carbs into cellular energy. Aphids even decompose own muscles to utilize as a source of energy when the going gets tough [7]. The precise gene control mechanisms behind this process, nevertheless, are still poorly

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understood.

Adachi *et al* [1]. first reported the human *Tmem258* gene on chromosomal 11q12.2, which contains two highly conserved transmembrane domains, as well as other orthologs. The subcellular location of Tmem258 is estimated to be in the endoplasmic reticulum (ER) membrane. It is a required component of the oligosaccharyltransferase complex and is essential for N-linked glycosylation. The results of expressed sequence tag (EST) analysis show that the *Tmem258* gene is widely expressed in human tissues. However, there is currently no data available on its expression in insects. The precise biological role of Tmem258 is still not well understood. To investigate how insects are able to endure and prevail over starvation, we analyzed the gene expression profiles of *GbTmem258* (a gene of *G. bimaculatus's Tmem258*).

Materials and Methods

The Rural Development Agency of Korea provided the two-spotted cricket (*G. bimaculatus*) fifth-instar larvae used in this investigation (Jeonju, Korea). The adults were raised in clear plastic cylinders (Ø 9 cm; h 10cm) at 28℃-30℃, 70% humidity, and a 10-14-hr light-dark cycle. Commercial meal designed for rats and rabbits (1:6) was provided to these crickets, along with an endless supply of water. The selection process focused on crickets with synchronized growth from the fifth instar stage to adult emergence. In the tests, only men were employed. Each vial only contained one male cricket due to cannibalism. The crickets were given unrestricted access to water for the duration of a 6-day fasting phase [16].

In order to dissect *G. bimaculatus*, it was anesthetized by CO_2 gas exposure. Its body was split open ventrally from the neck to the final abdominal section. Each tissue was collected using a computerized microscope (Nikon Eclipse E600). Each tissue's total RNA was isolated using the TRI^{zol} method (Invitrogen, Carlsbad, CA, USA), and any potential genomic DNA contamination was then removed before the RNA was further processed with RNase-free DNase-I. Then, using 1.5 mg of mRNA as a template, a cDNA library was built using a marathon cDNA amplification kit (Clontech, Palo Alto, CA, USA). The gene was identified using BLAST search. Primers created with Primer3 (http://simgene.com/Primer3) based on Conserved Domain Databases form National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) and Motif Databases (GenomeNet, Institute for Chemical Research, Kyoto University, Japan) were used to conduct PCR. Here are the forward and reverse primers for *GbTmem258*: *GbTmem258*-F 5'-CTC CGG CAC AAT GGC GGA CGA-3' and *GbTmem258*-R 5'-AGG TGA TTA ATT TTT GTA TTG CAA-3'. The ExPASy (http://www.expasy.org/) server's bioinformatics tools were used to estimate the open reading frame, molecular weight, theoretical isoelectric point, and predicted secondary & tertiary structure of protein. The NCBI server (http://www.ncbi.nlm.nih.gov/) was used to align several protein sequences. Under an Olympus SZ51 dissecting microscope, each tissue section from *G. bimaculatus* was excised and put into a 1.5 ml tube with 100 μl of TRI^{zol} reagent (Invitrogen). The TRI^{zol} reagent was used to extract total RNA according to the manufacturer's instructions. With the SuperscriptII^{TM} First Strand Kit, the samples' mRNA was reverse transcribed (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′-TTC CTG TGG ACA ATG GAT GG-3′) and *GbTmem 258*: F (5′-AGG ACT CTG GCT CCT CCA AT-3′) and R (5′-TGA CAT CAG CAG CAT TGA CA-3′). The following RT-PCR parameters (30 cycles) were used: 94℃ for 30 sec, 58℃ for 30 sec, and 72℃ for 1 min. By using RT-PCR, a single band of the *GbTmem258* amplicon was found. The *GbTmem258* cDNA's GenBank accession number is MN 205434. Five microliters of tunicamycin solution (0.05 mg) were intraperitoneally injected into the 11th abdominal cavity to induce ER stress. three times for two days using a syringe (10 μl).

Results and Discussion

A cDNA encoding transmembrane protein 258 (Tmen258) from *G. bimaculatus* was isolated and given by the name *GbTmem258* (GenBank # MN205434). It has 80 amino acids, which has a theoretical pI value of 5.5 and a molecular weight of 9.06 kDa, but none of its sites for N-glycosylation, N-terminal acetylation, C-terminal mannosylation, or GalNAc O-glycosylation are present. Instead, it has 5 potential phosphorylation sites, including 2 serines (S), 2 threonines (T), and 1 tyrosine (Y) (Fig. 1). Phosphorylation is a post-translational modification process that involves the addition of a phosphate group to specific amino acid residues of a protein. This process can affect the structure, stability, and activity of the protein, as well as its interactions with other proteins and cellular components [9]. In the case of GbTmem258, the presence of 5 phosphorylation sites suggests that this protein may be involved in various cellular signaling pathways. The addition

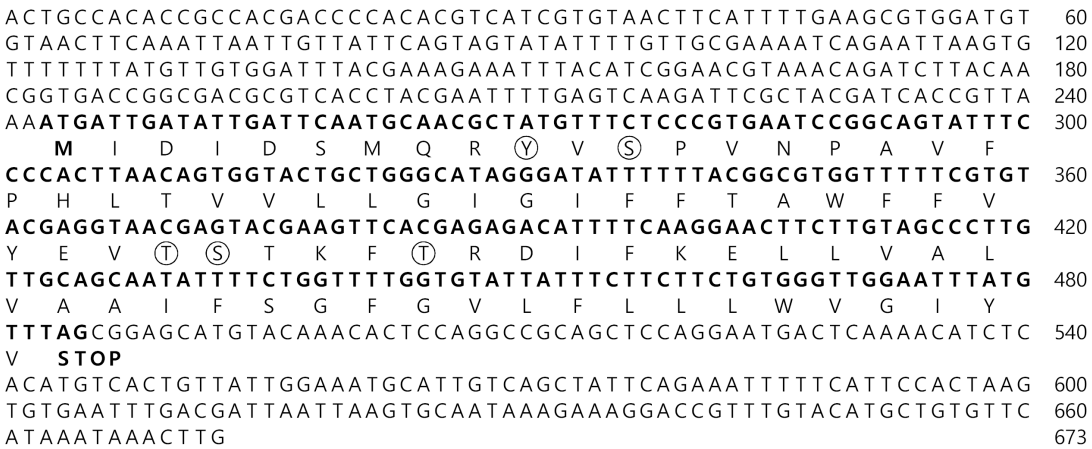

Fig. 1. The amino acid and nucleotide sequences of GbTmem258. An 80 amino acid long protein is encoded by the GbTmem258 cDNA. Underneath the nucleotide sequence within the open reading frame is the predicted amino acid sequence (represented by a single-letter abbreviation. There are no N-glycosylation sites, but there are five phosphorylation sites indicated by cycles for the two serines (S), two threonines (T), and one tyrosine (Y). The GbTmem258 protein's amino acid sequence has been deposited into GenBank with the accession number MN205434.

or removal of phosphate groups on these sites can modulate the function of the protein, potentially affecting its localization, stability, interactions, and downstream signaling events. The protein sequence of GbTmem258 was compared with those of other known Tmem258 homologs in the GenBank database, which showed a 52% homology with that 99% *Cryptotermes secundus* (PNF38811.1), 94% *Blattella germanica* (PSN47709.1), 85% *Nilaparvata lugens* (XP_022198957.1), 85% *Mus musculus* (NP_001156903.1), 84% *Bombyx mori* (XP_004934028.2), 82% *Homo sapiens* (AW73971.1), 78% *Rattus norvegicus* (EDM12795.1*), 78% Xenopus tropicalis* (NP_001005138.1), and 76% *Danio rerio* (NP_957063.1). Compared to the other regions of GbTmem258, it shows relatively high homology to the Tmem258 of other species compared (Fig. 2).

Different methods were used to calculate the evolutionary distances, which were then expressed as the number of amino acid differences per sequence. Among various Tmen258 homologs, the GbTmem258 displayed a notably high degree of evolutionary similarity to insect Tmen258, as illustrated in Fig. 3A. Given the relatively high homology (76%-99%) between GbTmem258 and other identified Tmen258 proteins, it is possible that the unique biological function of GbTmem258 is conserved across different species. Because they shed light on the evolution and functional characteristics of proteins, proteins with high homology and conserved biological function are particularly intriguing [8]. Understanding the function of these proteins and how mutations in particular amino acids might impact protein function will help us better understand how various animals have evolved and adapted to their environments. Furthermore, because these proteins are anticipated to play significant roles in disease processes, they could be potential targets for therapeutic development [10].

The tertiary structure of GbTmem258 was constructed based on the secondary structure information: alpha-helix (52.5%), random coils (22.5%), extended strands (16.25%), and beta turns (8.75%) (Fig. 3B). The GbTmem258 consists of 88 amino acids with P20-V42 and L55-G77 being the alpha-helix region that penetrates the ER membrane, T43-E54 being the cytosol region and M1-F19/I78-V80 being the ER lumen region. The GbTmem258 has 3 phosphorylation sites (T43, S44 and T48) in the cytosol region and 2 (Y10 and S12) in the ER lumen region. A phosphate group is added to a molecule, usually a protein or a nucleic acid, during the biological process of phosphorylation. The reversible process is catalyzed by enzymes known as kinases and reversed by phosphatases. Cellular signaling and control depend heavily on phosphorylation [3]. It can change the shape and operation of nucleic acids as well as the activity, localization, stability, and interactions of proteins. Numerous physiological activities, including metabolism, gene expression, cell cycle progression, cell differentiation, apoptosis, and response to stress and environmental stimuli, are affected by phosphorylation . The 5 phosphorylation sites are likely to play an important role in the various physiological functions of the GbTmem258.

The RT-PCR was used to analyze the mRNA expression

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Gryllus bimaculatus       ----------------------------MIDIDSMQRYVSPVNPAVFPHLTVVLLGIG 30
Cyryptotermes secundus    ------------------------------------MTRYVSPVNPAVFPHLTVVLLGIG 24
Blattella germanica       ----------------------------MIEIENMSRYVCPVNPAVFPHLTVVLLGIG 30
Nilaparvata lugens        --MLVELIQRSRLFSFLSQRHVLLIIINFTMVQIELMSRYVSPINQAVFPHLTIILLGIG 58
Homo sapiens              ------------------------------MEIEAMSRYTSPVNPAVFPHLTVVLLAIG 30
Mus musculus              ------------------------------------MSRYTSPVNPAVFPHLTVVLLAIG 24
Rattus norvegicus         MDHILFLLLNALAPADVFFRWRSVVYPVRGKMELEAMSRYTSPVNPAVFPHLTVVLLAIG 60
Xenopus tropicalis        ------------------------------MELEAMSRYTSPVNPAVFPHLTVVLLAIG 29
Bombyx mori               ----------------------------MSLEIESMIRYTSPINPAVFPHLTFLLLGIG 31
Danio rerio               ------------------------------MELEAMTRYTSPVNPAVFPHLTVVLLAIG 29
                                                          *  **.. *:**********.:**.**

Gryllus bimaculatus       IFFTAWFFVYEVTSTKFTRDIFKELLVALVAAIFSGFGVLFLLLWVGIYV 80
Cyryptotermes secundus    IFFTAWFFVYEVTSTKFTRDIFKELLVALVAAIFSGFGVLFLLLWVGIYV 74
Blattella germanica       IFFTAWFFVYEVTSTKFTRDIFKELLVALVAAIFSGFGVLFLLLWVGIYV 80
Nilaparvata lugens        IFFTAWFFVYEVTSTKLTRDPFKELLVSLVAAMFSGFGVLFLLLWVGIYV 108
Homo sapiens              MFFTAWFFVYEVTSTKYTRDIYKELLISLVASLFMGFGVLFLLLWVGIYV 79
Mus musculus              MFFTAWFFVYEVTSTKYTRDIYKELLISLVASLFMGFGVLFLLLWVGIYV 74
Rattus norvegicus         MFFTAWFFVYEVTSTKYTRDVYKELLISLVASLFMGFGVLFLLLWVGIYV 110
Xenopus tropicalis        MFFTAWFFVYEVTSTKYTRDVYKELLISLVASLFMGFGVLFLLLWVGIYV 79
Bombyx mori               IFFTAWFFVYEVTSTKASRDMFKELLLSLVASLFSGFGILFLLLWVGIYV 81
Danio rerio               MFFKAWFFVYEVTSTKYTRDVYKELLIALVAALFMGFGVHFLLLWVGIFV 79
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Fig. 2. Comparison of amino acid sequences of GbTmem258 protein. Alignment of the GbTmem258 with homologs from eight others species. Identical amino acid residues in this alignment are indicated by dark gray stars (*). Highly conserved amino acid residues are indicated by gray colons (:), and weakly conserved amino acid residues are indicated by light gray points (.). Deleted amino acid residues are indicated by dashes. Each homology is as follows; from 99% *Cryptotermes secundus* (PNF38811.1) to 76% *Danio rerio* (NP_957063.1).

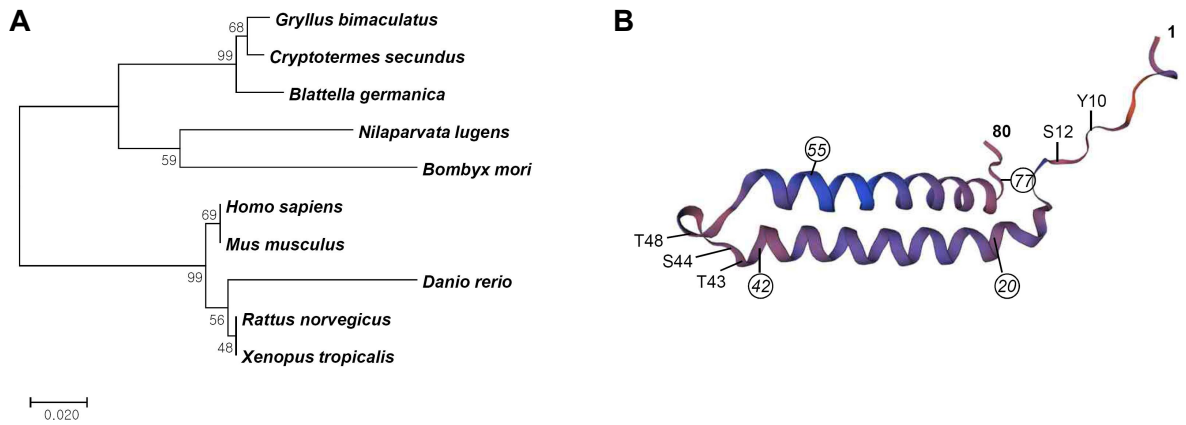

Fig. 3. Phylogenetic tree and tertiary structure of GbTmem258 protein. (A) Evolutionary tree of GbTmem258. The NCBI algorithm created a phylogenetic tree using homologs of GbTmem258 from 10 species. The section's length reveals how close together the segments are. (B) The GbTmem258 protein's tertiary structure created by the ExPASy software.

of *GbTmem258* in eight tissues of *G. bimaculatus*. Fig. 4A shows that the expression of *GbTmem258* mRNA is not significant in eight different tissues such as DL (dorsal longitudinal flight muscle), DV (dorsal ventral flight muscle), DW (dorsal wing flight muscle), FB (fat body), FG (foregut), MG (midgut), HG (hindgut), and MP (Malpighian tubules). However, a slightly higher expression of *GbTmem258* mRNA (~1.25 fold) was observed in the MP. When the need for protein synthesis outweighs the ER's ability to fold proteins, ER stress results [6]. Genetic abnormalities, viral infections, dietary deficiency, and exposure to toxins are just a few of the causes. The unfolded protein response (UPR), a signaling system, is activated when the ER is overloaded with unfolded or misfolded proteins [5, 14]. By boosting protein folding ability, lowering protein production, and destroying misfolded proteins, the UPR seeks to reestablish ER equilibrium. The UPR may, however, fail to restore ER function in cases of prolonged or extreme stress, which results in cell death or apoptosis. Here, we used tunicamycin injection to induce ER stress in *G. bimaculatus* (Fig. 4B). *GbTmem258* mRNA expression was significantly increased in the MP as a result of this stimulation (by about 1.25 fold). The Malpighian tu-

bules are an essential part of the insect excretory system, assisting in the maintenance of the correct fluid balance and the removal of waste products from the body. The active *GbTmem258* mRNA expression and ER stress-sensitive response in MP seems to be related to the maintenance of the excretory system through MP.

One of the most prevalent and coercive pressures on insects is starvation. Depending on the species and life stage of the insect, starvation can have a variety of impacts [15]. Insects use malnutrition to control their growth, development, reproduction, and survival in general [19]. The decline in growth and development of insects is one of the main impacts of hunger. An insect's metabolism slows down when it is starving, and it may go into a condition of hibernation or diapause when it saves energy and resources [9]. This can cause the insect's development to be postponed or even stopped entirely until food is once again available [4]. Next, we examined the impact of starvation (1, 3, and 6 days) and subsequent refeeding (1 and 2 days) after a 6-day starvation on the regulation of *GbTmem258* mRNA expression (Fig.

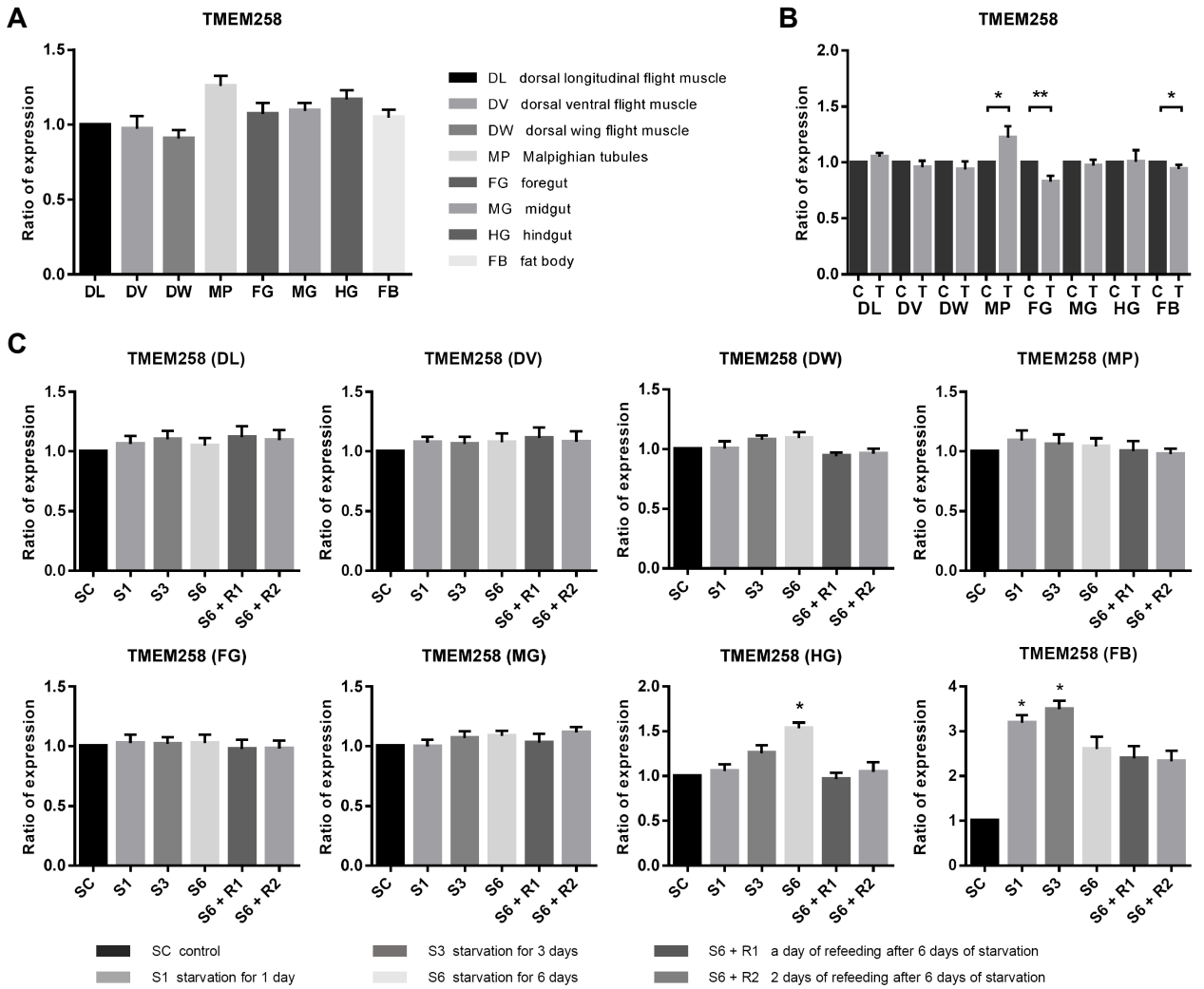

Fig. 4. Gene expression patterns of GbTmem258. (A) In the various tissues. (B) Under ER stress. Tunicamycin (0.05 mg) was intraperitoneally injected three times for 2 days to the *G. bimaculatus*. (C) The experimental conditions included S1-6, which denotes starvation for 1-6 days; S6+R1, which represents a day of refeeding after 6 days of starvation; and S6+R2, which indicates 2 days of refeeding after 6 days of starvation. The body tissues and organs measured were DL (dorsal longitudinal flight muscle), DV (dorsal ventral flight muscle), DW (dorsal wing flight muscle), FB (fat body), FG (foregut), MG (midgut), HG (hindgut), and MP (Malpighian tubules). The data were expressed as mean ± SEM with a sample size of n = 3. The statistical significance was determined using the one-way analysis of variance test, and the Graph Pad Prism 6 software (Graph Pad Software Inc.) was used for data analysis. The levels of significance were denoted as $*p<0.05$, $**p<0.005$, and $***p<0.001$.

4C). Our findings revealed that in HG, *GbTmem258* mRNA expression progressively increased with starvation, peaking at around 1.5-fold higher than control after 6 days. However, following refeeding (1 and 2 days) after the 6-day starvation period, *GbTmem258* mRNA expression returned to control levels. In FB, *GbTmem258* mRNA expression increased up to approximately 3-fold compared to control with starvation, but refeeding (1 and 2 days) following the 6-day starvation resulted in a reduction to approximately 2.5-fold increase. No significant changes in expression were observed in any other tissues during both the starvation and refeeding experiments.

Despite the current lack of knowledge about the precise biological function of GbTmem258, there is promising evidence to suggest that it plays a vital role in the survival of insects. For example, studies have shown that GbTmem258 has the ability to phosphorylate eIF2α, a key regulator of protein synthesis that is crucial for adaptation to external temperature changes and periods of starvation. While there is still much to be learned about the exact mechanisms by which GbTmem258 operates, continued research in this area holds significant potential for advancing our understanding of insect physiology and improving our ability to control pests. By gaining a deeper understanding of the biological function of GbTmem258 and related proteins, we may be able to develop more effective pest management strategies that are tailored to the specific needs and vulnerabilities of different insect species. Ultimately, such efforts could help to reduce the impact of insects on agriculture and other important ecosystems, improving food security and preserving biodiversity for future generations.

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 GbTmem258 cDNA 클로닝과 발현분석

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쌍별귀뚜라미(*Gryllus bimaculatus*)에서 분리한 막전단백질 258(transmembrane protein 258, Tmem258)을 코딩하는 cDNA를 *GbTmem258*로 이름 붙였다. 이 단백질은 80개의 아미노산으로 구성되어 있으며, N-glycosylation site가 없고, 각각 2개의 serine과 threonine, 1개의 tyrosine 잔기로 구성된 5개의 잠재적 인산화 부위를 가지고있다. GbTmem258 단백질은 분자량은 9.06 kDa이며 이론적 등전점은 5.5으로 계산되었으며, alpha-helix (52.5%), random coils (22.5%), extended strands (16.25%), beta turns (8.75%)의 2차 구조 정보를 기반으로 GbTmem258의 3차 구조가 작성되었다. 그리고, GbTmem258은 다른 종의 Tmem258와 아미노산 수준에서 높은 상동성을 보였다. 이 연구에서는 기아와 먹이 공급에 의해 *GbTmem258* 발현 조절이 어떻게 영향을 받는지 조사하였다. 기아가 지속되는 동안 hindgut에서 *GbTmem258* 발현이 점진적으로 증가하여 기아 6일 후 대조군보다 1.5배 높은 수준이 되었다. 그러나 6일간의 기아상태가 끝난 후 하루 또는 이틀 동안 다시 먹이를 주면 *GbTmem258* 발현이 대조군 수준으로 회복되었다. 지방에서는 기아 동안 대조군에 비해서 *GbTmem258* 발현이 최대 3배까지 증가했지만, 6일 기아 후 하루 또는 이틀 동안 다시 먹인 후에는 발현이 약 2.5배 증가로 감소되었다. 굶기고 다시 먹이는 실험 내내 각각의 조직에서 주목할만한 *GbTmem258* 발현은 관찰되지 않았다.