Journal of Stored Products Research 101 (2023) 102074

Contents lists available at ScienceDirect

Journal of Stored Products Research

journal homepage: www.elsevier.com/locate/jspr

The involvement of Antifreeze protein maxi-like and Cold-shock domain-containing protein genes in cold-induced larval diapause and cold-shock treatment of khapra beetle

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ARTICLE INFO

Handling Editor: Raul Guedes

Keywords:
Antifreeze protein
Cold-shock
Cold-shock domain-containing protein
Diapause
Khapra beetle

ABSTRACT

The khapra beetle, *Trogoderma granarium* (Everts) (Coleoptera: Dermestideae), is a cosmopolitan and one of the most destructive pests of various stored grains and grain products. This pest is categorized as a quarantine threat in many countries. The larvae undergo diapause that lasts for several years depending on different stress conditions such as temperature, insecticidal applications, starvation, humidity, crowding, and fecal pellets. When conditions are favorable for the development, diapause terminates and the larva continues its development. In the present study, two cold-regulated genes Antifreeze protein maxi-like (*TgAFP*) and Cold-shock domain-containing protein E1 (*TgCSDP*) were identified in the cDNA library of *T. granarium*. Nucleotide sequences of two unigene cDNAs encoding *TgAFP* and *TgCSDP* were verified using RT-PCR and RACE-PCR amplifications. Transcriptional regulations of *TgAFP* and *TgCSDP* were examined in cold-induced diapause larvae, cold-shocked larvae, and at different developmental stages. Gene expression pattern of *TgAFP* revealed the highest mRNA levels during pre-diapause (25 °C) followed by the larvae exposed to 5 °C during the diapause phase. This can be attributable to the protective role of *TgAFP* against temperature fall. Significant upregulation of *TgCSDP* at 15 °C might indicate its probable chaperone role in toleration for cold-induced diapause. *TgAFP* level was downregulated after cold-shock treatment (CST), while it was slightly upregulated by recovery, indicating that it might confer tolerance for the recovery period (RP). The highest *TgCSDP* expression after the CST might suggest its involvement in response to acute CST. Low abundance of *TgAFP* expression in each developmental stage might suggest that 33 °C temperature does not induce the synthesis of *TgAFP*. Significant amount of *TgCSDP* levels in adults might indicate its putative role in development. These findings suggest that *TgAFP* and *TgCSDP* genes might be crucial for cold survival where the *T. granarium* undergoes its facultative diapause.

1. Introduction

The distribution and abundance of insects are significantly affected by climatic factors such as temperature (Bale et al., 2002; Advani et al., 2016; Pan et al., 2018). While the lower temperatures decrease the insect population growth, it can also trigger the insects to undergo diapause, which is formed by pre-diapause, diapause, and post-diapause phases at various developmental stages depending upon the species (Koštál, 2006; Hahn and Denlinger, 2007; Dageri et al., 2021). On the other hand, it might also cause an acute cold-shock with a sudden temperature fall. In such cases, a survival mechanism in insects keeps them alive by up- or down regulation of some specific genes.

Antifreeze proteins (AFPs) are a class of proteins that provide organisms the ability to survive in very low-temperature conditions (Venketesh and Dayananda, 2008). These proteins can remarkably change the freezing and melting points of the water by manipulating its thermal hysteresis (DeVries et al., 1970; Walters et al., 2009). They can also absorb and depress ice crystals until the freezing temperature

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https://doi.org/10.1016/j.jspr.2022.102074
Received 18 September 2022; Received in revised form 27 December 2022; Accepted 30 December 2022
Available online 3 January 2023
0022-474X/

maintains a tolerable level (Huang et al., 2002; Ustun and Turhan, 2015). Since the initial discovery of AFPs in the Antarctic teleost fish (DeVries et al., 1970), identification of AFPs and their expressions in response to cold temperatures have been revealed in various organisms, including invertebrates, vertebrates, plants, and microbes (Venketesh and Dayananda, 2008). Furthermore, the biological roles of AFPs have been briefly studied in various areas including biotechnology, agriculture, food processing, cryopreservation etc. (Białkowska et al., 2020).

Cold-shock domain containing proteins (CSDPs) are well-known to function as an RNA chaperone by destabilizing mRNA structures and were primarily detected in bacterial cold-responsive Cold-shock proteins (CSPs) (Graumann and Marahiel, 1996; Mihailovich et al., 2010). Cold-shock domain-containing protein E1 (CSDE1) is a member of CSDPs comprising five cold-shock domains, referred to as *upstream of N-RAS (UNR)*, and discovered in the 5' flanking region of the *N-RAS* gene (Anderson and Catnaigh, 2015; Lindquist and Mertens, 2018). It is a single-stranded DNA- or RNA-binding protein commonly positioned in the cytoplasm to regulate mRNA translation and steadiness (Anderson and Catnaigh, 2015; Liu et al., 2020). Besides the role CSDE1 takes in the cell cycle (Schepens et al., 2007), differentiation (Elatmani et al., 2011), apoptosis (Mitchell et al., 2001), and dosage compensation (Patalano et al., 2009), it has been reported in many organisms that the transcript level of *CSDE1* is inducible after cold treatment (Goldstein et al., 1990; Thieringer et al., 1997; Yang et al., 2012; Li et al., 2018). The studies related to *CSDP* genes in eukaryotes are limited, and also no exhaustive research is present on *CSDP*s from insects.

The khapra beetle, *Trogoderma granarium* (Everts) (Coleoptera: Dermestideae), is a cosmopolitan and one of the most destructive pests of various types of stored grains and grain products (Mohammadzadeh and Izadi, 2018). This pest is categorized as an A2 quarantine organism (EPPO, 2021). It is widely distributed in West Asia, the Sahel region of Africa, the Mediterranean regions, and in some other countries across the world (Ahmedani et al., 2007; EPPO, 2021). The long-term diapause in response to various stresses in *T. granarium* makes its control difficult. Since larvae of the *T. granarium* might persist into the diapause phase for up to eight years (Burges, 1962), it is needed to be investigated which genes function in favor of this pest. An increased tolerance level of *T. granarium* has been reported when larvae enter the diapause phase due to different biotic and abiotic stresses (Lindgren and Vincent, 1959; Burges, 1963; Stanic et al., 1963; Bell et al., 1984; Wilches et al., 2016). This tolerance characteristic could be attributed to the synthesis of some diapause-specific genes (Wilches et al., 2016).

To our knowledge, no comprehensive study exists based on the effects of cold-induced diapause and cold-shock treatment (CST) related to any genes from *T. granarium* thus far. In this study, we identified two critical genes in the cDNA library of *T. granarium*, which might contribute to the cold-induced diapause phase of the larvae. Transcriptional regulation of Antifreeze protein maxi-Like (*TgAFP*) and Cold-shock domain-containing protein E1 (*TgCSDP*) in cold-induced diapause phase and cold-shocked larvae were analyzed by measuring their mRNA levels. In addition, the expression profiles of both genes were monitored during the developmental stages of the pest.

2. Materials and methods

2.1. Trogoderma granarium culture

Larvae of *T. granarium* were reared on wheat grains (*Triticum aestivum* L.), which were first sterilized at 55 °C for 6 h. Insect colonies were kept in translucent plastic containers with a lid hole covered with a mesh net for ventilation. Insects were maintained in the incubator set at 33 ± 1 °C with 65 ± 5% RH in continuous darkness.

2.2. Induction of diapause

Diapause was triggered by performing cold acclimation. Individuals of fifth-instar larvae were placed in a translucent plastic container. The lid hole of the container was covered with a mesh net for ventilation under rearing conditions. The adults were fed for about one week until they start laying eggs. The first to fifth-instar larvae and one-week-old male and female adults were obtained from hatched eggs for gene expression analyses. Samples were directly shifted to 1.5 ml microcentrifuge tubes containing NucleoZOL reagent (Machery-Nagel GmbH, Düren, Germany). At fifth-instar, larvae were transferred to another container in order to carry out the cold-induced diapause experiment. The temperature was cooled from 33 ± 1 °C to 5 ± 1 °C by lowering ±5 °C per week to induce diapause. The larvae showed signs of diapause such as immobilizing, feeding cessation, and retardation of pupariation as described in previous studies (Burges, 1959; Wilches et al., 2019). After maintaining the temperature at 5 °C for four weeks, diapause was terminated by adjusting the temperature to 33 °C. Post-diapause group was formed by the samples kept at 33 °C for 1, 3, and 6th days. Collected samples from the cold-induced diapause (pre- and diapause) and post-diapause phases were immediately transferred to the 1.5 ml microcentrifuge tubes containing NucleoZOL Reagent for RNA isolation. The larvae considered as non-diapause were not included in the experiments (Burges, 1963). Indication of larvae collected at a different time interval during the pre-diapause, diapause, and post-diapause phases is illustrated in Fig. 1. Control groups were established in an incubator set at 33 ± 1 °C with 65 ± 5% RH in continuous darkness, and insects were reared up to the fifth-instar larvae on wheat grains in translucent plastic containers with a lid hole covered by a mesh net for ventilation. After collecting fifth-instar larvae from control groups, they were immediately shifted to the 1.5 ml microcentrifuge tubes containing NucleoZOL reagent for RNA isolation. All the experiments were performed in three biological replicates.

2.3. Cold-shock treatment (CST)

To measure the gene expression levels of *TgAFP* and *TgCSDP* during acute CST and following recovery period (RP), two hundred forty fifth-instar larvae were placed in thin-layered translucent plastic containers with a lid hole covered with a mesh net for ventilation and were kept at 0 °C in a cooled incubator. A sample of ten insects was collected after 0.5, 1, 2, and 3 h of CST, respectively. Following CST, the rest of the insects was recovered at 33 ± 1 °C and ten larvae were collected after 0.5, 1, 2, and 3 h. A corresponding control at 33 ± 1 °C was used for each sampling time of CST and RP for the same period and same number of insects. Treated and control samples were collected and immediately transferred to the 1.5 ml microcentrifuge tubes containing NucleoZOL Reagent for the gene expression analyses. Experiments for each sample were performed in three biological replicates.

2.4. RNA Isolation and cDNA synthesis

Total RNA was extracted from cold-induced diapause and cold-shock treated larvae, control groups, and developmental stages using NucleoZOL Reagent according to the manufacturer's specifications. DNase digestion was conducted using RNase-Free DNase (Ambion). RNA quantification was performed by measuring the absorbances at 260 nm and 280 nm utilizing a Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific, MA, USA). OneScript® Plus cDNA Synthesis Kit (ABM Good, Canada) was used to synthesize cDNAs from 1 μg total RNA according to the manufacturer's instructions.

2.5. RT-PCR, RACE-PCR, and sequencing

Two unigene cDNAs encoding *TgAFP* and *TgCSDP* were identified based on the transcriptome database of *T. granarium* (Unpublished data). PrimerQuest Tool (https://eu.idtdna.com/Primerquest/Home/Index) was used to design gene specific primers for RT (Reverse Transcription)-PCR and RACE (Rapid Amplification of cDNA Ends)-PCR amplifications

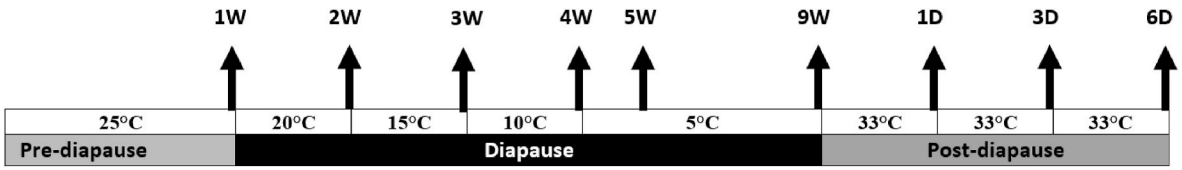

Fig. 1. Diagrammatic representation of cold-induced diapause process. 1W, 2W, 3W, 4W, 5W, and 9W represent sampling time (in weeks) of pre-diapause, diapause phases, and 1D, 3D, and 6D indicate sampling times (in days) of post-diapause development.

Table 1
Primers sequences utilized in the study.

Gene	Primer Sequence (5′-3′)	Application
TgAFP	F: GAAGCAGCCAACGAATTAGC	qRT-PCR
	R: CGTAATCGATCCGTACACCTTT	
TgCSDP	F: CTCTGAAGCGAAGACCAAAGA	qRT-PCR
	R: TACAGTTCCAGGTGCTAAACG	
***β*-Actin**	F: ATGGCGTGTGGCAAAGCGTAA	qRT-PCR
	R: ACCTTCAACACACCAGCTATGT	
TgAFP	F: AACAACTGCTGCAAGCCGAAC	RT-PCR
TgAFP	R: CGGGACTTTCTACTTTTCTAGG	RT-PCR
***TgCSDP* SP1**	ACGCTCACAGCACTGGATGAAG	RACE-PCR
***TgCSDP* SP2**	TCAAGAGAGAAAGTGCCGATCGG	RACE-PCR
***TgCSDP* SP3**	GTAAAGGAAAGTCTTGCCCGAACC	RACE-PCR
***TgCSDP* SP5**	TCGTGAGGCCTTTGAGATCGGT	RACE-PCR

(Table 1). Confirmation of partial sequence of *TgAFP* was carried out by RT-PCR using specific primers. Amplification conditions were as follows: 94 °C for 3 min; 35 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min and a final step at 72 °C for 10 min. Confirmation of full-length cDNA sequence of *TgCSDP* was performed by RACE-PCR using 5'/3′ RACE Kit, 2nd Generation (Roche, Mannheim, Germany) according to manufacturer's directions. PCR products of both applications were run on a 2% agarose gels and purified using Qiaquick Gel Extraction Kit (Qiagen, Santa Clarita, CA) according to manufacturer's recommended protocol. Sanger sequencing was performed using CEQ 8800 Genetic Analysis System (Beckman Coulter, Fullerton, CA) as described by Guz et al. (2020). Nucleotide sequences of *TgAFP* and *TgCSDP* were deposited in GenBank under accession numbers OP373183 and OP373182, respectively.

2.6. Quantitative real-time PCR (qRT-PCR) analysis

Transcriptional regulation of *TgAFP* and *TgCSDP* in diapause phases, cold-shocked larvae, and developmental stages was analyzed by qRT-PCR. Specific primers for qRT-PCR were designed using Integrated DNA Technologies (IDT) (https://eu.idtdna.com/Primerquest/Home/Index) (Table 1). qRT-PCR analyses were conducted using Fast-Start Essential DNA Green Master Mix (Roche, Mannheim, Germany). Cycling conditions included an initial 95 °C for 10 min preincubation, followed by amplification for 40 cycles at 95 °C for 10 s, annealing temperature for 10 s (52 °C for *TgAFP*, 55 °C for *TgCSDP*), and 72 °C for 10 s with the QuantStudio 3 Real-Time PCR System (Applied Biosystems). The *β*-actin gene was used as an internal control for normalization. The efficiency of each reaction for all sets of primers was determined by measuring the standard curve. The accuracy of each amplicon was confirmed by conducting a melting curve analysis for each reaction. The relative expression level of the *TgAFP* and *TgCSDP* was calculated using the relative quantitative method ($2^{-\Delta\Delta Ct}$) (Livak and Schmittgen, 2001). A modified comparative CT approach was used to quantify the relative expression levels of the two genes for developmental expression analysis (Pfaffl, 2001). Three biological replicates were tested for each treatment. The reactions were run in three technical repeats.

2.7. Bioinformatics and phylogenetic analysis

NCBI Open Reading Frame (ORF) Finder (https://www.ncbi.nlm.nih.gov/orffinder/) tool was used to visualize the ORF of each putative protein. The partial sequence of TgAFP and the full-length sequence of TgCSDP were submitted to the BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to check for the similarities. Estimation of the putative molecular weight and isoelectric point (pI) of TgCSDP was carried out using The Compute pI/Mw tool (https://web.expasy.org/compute_pi/). ScanProsite (https://prosite.expasy.org/) was used for scanning the conserved domains of putative TgCSDP. JalView software (Waterhouse et al., 2009) was used to align the sequences for secondary structure analysis (SSA) by submitting the aligned CSDP amino acid sequence of three different Coleopteran insects and TgCSDP using the Clustal OMEGA tool (https://www.ebi.ac.uk/Tools/msa/clustalo/). JNetPRED in the JalView was utilized to perform secondary structure analysis (Cole et al., 2008). Prediction of the 3D structure of TgCSDP was conducted by using Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/html). To create phylogenetic trees, the amino acid sequences of 9 AFPs and 12 CSDPs from different insects were retrieved from the NCBI Database. The Neighbour-joining method was utilized to infer the evolutionary history (Saitou and Nei, 1987). Evolutionary distances, which were in the units of the number of amino acid substitutions per site, were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965). All locations that contain missing data and gaps were discarded. The MEGA 6 was used to conduct the evolutionary analyses (Tamura et al., 2013).

2.8. Data analysis

Statistical significance among treatments was determined through one-way analysis of variance (ANOVA) followed by a least significant difference test to compare means. Less than <0.05 *P*-value was considered significant. Statistical analyses were performed using Minitab 17.0 (Minitab Ltd, Brandon Court, United Kingdom) software.

3. Results

3.1. Identification and phylogenetic analysis of TgAFP and TgCSDP from T. granarium

In the present study, two novel putative cold-related genes, *TgAFP* and *TgCSDP*, were determined from the transcriptome data of the cold-induced diapause larvae of *T. granarium* and their nucleotide sequences were confirmed using RT-PCR and RACE-PCR sequencing. Nucleotide length of partial *TgAFP* and full-length *TgCSDP* are 400 and 4805 bp, cDNAs contain ORFs of 396 and 2745 bp encoding 131 and 914 amino acids, respectively. The predicted amino acid sequence of TgAFP and TgCSDP displayed 58.33% and 85.06% identity to Antifreeze protein maxi-like (XP_019867935.1) from *Aethina tumida* Murray (Coleoptera: Nitidulidae) and Cold-shock domain-containing protein (XP_008196887.1) from *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae), respectively. Theoretical pI and molecular weight of TgCSDP were found 6.28 and 102.26 kDa, respectively. Six characteristic CSD domains were determined in the putative amino acid sequence of TgCSDP (Fig. S1). The putative amino acid sequence of TgCSDP

contained characteristic (Y/F)GFI and (V/F)(V/F)H motifs. Prediction of the tertiary structure of TgCSDP was performed using the template "c6y6eA" model (PDB accession nr: 6Y6E) with 100% confidence, and 64% identity, respectively (Fig. 2).

The phylogenetic tree, constructed based on the alignment of the deduced AFP amino acid sequences from various insects showed that TgAFPs were clustered in two main branches. While one main branch consisted of Coleopteran and Hymenopteran AFPs, another main branch comprised Hemipteran and Dipteran AFPs (Fig. 3). TgAFP was phylogenetically close to AFP from *A. tumida,* which is consistent with the BLAST results. CSDPs from different insects formed two major branches in the phylogenetic tree. One main branch only consisted Dipteran CSDPs, another branch was formed by Coleopteran, Isopteran, Hymenopteran, and Hemipteran CSDPs. Depending on the phylogenetic tree, the deduced amino acid sequence of TgCSDP was found to be phylogenetically close to CSDP from *T. castaneum,* which is consistent with BLAST analysis (Fig. 4).

3.2. Diapause-associated expression levels of TgAFP and TgCSDP

Gene expression patterns of *TgAFP* and *TgCSDP* in relation to diapause triggered by cold acclimation were evaluated in last-instar larvae (Fig. 5). *TgAFP* was expressed at the highest level after decreasing the temperature from 33 °C to 25 °C in pre-diapause larvae. Transcript levels were dramatically downregulated at 20 °C and started to elevate at 15 °C in diapause larvae. The highest expression of *TgAFP* during diapause phase was found to be at 5 °C (fifth week). The first day of the post-diapause development phase showed significant expression of *TgAFP* compared to the third and sixth days, which demonstrated a significant downregulation. The transcript level of *TgCSDP* was found to be slightly downregulated in pre-diapause larvae which were formed by the first week of the collected samples at 25 °C. Although the larvae at 20 °C represented a downregulation for *TgCSDP*, the highest abundance of *TgCSDP* was detected at 15 °C in diapause larvae. Furthermore, a high abundance of *TgCSDP* was observed at 10 °C and 5 °C (fifth week) for diapause larvae. *TgCSDP* was remarkably downregulated in diapause larvae kept at 5 °C for four weeks. The highest *TgCSDP* expression level in the post-diapause development phase was detected on the first day followed by the third day, while it was found almost insensitive on the sixth day (Fig. 5).

3.3. Cold-shock and recovery period expression levels of TgAFP and TgCSDP

Temporal expression profiles of both *TgAFP* and *TgCSDP* during CST and the RP were analyzed using qRT-PCR (Fig. 6). The expression level of *TgAFP* was found to be downregulated during CST, first and the second hour of RP, while it was found slightly overexpressed in the first half and the third hour of RP. The expression profile of TgCSDP showed that CST caused a significant upregulation at first half hour compared to other time points of CST and RP.

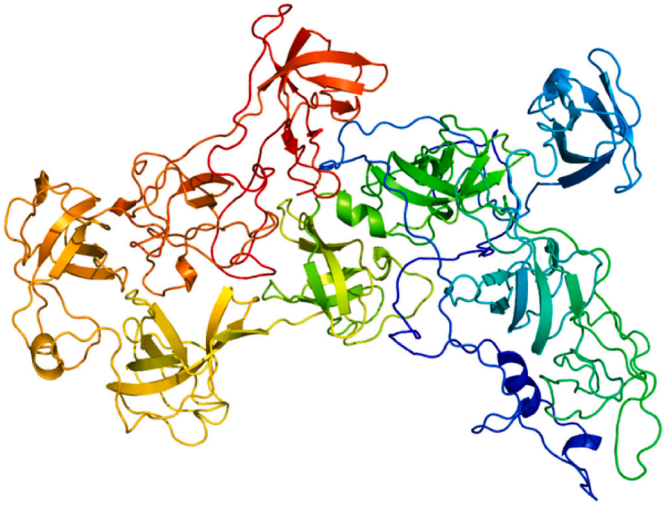

Fig. 2. Predicted 3D structure of the TgCSDP.

3.4. Developmental stage-specific expression of TgAFP and TgCSDP

Expression levels of *TgAFP* were lower throughout the developmental stages of *T. granarium*, whereas its amount was found higher in adults than in larval stages. Remarkable expression of *TgCSDP* was observed in female adults and followed by male adults. Larval stages exhibited lower expression levels of *TgCSDP* compared to adults (Fig. 7).

4. Discussion

Domain structure analysis demonstrated that TgCSDP has six CSD domains unlike CSDPs from many other organisms which include five CSD domains. Furthermore, TgCSDP includes both (Y/F)GFI and (V/F)(V/F)H motifs, and they are recognized as the ribonucleoprotein (RNP)-1 and RNP-2 motifs and participate in RNA/DNA binding (Kloks et al., 2002; Max et al., 2006; Goroncy et al., 2010). The presence of these motifs might imply its RNA/DNA binding role.

The phylogenetic tree analysis showed that TgAFP was detected to be closer to the AFPs of *Leptinotarsa decemlineata* (Say), *Diabrotica virgifera virgifera* LeConte, and *A. tumida* from the Coleopteran clade in the phylogenetic tree. TgCSDP was found to be closer to the AFPs of *T. castaneum* and *Photinus pyralis* (Linnaeus) and clustered within the Coleopteran clade. The placing of each insect species in each tree has been found to be consistent with their taxonomical order.

Organisms produce a variety of specific genes to cope with unsuitable circumstances. In this study, the expression level of *TgAFP* initially reached a peak point during the pre-diapause phase, which is consistent with similar expression profiles prior to diapause in previous studies. Guz et al. (2014) showed the highest expression of *AFP* in adults of sunn pest *Eurygaster maura* (Linnaeus) prior to diapause entry during the aestivation and pre-diapause phases. Similarly, a maximum point of *AFP* production from *Dendroides canadensis* (Latreille) was detected in early winter before passing to the colder months (Andorfer and Duman, 2000). The highest expression of *TgAFP* at 25 °C might indicate the possible roles of this gene such as water conservation prior to diapause and protection against sudden temperature fall other than freeze avoidance. The reason for declined *TgAFP* amounts during diapause entry at 20 °C compared to pre-diapause could be due to enough production of AFP before entering the diapause phase. Also, the long half-life of protein during the coldest circumstances could be another reason for the non-continuous expression of *TgAFP* (Qin et al., 2019).

In many studies, the most abundant expression of *AFPs* has been demonstrated in insects and mites, such as diapausing second instar larvae of spruce budworm (Qin et al., 2007), overwintering stag beetle (Arai et al., 2021), diapausing mites (Bryon et al., 2013) providing these organisms to keep alive under very low temperatures. In the present study, the highest mRNA levels of *TgAFP* were detected in diapause larvae of *T. granarium* exposed to 5 °C for one week, and a comparatively lower overexpression was detected for 4 weeks at 5 °C. Likewise, Graham et al. (2000) showed more than a 20-fold expression of *AFP* in small larvae of *Tenebrio molitor* L. which was exposed to 4 °C for 4 weeks. We can suggest that overexpression of *TgAFPs* during the diapause phase might allow *T. granarium* to survive at lower temperatures. The expression of *TgAFP* was found to be higher on the first day of recovery than the diapause larvae held at 5 °C for five weeks. This is similar to *Apis mellifera ligustica* Spin. in which *AFP* expression levels elevated in March when the temperature recovered (Qin et al., 2019). Moreover, *MpAFP* gene from *Microdera punctipennis* Kasz was found to be expressed in hot temperatures and thought to be assisting in protection against a

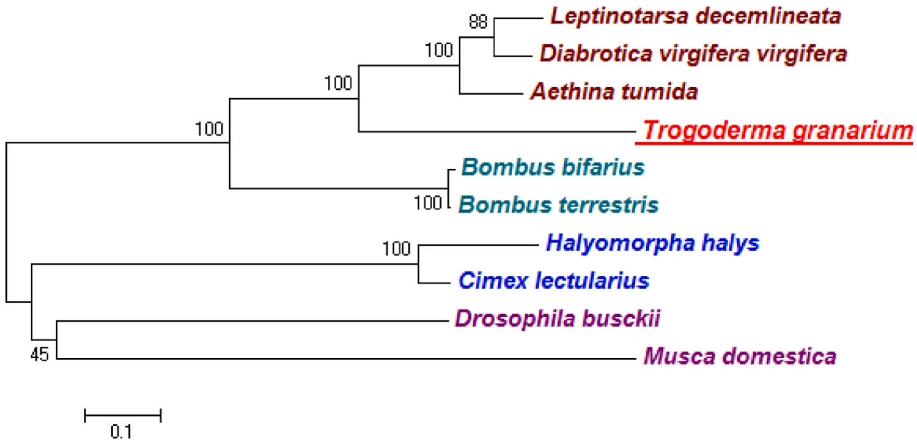

Fig. 3. Phylogenetic analysis of AFPs from different insects. Insect species and GenBank accession numbers used to create a phylogenetic tree are as follows: *Leptinotarsa decemlineata*, XP 023027979; *Diabrotica virgifera virgifera*, XP 028135954.1; *Aethina tumida* XP 019867935.1; *Bombus bifarius* XP 033316619.1; *Bombus terrestris*, XP 003398647.1; *Halyomorpha halys*, XP 014291522.1; *Cimex lectularius*, XP 014260537.1; *Drosophila busckii*, XP 017846674.1; *Musca domestica*, XP 019892706.1.

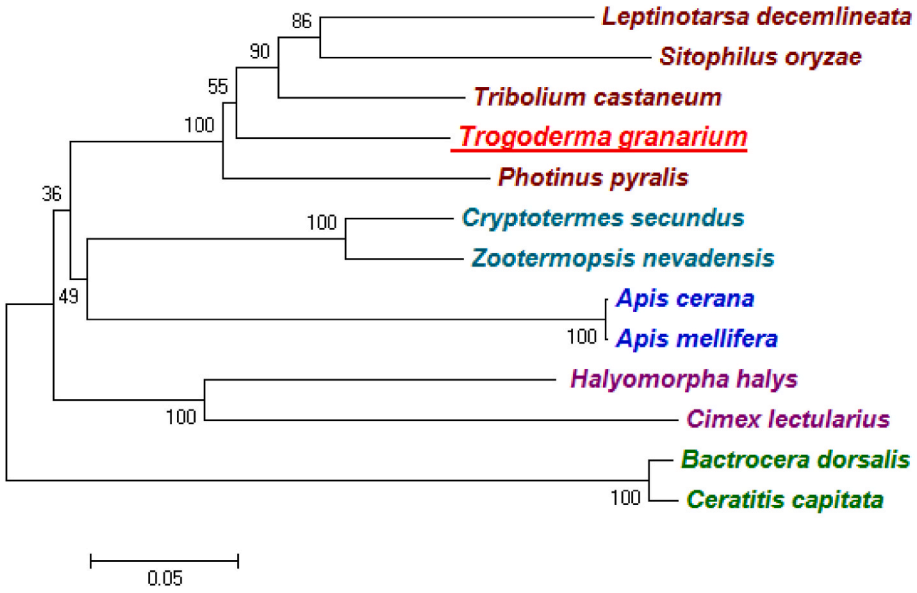

Fig. 4. Phylogenetic analysis of CSDPs from different insects. Insect species and GenBank accession numbers used to create a phylogenetic tree are as follows: *Leptinotarsa decemlineata*, XP 023030355.1; *Sitophilus oryzae*, XP 030765658.1; *Tribolium castaneum*, XP 008196887.1; *Photinus pyralis*, XP 031348080.1; *Cryptotermes secundus*, XP 023710927.1; *Zootermopsis nevadensis*, XP 021919017.1; *Apis cerana,* XP 016914731.1; *Apis mellifera*, XP 016768618.1; *Halyomorpha halys*, XP024217243.1; *Cimex lectularius,* XP 014261453.1; *Bactrocera dorsalis*, XP 011201977.1; *Ceratitis capitata*, XP 012157649.1.

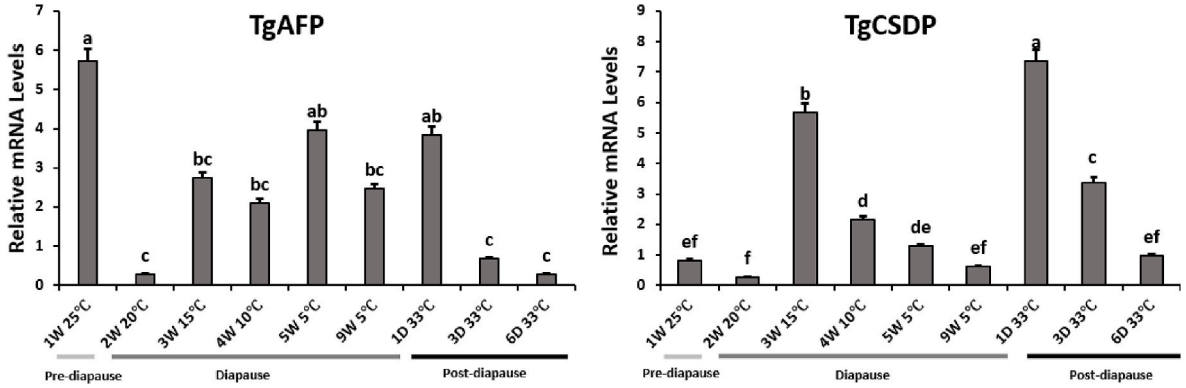

Fig. 5. Expression profiles of *TgAFP* and *TgCSDP* during different diapause phases *of T. granarium*. Pre-diapause (1W 25 °C), Diapause (2W 20 °C, 3W 15 °C, 4W 10 °C, and 5W 5 °C – 9W 5 °C), Post-diapause (1D, 3D, and 6D 33 °C).

rapid temperature fall or conserving water (Qiu et al., 2013). Furthermore, a remarkable expression of *TgAFP* was detected during the first day of the post-diapause at 33 °C, probably indicating pre-emptive protection before temperature decreases.

The information about the function of the *CSDP* genes in eukaryotes is limited, and no comprehensive study is available on *CSDPs* from insects. The upregulated expression of *TgCSDP* at 15 °C could indicate its possible chaperone role in toleration of cold-induced diapause. CSPA from *Escherichia coli* was synthesized following the low-temperature treatment and composed more than 10% of the total proteins inside

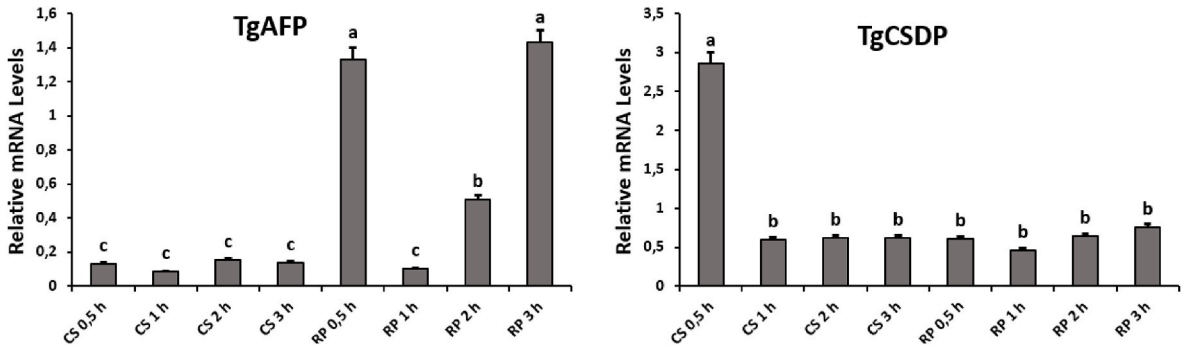

Fig. 6. *TgAFP* and *TgCSDP* mRNA expressions after 0.5, 1, 2, 3 h cold-shock treatment (CST) and 0.5, 1, 2, 3 h of recovery period (RP).

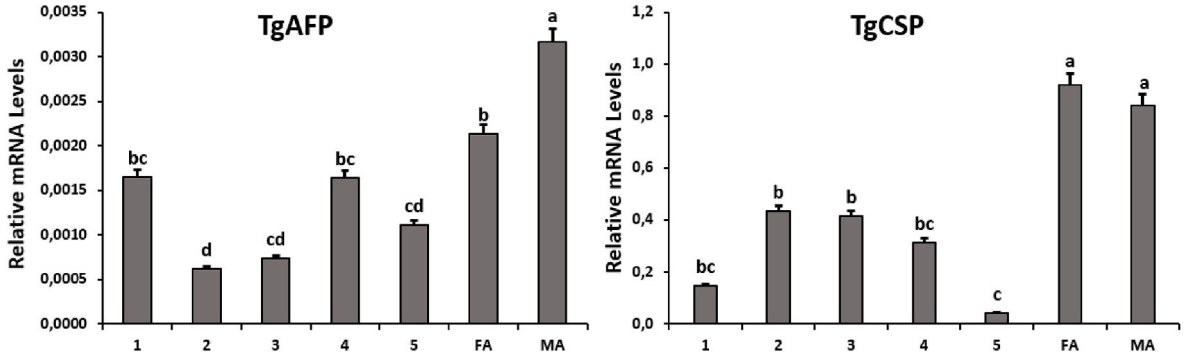

Fig. 7. Developmental stage-specific expression profiles of *TgAFP* and *TgCSDP*: 1: First-instar larvae, 2: Second-instar larvae, 3: Third-instar larvae, 4: Fourth-instar larvae, 5: Fifth-instar larvae, FA: Female adults, MA: Male adults. Expression levels were normalized to β-actin levels using ΔCT method.

the cells (Goldstein et al., 1990). A sufficient amount of *TgCSDP*, which has the ability to sense the decrease in temperature (Bae et al., 2000) might be synthesized around the beginning of the diapause at 15 °C, before being exposed to the colder temperatures. Furthermore, pleiotropic roles of CSDPs including development have been found in many organisms (Brandt et al., 2012; Behl et al., 2020). We can suggest that its elevated expression after the diapause phase might be relevant to post-diapause development. Since there is no study on the regulation of *CSDP*s in diapause insects, our study will be the first contribution to the literature for further research.

There are different results in various insects regarding AFP providing tolerance to CST. An *AFP* gene from spruce budworm *Choristoneura fumiferana* (Clem.) was transferred to *Drosophila melanogaster* Meigen to investigate its expression after CST; however, it has not provided any tolerance to the cold in the transgenic flies (Tyshenko and Walker, 2004). On the contrary, *AFP*s from *D. canadensis* (DAFPs) and an anti-freeze glycoprotein (*IAFGP*) from *Ixodes scapularis* Say expressed by transgenic *D. melanogaster* ensured the capability of surviving cold (Nicodemus et al., 2006; Lin et al., 2010; Neelakanta et al., 2012). *TgAFP* level was downregulated after CST, while it was slightly upregulated by recovery. This indicates that it might not provide any tolerance after cold-shock. But, it could take part in RP.

Similar to our result, a slight increased expression of the transcript level of Y-box binding protein 1 (YB-1) which is a member of the CSDP superfamily was reported after 0.5 h CST in *Drosophila melanogaster* (Thieringer et al., 1997). The upregulation of AccYB-1 from *Apis cerena cerana* Fabricius was detected by 1 h and it reached to the highest level at 2 h under 4 °C stress (Li et al., 2018). Moreover, two different studies have been conducted on mollusks that showed overexpression of CSDP after acute CST, indicating their contribution to cold tolerance (Yang et al., 2012; Dong et al., 2020). The significant expression elevation of TgCSDP after the CST might imply its involvement in response to acute cold-shock, but not to the RP.

Depending on the developmental stage in which the insect undergoes diapause or overwintering, the expression level of the *AFP* transcripts might vary. The majority of the isoforms from seventeen *AFP* transcripts were found most abundant in the second instar overwintering stage of *Choristoneura fumiferana* (Qin et al., 2007). Profiling of *AFP* expression in developmental stages of *E. maura* showed that *AFP*s were notably specific to the overwintering adults (Guz et al., 2014). In this study, mRNA levels of *TgAFP* were found to be very low in each developmental stage, while its level was detected to be higher in male and female adults. This is likely due to the temperature, which does not induce the synthesis of *TgAFP* in the developmental stages of insects at 33 °C.

It has been reported that CSDPs have pleiotropic roles (Behl et al., 2020) including developmental regulation (Moss et al., 1997). Silencing of YB-1 has resulted in a reduction of some development and growth-related genes in *A. cerena cerena* (Li et al., 2018). Furthermore, a Y-box protein-encoding gene from *D. melanogaster* was expressed throughout the developmental stages in which adults were the most significant (Thieringer et al., 1997). Likewise, the most abundant amount of *TgCSDP* was detected in adults, which might be attributed to its possible role related to development. Since the role of *CSDP*s is not fully understood in insects, further functional studies are needed to better understand this phenomenon.

5. Conclusions

Two important cold-related proteins, *TgAFP* and *TgCSDP,* have been identified in the cDNA library of *T. granarium*. Their gene expression patterns were profiled using qRT-PCR under a diapause induced by cold and after cold-shock treatment, and at developmental stages. In addition to the function of these protein genes in cold survival, they may serve as a critical target for methods such as RNA interference in the control of the pest. Further, the findings of this study expand our understanding of molecular mechanisms of insect diapause, freeze avoidance, and

responses to rapid environmental changes in temperature.

Contributions

Asli Dageri: Conceptualization, Methodology, Formal analysis, Investigation, Writing—original draft preparation, Writing—review and editing, Funding acquisition, Resources, Supervision, Data curation, Project Administration, Validation, Visualization.

Mohammed Lengichow Kadir: Investigation, Writing—original draft preparation, Writing—review and editing, Data curation, Visualization.

Nurper Guz: Conceptualization, Methodology, Writing—original draft preparation, Writing—review and editing, Supervision, Data curation.

Ayhan Ogreten: Conceptualization, Methodology, Investigation, Writing—review and editing, Data curation, Validation.

Muhammad Arshad: Formal analysis, Writing—original draft preparation, Writing—review and editing, Data curation, Visualization.

Funding

This study was funded by the Scientific Research Projects Coordination Unit of Necmettin Erbakan University (BAP Project no: 181215005).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgment

We would like to express our sincere thanks to Türkiye Scholarships for supporting M. L. K. during his research in Turkey and Kit-ARGEM R&D Center Konya Food and Agriculture University for providing lab facilities.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jspr.2022.102074.

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