Expression of mRNA for the t-complex polypeptide–1, a subunit of chaperonin CCT, is upregulated in association with increased cold hardiness in *Delia antiqua*

Takumi Kayukawa,¹ Bin Chen,^{1,2} Shoichiro Miyazaki,¹ Kyo Itoyama,³ Tetsuro Shinoda,⁴ and Yukio Ishikawa¹

¹Laboratory of Applied Entomology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo 113-8657, Japan

²Department of Biological Sciences, University at Buffalo, Buffalo, NY 14260, USA

³Akita Prefectural Agricultural Experiment Station, Genpachizawa 34-1, Aikawa, Yuwa, Kawabe, Akita 010-1231, Japan

⁴Insect Gene Function Research Center, National Institute of Agrobiological Science, Oowashi 1-2, Tsukuba, Ibaraki 305-8634, Japan

Abstract Summer-diapause and winter-diapause pupae of the onion maggot, *Delia antiqua* (Diptera: Anthomyiidae), were significantly more cold hardy than nondiapause, prediapause, and postdiapause pupae. Moreover, cold acclimation of nondiapause pupae conferred strong cold hardiness comparable with that of diapause pupae. Differential display analysis revealed that the expression of a gene encoding TCP-1 (the t-complex polypeptide–1), a subunit of chaperonin CCT, in *D. antiqua* (*DaTCP-1*) is upregulated in the pupae that express enhanced cold hardiness. Quantitative real-time polymerase chain reaction analyses showed that the levels of *DaTCP-1* messenger RNA in pupal tissues, brain, and midgut in particular, are highly correlated with the cold hardiness of the pupae. These findings suggest that the upregulation of *DaTCP-1* expression is related to enhanced cold hardiness in *D. antiqua*. The upregulation of CCT in response to low temperature in an organism other than the yeast is newly reported.

INTRODUCTION

Chaperonins are molecular chaperones responsible for the folding and assembly of newly synthesized proteins and refolding of denatured proteins in cells (Sonnen et al 2002). Chaperonins are classified into 2 groups (I and II): group I includes bacterial GroEL (Georgopoulos et al 1973) and the rubisco subunit-binding protein in higher plant chloroplasts (Hemmingsen et al 1988). Group II includes CCT (chaperonin containing the t-complex peptide–1 [TCP-1]) (Lewis et al 1992) and thermophilic factor 55 (Trent et al 1991).

CCT is a hetero-oligomeric chaperonin composed of 8 to 9 different subunit species of approximately 60 kDa (Rommelaere et al 1993) and has a central cavity that me-

diates the folding of polypeptides (Gómez-Puertas et al 2004). Each subunit species of CCT is encoded by an independent gene, and although they have common adenosine triphosphate (ATP)-binding motifs (SLGPVG, TI/VTNDG, and GDGTT) (Kim et al 1994), their amino acid sequence identities are only approximately 30% (Yokota et al 1999; Valpuesta et al 2002). Whereas GroEL in group I takes various substrates (Georgopoulos et al 1973), only actin and tubulin are known as the major substrates of CCT in vivo (Sternlicht et al 1993), although α -transducin and firefly luciferase are folded by CCT in vitro (Farr et al 1997; Gebauer et al 1998).

Despite the importance of molecular chaperones in stress response, the role of CCT is not clearly understood. We have long been interested in the unusually strong cold hardiness of the diapause pupae of the onion maggot, *Delia antiqua* (Diptera: Anthomyiidae). To explore the molecular mechanisms underlying the strong cold hardiness in *D. antiqua*, we sought differentially expressed genes in

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Correspondence to: Yukio Ishikawa, Tel: 81-3-5841-5061; Fax: 81-3-5841-5060; E-mail: ayucky@mail.ecc.u-tokyo.ac.jp.

the diapause pupae. As a consequence, the expression of *DaTCP-1*, which encodes a subunit species of CCT, was found to be upregulated in the cold hardy pupae. This study reports the expression levels of *DaTCP-1* in the Malpighian tubules, brain, fat body, and midgut of the pupae in different physiological states with varying cold hardiness and examines the relationship between cold hardiness and *DaTCP-1* expression.

MATERIALS AND METHODS

Insects

The onion maggots were originally collected at Sapporo, Japan, and their offspring have been reared on an artificial diet (Ishikawa et al 1983) for about 40 generations in the laboratory. Adults were reared in fabric screen cages (30 × 30 × 30 cm) with a supply of water, sugar, and yeast extract (Dried Yeast Extract D-3, Wako Pure chemicals, Kyoto, Japan). The cages were maintained in an environmental chamber controlled at 23 ± 1.0°C, under a 16 hour light:8 hour dark photoperiod (16L:8D) and 50–70% relative humidity. A plastic dish (8 × 5 cm diameter) containing damp glass beads and several pieces of onion was placed in the cage as the oviposition substrate. Eggs were inoculated on a piece of artificial diet placed on moistened fine sand in a plastic container (13 × 8 cm diameter). To obtain pupae with varied cold hardiness, larvae were reared under the conditions described below (Nomura and Ishikawa 2001). In this study, the age of pupae is expressed by the days after pupariation, with the day of pupariation being day 0.

Cold-acclimated nondiapause pupae

Larvae were reared on the artificial diet at 20 ± 0.2°C and 16L:8D. After pupariation, the nondiapause pupae were maintained at 17°C ± 0.2°C and 16L:8D for 5 days (ND5). ND5 pupae were then housed in a Styrofoam box and placed in an incubator adjusted to 5.0 ± 0.2°C for 0, 2, 8, or 32 days (CA0, CA2, CA8, or CA32). Because the lower threshold temperature for pupal development in *D. antiqua* is 5.7°C (Nomura and Ishikawa 2000), no development was considered to have occurred during acclimation. Hence, all cold-acclimated pupae were regarded as ND5, irrespective of the duration of acclimation.

Summer-diapause pupae

Larvae were reared at 25 ± 0.2°C and 16L:8D. Under these conditions, more than 95% of pupae entered summer diapause (SD). Puparia were maintained under the same conditions. For obtaining pupae that have termi-

nated summer diapause (SDT), the 10-day-old SD pupae (SD10) were transferred to 17 ± 0.2°C and 16L:8D for 10 days. In more than 95% of pupae, the diapause was terminated with this treatment.

Winter diapause

Larvae were reared at 15 ± 0.2°C and 12L:12D. Under these conditions, more than 95% of pupae entered winter diapause (WD). Puparia were maintained under the same conditions.

Cold-hardiness assay

The cold hardiness of the cold-acclimated nondiapause pupae (CA0, CA2, CA8, and CA32), WD pupae (WD3, WD10, and WD50), SD pupae (SD3, SD20), and the SDT pupae were evaluated from the survival rate after exposure to -20 ± 0.2°C for 5 days (Kayukawa and Ishikawa 2005).

Dissection and total RNA extraction

The thin outermost shell of the puparia (pupal case) was removed using fine forceps, and the pupa was dissected in phosphate-buffered saline (137 mM NaCl, 8 mM Na₂HPO₄·12H₂O, 2.7 mM KCl, and 1.5 mM KH₂PO₄, pH 7.4) on ice. The brain, Malpighian tubules, fat body, and midgut were isolated from 25 to 30 animals and snap frozen in liquid nitrogen. Total RNA was extracted from these tissues using an RNeasy Mini Kit (QIAGEN, Valencia, CA, USA) with RNase-free DNase I (QIAGEN).

Differential display analysis

Differential display (DD) was performed with a Gene Fishing DEG Kit (Seegene, Seoul, South Korea). The total RNA of the brain from CA0, CA2, CA32, and WD50 pupae was reverse-transcribed using an RNA PCR Kit (AMV) (V3.0, TAKARA BIO, Otsu, Shiga, Japan) and dT-ACP1 primer (Seegene; 5'-CTGTGAATGCTGCGACTACGATXXXXX(T)₁₈-3'). Using the first-strand complementary DNA (cDNA) as the template, polymerase chain reactions (PCRs) were performed with combinations of an arbitrary ACP (ACP01-ACP20) and dT-ACP2 (5'-CTGTGAATGCTGCGACTACGATXXXXX(T)₁₅-3') at 94°C for 5 minutes, 50°C for 3 minutes, and 72°C for 1 minute, 40 cycles of 94°C for 40 seconds, 65°C for 40 seconds, and 72°C for 40 seconds, and 72°C for 5 minutes. The PCR products were separated by 2% agarose gel electrophoresis and stained with SYBR[®] Green I (Molecular Probes, Eugene, OR, USA). One of the differentially expressed gene fragments, the *DaTCP-1* fragment, was extracted from the agarose gel, reamplified by PCR (94°C for 3 min-

Fig 1. Cold hardiness of *Delia antiqua* pupae assessed by survival after a 5-day treatment at -20°C . After the -20°C treatment, pupae were maintained at 17°C . (A) Nondiapause pupae acclimated at 5°C for 0, 2, 8, and 32 days (CA0, CA2, CA8, and CA32) ($n = 150$). (B) The pupae obtained under winter diapause-inducing conditions were maintained under the same conditions for 3, 10, and 50 days (WD3, WD10, and WD50) ($n = 200$). (C) Pre-summer diapause (SD) pupae (SD3), SD pupae (SD20), and pupae that had terminated summer diapause (SdT) ($n = 150$). Bars with the same letter are not significantly different (Ryan's multiple comparison test of proportions, $P < 0.05$).

utes, 35 cycles of 94°C for 40 seconds, 65°C for 40 seconds, and 72°C for 40 seconds, and 72°C for 5 minutes) with 5' and 3' universal primers (Seegene), and sequenced using a BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and 5' universal primer on an ABI PRISM 377HN Sequencer (Applied Biosystems).

Cloning of full-length cDNA by RACE

The 5' and 3' ends of the *DaTCP-1* cDNA were obtained by a modified rapid amplification of cDNA ends (RACE) method using a FirstChoice[®] RLM RACE Kit (Ambion, Austin, TX, USA). The template first-strand cDNA was synthesized from the total RNA extracted from the brain of WD50 pupae. The first PCR in the 5' RACE was performed using 5' RACE outer primer (Ambion, Austin, TX, USA) and SP-R1 (5'-AGAAGCAGCAGCTCTGGCTTTAG-3'). The first and second nested PCRs were performed using 5' RACE inner primer and SP-R2 (5'-GCAGCACAGTTGCCTTAGC-3') and the inner primer and SP-R3 (5'-AATTCACGGGCACGGATACC-3'), respectively. The first PCR in the 3' RACE was performed using 3' RACE outer primer and SP-F1 (5'-TCGCAACAGATGCCCAAGA-3'). The nested PCRs were performed using 3' RACE inner primer and SP-F2 (5'-CGGCGGTGTTGATGATTTGT-3'), and the inner primer and SP-F3 (5'-GCTGCTGAGGTGGCTCAAGA-3'). The PCR products obtained by 5' and 3' RACE were subcloned using pGEM[®]-T Easy Vector System I (Promega, Madison, WI, USA) and sequenced.

Quantitative real-time PCR

The complementary DNAs (cDNAs) prepared from the Malpighian tubules, brain, fat body, and midgut using the SYBR[®] RT-PCR Kit (TAKARA BIO) were diluted to 1/50 for detection of *DaTCP-1* detection and 1/1000 for detection of the 18S ribosomal RNA (rRNA) gene. The primers used for quantitative real-time PCR (*qr-PCR*) of *DaTCP-1* were SP-F2 and SP-R2. The primers for the 18S rRNA gene were 5'-TTAAGCCATGCATGTCTAAGTAC-3' and 5'-TCTCAGGCTCCCTCTCCGGAATCG-3'. The *DaTCP-1* transcript was quantified on an ABI PRISM[®] 7700 Sequence Detection System (Applied Biosystems). The *qr-PCR* was carried out in a 20- μL reaction volume containing 1 μL of template cDNA, SYBR Premix Ex Taq, ROX Reference Dye (TAKARA BIO), and 0.2 μM of each primer. Shuttle PCR conditions were 95°C for 10 seconds followed by 40 cycles of 95°C for 5 seconds and 60°C for 30 seconds. The level of *DaTCP-1* messenger RNA (mRNA) was normalized to that of 18S rRNA.

RESULTS

Cold hardiness

Nondiapause (CA0) pupae of *D. antiqua* could not survive a 5-day exposure to -20°C (Fig 1A). However, the survival rate increased as the period of cold acclimation was extended (Fig 1A). Most prediapause pupae (WD3 and SD3) were not able to survive the -20°C treatment; however, the pupae in the midst of diapause (WD10, WD50,

Fig 2. Differential display of messenger RNA derived from the brain of CA0, CA2, CA32, and WD50 pupae. The arrow indicates a band corresponding to the *DaTCP-1* complementary DNA fragment, the size of which is 1.5 kbp.

and SD20) had marked cold hardiness (Fig 1 B,C). Moreover, the SDT pupae could not survive the -20°C treatment (Fig 1C).

Differential display

The cDNAs prepared from CA0, CA2, CA32, and WD50 pupae were subjected to DD analysis. DD analyses using 20 arbitrary primers revealed 3 bands that appear in association with the increase in cold hardiness (data not shown). In this study, only the band that showed the clearest change (Fig 2) was analyzed further. Analysis of the sequence of the DD product by BLAST (Altschul et al 1997) for the nucleotide and for the deduced amino acid sequence showed high homology to TCP-1s in *Drosophila* and *Anopheles*.

Full-length cDNA

The full-length cDNA (*DaTCP-1*) has a predicted ORF encoding a protein of 555 amino acids (Fig 3). The protein is estimated to have a molecular weight of 59.4 kDa and a *pI* of 6.45. In the 3' UTR, 4 ATTTA-motifs specific to mRNAs that are susceptible to degradation were observed. In addition, 3 characteristic ATP-binding motifs are present in *DaTCP-1* (SLGPVG, TVTNDG, and GDGTT) (Fig 3). The homology analyses showed 93% amino acid identity to *D melanogaster* TCP-1- α (GenBank accession no. P12613), 78% identity to *Apis mellifera*

(XP_392660), 74% identity to *Homo sapiens* TCP-1 (AAH00665), and 61% identity to *Neurospora crassa* (XP_323801).

Expression of *DaTCP-1* mRNA

The changes in levels of *DaTCP-1* mRNA in different tissues of the pupae that occur in association with cold acclimation, SD, and WD were analyzed by *qr*-PCR (Fig 4). The expression of *DaTCP-1* was increased in the brain, midgut, and Malpighian tubules as the period of cold acclimation was extended (Fig 4A). In contrast, the expression in the fat body was decreased to half after a 32-day cold acclimation (Fig 4A). In WD pupae, the expression levels in all tissues examined were increased 2- to 6-fold (Fig 4B). In SD pupae, levels in all tissues examined were increased 2- to 6-fold in the midst of diapause (SD20). Interestingly, when the SD was completely terminated (SDT), the level decreased to that of the prediapause pupae (SD3) (Fig 4C).

DISCUSSION

Cold hardiness in insects has been discussed in terms of cryoprotectants, supercooling point, antifreeze proteins, homeoviscous adaptation, and heat shock proteins (Lee 1991; Storey and Storey 1991; Graether et al 2000; Yocum 2001; Košťál et al 2003). This study clearly demonstrated that expression levels of *DaTCP-1* are highly correlated with the cold hardiness of the *D. antitqua* pupae.

The roles of molecular chaperones in response to various stresses have been widely studied in organisms from microbes to plants and animals. GroEL, a chaperonin in group I, was reported to play important roles in response to temperature stress, osmotic stress, and chemical stress (Fayet et al 1989; Ben-Zvi and Goloubinoff 2001; Hennequin et al 2001). In contrast, CCT, a chaperonin in group II, was considered not to be induced by stress until the early 1990s (Ursic and Culbertson 1992; Leroux and Candido 1995). Recently, however, CCT was shown to be induced by heat shock in human cells (Schena et al 1996), chemical stress (CdCl_2) in *Oxytricha granulifera* (Palmedo and Ammermann 1997), and cold shock in the yeast *Saccharomyces cerevisiae* (Somer et al 2002).

In general, the mechanisms that cause cold injuries in cells are not well understood, although the involvement of phase transitions in the cell membrane lipid and a complex metabolic disorder have been proposed (Drobnis et al 1993; Nedvĕd et al 1998). Recently, depolymerization of actin and tubulin at low temperature has been recognized as an important factor for the occurrence of cold injuries. In a wide range of plant and animal species, the significance of the recrystallization of cytoskeletons in the recovery from cold stress has been reported (Upadhy

1 CTAATTGT 8
 9 GAGTCAGCGCGTGTAGCGGCTGAAGAAAATTTCAATAAAACAAATTTAATAAATTTAAATAAAATTTAAATTAATAAACAAA 98
 99 ATGTCACAATTTGTAGTCATTATCTAGCTGGTACCGGTCACCGGTCACACAAATTTATGGCTGCTAGTTCC 188
 1 M S T I V S P L S I A G T R Q S G Q P V R T Q N V M A A S S 30
 189 ATAGCCAACATGTTAAAGTTCCTTGGGCTCTGGGTTTGGCAAAATTTGGTCGATGATTTGGTGATTTACCGTTACCAATGAT 278
 31 I A N I V K S S L G P V G L D K M L V D D I G D V T V T N D 60
 279 GCGGCCACCATTTTAAGATTATTGGAAGTTGAACATCCTGCTGCTAAAGTCTTAGTTGAGCTGGCTCAATTGCAAGATGATGAAGTTGGT 368
 61 G A T I L R L L E V E H P A A K V L V E L A Q L Q D D E V G 90
 369 GATGGTACCACCTCTGTGGTCAATTTAGCCGAGAACTCTTAAGAATGCCATGAATTTGGTTAAACAAAATTCATCTACTCTCCATC 458
 91 D G T T S V V I L A A E L L K N A D E L V K Q K I H P T S I 120
 459 ATCTCTGGCTATCGTTTGGCTTGCAAGAAAGCTTGCAATACATCTCAGAACATTTGACGGCTCCGGTAGATGAATGGGACGTGAACA 548
 121 I S G Y R L A C K E A C K Y I S E H L T A P V D E L G R E T 150
 549 TTGATTAACATCGCCAAACCTCCATGAGTTCCAAAATTTGGAGCTGATGCTGATTCTTCTCAGCCATGGTGGTAGATGCTGCTCAG 638
 151 L I N I A K T S M S S K I I G A D A D F F S A M V V D A A Q 180
 639 GCGGTCAAAATCACGATCCTAAAGCAATGCTGGCTATTCCATTAAAGCCGTTAATGTTTAAAGGCTCATGGCAAGTGGCCCGCGAA 728
 181 A V K I T D P K G N A A Y S I K A V N V L K A H G K S A R E 210
 729 TCTCTTTGATCCCGGTTATGCTTTGAAGTGTACCATTTGCTTCGCAACAGATGCCAAGAAAATTTGCAATGCCAAGATGCTGCTTTA 818
 211 S L L I P G Y R I A L N C T I A S Q Q M P K K I V N A K I A C L 240
 819 GATTTCTTTTGGCAAAAGCAAAATGAAATGGGTGCCAAGTTTGTATCAATGATCCGATAAGTTGGAAGGTATCCGTGCCCGTGAA 908
 241 D F S L Q K T K M K M G V Q V L I N D P D K L E G I R A R E 270
 909 TTGGACATTACCAAGAACATCAATGAAGTTTGGAGTACCGGTGCAATGTTGTTTGTGTTCCGGCGGTGTTGATGATTGTCATG 998
 271 L D I T K E R I N K I L S T G V N V V L C S G G V D D L C M 300
 999 AAATATTTTGTGAAGCTGGTCTATGGCTGTGACAGCTGTAAGAAGGGTGATTGAAAATTTGCTAAGGCACTGGTGTCTTTC 1088
 301 K Y F V E A G A M A V R R V K K G D L K I I A K A T G A A F 330
 1089 CTTACATCGCTAACCAACATGAAGGTGAAGAAAGTTTCGATGCCCTCCATGCTAGGTGAAGCTGCTGAGGTGGCTCAAGAACGTATCTGT 1178
 331 L T S L T N M E G E E S F D A S M V G E A A E V A Q E R I C 360
 1179 GATGATGAATTGATAATTATCAAGGTACTAAAGCCAGAGCTGCTGCTTCTATTATCTTACGTGGTCCCAATGATTCTATTGTGATGAA 1268
 361 D D E L I I I K G T K A R A A A S I I L R G P N D F Y C D E 390
 1269 ATGGAAACATCTATACATGCTTTGTGTGTTTAAAGGCTGTTTGGAAAGCAAGAGTAGTACGTGGAGGTGGCTGTGTGAAGCT 1358
 391 M E R S I H D A L C V V K R V L E S K K V V A G G G C V E A 420
 1359 GCTTGTCTATTACTTGGAAACCTTGCCACTTCATTGAGCTCCCGCAACAAATTTGGCTATTGCTGAATTTGCCAAATCTTTGTGTGTT 1448
 421 A L S I Y L E N F A T S L S S R E Q L A I A E F A K S L L V 450
 1449 ATTCGCAAAACCTTATCGGTAATGCTCTAAAGATGCTACCGATTGGTGGCAAAATTTGGTGGCTATCACAATTCAGTCAACAAAA 1538
 451 I P K T L S V N A A K D A T D L V A K L R A Y H N S S Q T K 480
 1539 ACCGAACATGCCCAATTAATATGACCGGTTAGATTAAATCGAGGTGTAGTGGTGACAACAAAAGGCTGGTGTTTAGAGCTGCC 1628
 481 T E H A Q L K W T G L D L I E G V V R D N K K A G V L E P A 510
 1629 ATGTCTAAAATAAATCATTGAAATTTGCCACGAGCGCCATTACCAATTTTGGCTATTGATGATATGATTAAATTAATCTGATGAG 1718
 511 M S K I K S L K F A T E A A I T I L R I D D M I K L N P D E 540
 1719 AAGCCCGCAAGTCGTATGGTGTGCTGCTGGTGAACCTTAAGTTGTATCATTTTAAATCGTCGATTATTAAGTTTAATTAATC 1808
 541 K P G K S Y G D A C A A G E L * 555
 1809 ATCATCTTTGTTAATTTGCTACTATCCAACAAAACAAAAGCACTTTATTATTAACAATTTAATTTGTTAAAAAACATCCACCTT 1898
 1899 TTTTCATTAAATTTTAAGTTGTTTCTTTATTTAACTTTTCTTAACATAATATTTATTAACTTTACAAATTTGATTAATTCACA 1988
 1989 CGCCCTCAATTTAAATACAAATCCATAATTTGATATTATGAAAGAAGTCCTGTGTAACTGTAAGTTTGAATAAGGATTTT 2078
 2079 GATAAATAAGAAACAAACAAAAACAC (A)n

Fig 3. Nucleotide and deduced amino acid sequences of *DaTCP-1* complementary DNA. The underlined amino acids represent adenosine triphosphate-binding motifs. Double-underlined nucleotides represent a presumable polyadenylation signal (AATAAA) and RNA instability motif (ATTTA). This sequence has been deposited in the GenBank database (Accession no. AB194475).

and Strasberg 1999; Egierszdorff and Kacperska 2001). Pokorná et al (2004) investigated changes in the organization of actin filaments and microtubules in tobacco cells during cold treatment and subsequent recovery and proposed a model for the mode of reorganization of the cytoskeletons. In wheat, an actin-binding protein induced by cold acclimation is suggested to facilitate cytoskeletal rearrangements, resulting in the enhancement of cold tolerance (Ouellet et al 2001). As mentioned in the Introduction, actin and tubulin are the major substrates of CCT. On the basis of the finding that CCT was upregulated in response to low temperature in the yeast *S cerevisiae*, Somer et al (2002) hypothesized that upregulation of CCT expression is related to the reorganization of actin and tubulin monomers during the recovery from cold stress. In view of these findings, the upregulation of *DaTCP-1* expression in *D antiqua* is likely to be related to the recrystallization or rearrangement of actin and tubulin and hence contribute to the reinforcement of cold hardiness.

D antiqua is known to pass the coldest and hottest seasons as diapause pupae. WD and SD pupae as well as cold-acclimated nondiapause pupae have significantly higher tolerance to both low and high temperatures than nondiapause pupae (Nomura and Ishikawa 2001). However, the ecological significance of cold tolerance in SD pupae and heat tolerance in WD pupae has been unclear. We suggest that diapause pupae acquire both low- and high-temperature tolerance because common mechanisms underlie these 2 kinds of tolerance. Although the conditions that induce SD and WD are different (Nomura and Ishikawa 2001), it is likely that common mechanisms are activated in the 2 diapauses. Actually, in addition to *DaTCP-1*, the expressions of other chaperones, *Hsp70* and *Hsp90*, are also upregulated in both SD and WD pupae (B. Chen et al, unpublished data). A curious finding in this study is that the expression of *DaTCP-1* in the fat body was decreased by extended cold acclimation. This finding strongly suggests that cold acclimation and diapause confer tolerance to pupae through different processes.

Fig 4. Quantitative real-time PCR analysis of *DaTCP-1* messenger RNA (mRNA) levels. The levels of *DaTCP-1* mRNA were normalized to those of the internal standard, 18S ribosomal RNA (3 replicates). *DaTCP-1* mRNA levels in cold-acclimated nondiapause pupae (A), winter-diapause pupae (B), and summer-diapause pupae (C). Br, brain; MG, midgut; MP, Malpighian tubules; FB, fat body.

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