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DaTrypsin, a novel clip-domain serine proteinase gene up-regulated during winter and summer diapauses of the onion maggot, *Delia antiqua*

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Abstract

Diapause prepares insects and other arthropods to survive in harsh environments. To explore the molecular basis of winter (WD) and summer diapauses (SD), we screened for diapause-specific genes in the onion maggot, *Delia antiqua*, that diapauses as a pupa in both summer and winter. A diapause-induced transcript, *DaTrypsin*, was identified through differential display, and examined by Northern blot, quantitative real-time PCR and sequence analyses. The full-length cDNA, 1379 bp long, encodes 384 a.a. with a molecular mass of 43,005 Da. The protein contains a 20-a.a. secretion peptide, followed by an amino-terminal clip domain and a carboxyl-terminal serine proteinase domain. With Ser, His and Asp as catalytic residues and Asp, Gly and Ser as specificity determinants, DaTrypsin is anticipated to be a trypsin-like enzyme. *DaTrypsin* transcription is up-regulated in both SD and WD pupae with higher mRNA levels during WD than SD. Heat shock further elevated gene transcription in both SD and WD pupae, whereas cold shock reduced *DaTrypsin* expression in SD pupae and had no significant effect on WD pupae. In SD pupae, *DaTrypsin* transcripts gradually build up during diapause, and after temperature shocks, whereas in WD pupae *DaTrypsin* mRNA levels are high at the beginning of diapause and immediately after a temperature shock and then gradually decrease with time. *DaTrypsin* represents the first serine proteinase gene expressed during diapause as well as the first gene up-regulated in both SD and WD. It may participate in the host's immune defense and/or maintain the developmental status in the diapausing pupae.

Keywords: Trypsin-like; Gene expression; Stress response; Protein structure; Insect immunity

1. Introduction

Diapause is a developmental strategy widespread among insects and their arthropod relatives. It allows insects to survive in harsh summers, winters, dry seasons and other unfavorable conditions, to exploit seasonal resources, and to synchronize the growth pace of their populations (Denlinger, 2002). The light cycle and environmental temperature usually combine to dictate whether the insect will enter diapause some time later. Long day-length and high temperature channel the insect toward summer diapause (SD), whereas short day-length and low temperature lead to winter diapause (WD). The diapause program involves behavioral, morphological and physiological changes that prepare the diapause-destined insect for a period of developmental arrest. All of these preparative steps are

Abbreviations: SP, serine proteinase; SD, summer diapause; WD, winter diapause; DIG, digoxigenin; RAPD, random amplification of polymorphic DNA; PCR, polymerase chain reaction; Q-RT-PCR, quantitative real time-PCR; RACE, rapid amplification of cDNA ends; HSP, heat-shock protein.

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expected to be associated with gene expression patterns not observed in nondiapausing individuals. Many genes cease expression during diapause, while others are uniquely expressed at this time (Denlinger, 2002). Northern blot analysis suggested that diapause-specific genes represent about 4% of all genes expressed during diapause in the flesh fly, *Sarcophaga crassipalpis* (Flannagan et al., 1998). Understanding the molecular mechanisms of diapause is of great importance in basic biology, developmental biology and management of insect pests.

During diapause insects are especially vulnerable to bacterial and fungal infections and rely on their innate immune system for survival (Khush and Lemaitre, 2000). Serine proteinases (SPs) of the chymotrypsin family and serine proteinase inhibitors of the serpin family mediate/coordinate various immune responses that include hemolymph coagulation, melanotic encapsulation, activation of cytokine precursors and induced synthesis of antimicrobial peptides (Iwanaga et al., 1998; Söderhäll and Cerenius, 1998; Jiang and Kanost, 2000; Kanost et al., 2001; Ahn et al., 2003; Theopold et al., 2004). This family of SPs and their homologues is quite extensive, and *Drosophila* sequences have been recently categorized by sequence features, domain structures, chromosomal locations and phylogenetic relationships (Ross et al., 2003). Biological functions, however, are known for only a few family members.

The onion maggot (*Delia antiqua*), widely distributed over Asia, Europe and North America, and a serious pest of onion (*Allium cepa*), is an excellent model for diapause research. It can be easily reared in the lab on an artificial diet (Ishikawa et al., 1983) and long-term storage (e.g. 1 year) of the pupae at 4 °C does not seriously influence its survival rate (Ishikawa et al., 2000). *D. antiqua* overwinters and oversummers as diapausing pupae (Tomioka, 1977; Park et al., 1990), and both diapauses can be easily induced in the laboratory (Ishikawa et al., 1987, 2000). This insect has extraordinarily strong cold hardiness and high-temperature tolerance (Nomura and Ishikawa, 2000, 2001; Ishikawa et al., 2000). Finally, because *D. antiqua* is phylogenetically relatively close to *Drosophila melanogaster* it can profit from its genomic information. In this study, we isolated a diapause-specific gene that encodes a trypsin-like SP most similar to *D. melanogaster* SP33 (CG2056). A significant level of the transcript was detected during both SD and WD.

2. Material and methods

2.1. Experimental insects

The colony of *D. antiqua* was reared on an artificial diet at 20 °C with a 16L:8D cycle and relative humidity 50–70%, as described by Ishikawa et al. (1983). Larvae were maintained at 25 °C with a 16L:8D photocycle to induce SD. Newly formed puparia (white or pale-orange) were used for experiments. They were kept under the same conditions as the larvae until day 15 after pupariation (D15) and then transferred to 16 °C and 16L:8D to trigger diapause termination (Ishikawa et al., 2000). A few co-occurring non-diapausing pupae, which could be discriminated by direct observation through the semi-transparent pupal case (Nomura and Ishikawa, 2001), were eliminated at D8. D5 diapausing pupae were subject to cold shock at −15 °C with 0L:24D for 2, 4 or 6 days, or heat shock at 35 °C with 16L:8D for 2, 4 or 6 days. To induce WD, larvae were reared at 15 °C with 12L:12D (Nomura and Ishikawa, 2000), and pupae were kept under this condition throughout. The pupae at D40 were treated for 5, 10 or 15 days either at −20 °C with 0L:24D or 35 °C with 16L:8D. Pupae collected at various stages were snap frozen in liquid nitrogen and stored at −80 °C prior to RNA extraction.

2.2. Preparation of RNA and cDNA

Total RNA and mRNA were isolated from the pupae using a RNeasy Midi/Maxi kit (Qiagen) and a Micro-Poly(A)Pure™ kit (Ambion), respectively. To eliminate genomic DNA, the RNA samples were treated with RNase-Free DNase I according to the manufacturer's protocols (Qiagen). cDNA was reverse-transcribed from total RNA and from mRNA using random nanomers and oligo(dT)-adaptors with a RNA PCR kit (Takara), respectively. cDNA purification was carried out using a SUPREC™-02 kit (TaKaRa).

2.3. Isolation, cloning and sequencing of DaTrypsin cDNA fragment

A *DaTrypsin* cDNA fragment was isolated by screening for diapause-induced genes through differential display (Liang and Pardee, 1992; Nakazono and Yoshida, 1997). A small set of cDNAs from prediapause, diapause and postdiapause was used in the screening, and the screening was performed by PCR using 20 arbitrary dodecamers (Bex). The PCRs were conducted using Ex Taq™ (TaKaRa) at 95 °C for 5 min, 35 cycles of 95 °C for 1 min, 35 °C for 1 min and 72 °C for 2 min, and an additional 5 min at 72 °C in the last cycle. The PCR products were separated by 2% agarose gel electrophoresis and stained with SYBR™ Green I (Molecular Probes). The *DaTrypsin* fragment was recovered using a SUPREC™-01 Kit (TaKaRa) and cloned into a pGEM-T vector (Promega). DNA inserts of the resulting clones were amplified by PCR with vector-specific primers and sequenced using BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) on an ABI PRIM 377HN Sequencer (Perkin-Elmer).

2.4. Northern blot analysis

Denatured total RNA samples (8 μg each) were separated by electrophoresis on a formaldehyde/MOPS agarose gel

(1.5%), along with digoxigenin (DIG)-labeled molecular weight markers (Roche Applied Sciences). The RNAs were transferred onto Hybond-N+ nylon membrane (Amersham Pharmacia) and separately hybridized with labeled *DaTrypsin* and 18S rRNA cDNA probes in DIG Easy Hyb (Roche Applied Sciences). The probe-target hybrids were reacted with anti-DIG, developed with NBT/BCIP (DIG Nucleic Acid Detection Kit, Roche Applied Sciences), and photographed using a digital camera.

DaTrypsin and 18S rRNA cDNA probes were individually labeled with DIG in a PCR using specific primers (PCR DIG Probe Synthesis Kit). A *DaTrypsin* cDNA fragment (186 bp) was amplified using gene-specific primers 5′-AGA ACG GCC ATA AAC GGA AAT TCA T-3′ and 5′-GCA GCA AAA TCA AAC TGC TAA AG-3′. Primers for 18S rRNA were: 5′-TTA AGC CAT GCA TGT CTA AGT AC-3′ and 5′-TCT CAG GCT CCC TCT CCG GAA TCG-3′, which amplified a 333 bp fragment. The rRNA primers were designed based on an alignment of insect 18S gene sequences from *D. melanogaster*, *Anopheles gambiae*, *Bombyx mori*, *Dolichopeza subalbipes*, *Tanyptera dorsalis*, *Chrysops niger*, *Anastrepha fraterculus* and *Epiphragma fasciapenne* (data not shown).

2.5. Quantitative real-time PCR (Q-RT-PCR)

DaTrypsin transcripts in total RNA were quantified by real time-PCR on an ABI PRISM 7700 thermal cycler (PE Biosystems). The reverse transcribed cDNA samples were used as PCR templates. *DaTrypsin*- and 18S rRNA-specific primers were the ones described above. The 18S rRNA gene was chosen as a reference for normalizing the templates. One cDNA sample, diluted to 1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}, was employed as an internal standard. Q-RT-PCR was carried out in 20 μl reactions containing 1 μl of template cDNA or the standard, 1× QuantiTect SYBR Green PCR premix (Qiagen), and 0.3 μM of each primer. Thermal cycling conditions were: 95 °C for 15 min, 45 cycles of 95 °C for 15 s, 58 °C for 30 s and 72 °C for 30 s, then 40 °C for 30 s and 95 °C for 1 min with ramp time of 19′59″. After Q-RT-PCR, the absence of unwanted by-products was confirmed by automated melting curve analysis and agarose gel electrophoresis of the PCR product. The relative molar amounts of *DaTrypsin* and 18S rRNA transcripts were calculated based on crossing point analysis, using standard curves generated from the cDNA standards. *DaTrypsin* mRNA levels were normalized with those of 18S rRNA in the same samples quantified in the same manner, and the final relative mRNA levels of *DaTrypsin* were averages of four replicates.

2.6. Cloning and sequencing of the 5′ and 3′ cDNA ends

For 3′-RACE (rapid amplification of cDNA ends), the first-strand cDNA was synthesized from mRNA using the oligo(dT)-anchor primer from a 5′/3′ RACE Kit (Roche Applied Science). After purification using the SUPREC™-02 Kit, the cDNA was amplified with the anchor primer and a *DaTrypsin*-specific forward primer (5′-TGA CAG TAC AAT TTG GTA TCG-3′). The PCR product was reamplified using the anchor primer and another gene-specific forward primer (5′-CGG ACA CTC ATC TTT GTG CCG GTG A-3′). The PCRs consisted of five cycles of 94 °C for 30 s, 45 °C for 30 s and 72 °C for 2.5 min, followed by 30 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 2.5 min, and an additional 7 min at 72 °C on the last cycle. The product from the second-round PCR was cloned and sequenced as described above.

For 5′-RACE, template cDNA was synthesized with a *DaTrypsin*-specific reverse primer (5′-TCA CCG GCA CAA AGA TGA GTG TCC G-3′). After purification, the 3′ end of the cDNA was attached with a poly(A)-tail using dATP and terminal deoxynucleotidyl transferase. Then, the cDNA was amplified by PCR using an oligo(dT)-anchor primer and a gene-specific primer (5′-TGG ATT TGT TGA CTT CTC TGT CC-3′). The PCR product was amplified once more with the anchor primer and another gene-specific primer (5′-AGA ACG GCC ATA AAC GGA AAT TCA T-3′). Thermal cycling conditions were: 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 60 s, and additional 7 min at 72 °C on the last cycle. The second-round PCR product was cloned and sequenced.

2.7. Bioinformatics analyses

The initial *DaTrypsin* cDNA fragment and cDNA ends derived from the 5′ and 3′ RACE were edited and assembled with BioEdit. A BLAST search was performed to identify similar sequences in GenBank (http://www.ncbi.nlm.nih.gov/). Similar sequences retrieved from the repository were aligned using Clustal X (Thompson et al., 1997). The size of the mature protein was calculated after removing the predicted signal peptide using SignalP v2.0 (Nielsen et al., 1997). The clip-domain, the proteolytic activation site, catalytic and specificity residues were predicted from comparison with *Drosophila* SP sequences (Ross et al., 2003).

3. Results

3.1. cDNA and protein structure

We identified an abundant transcript, designated as *DaTrypsin*, in the screening for differentially expressed genes during diapause. Cloning and sequencing of the cDNA fragment yielded a 675 bp expressed sequence tag. Based on that fragment, we designed five gene-specific primers and amplified both the 5′ and 3′ ends of its cDNA. A 1379 bp full-length sequence, assembled from the fragments, contained an open reading frame spanning

nucleotides 112–1263. The cDNA and its deduced protein sequences were deposited in GenBank with an accession number AY728056.

The amino acid sequence of *DaTrypsin* includes 384 residues, the first 20 of which represent a predicted signal peptide for extra-cellular secretion. The molecular mass of the mature protein beginning at residue 21 is 40,731 Da. *DaTrypsin* is likely a glycoprotein because of six potential glycosylation sites-N^{78}QT (code beginning at residue 21), N^{184}LT, N^{205}VT, N^{223}KS, N^{239}VT (N-linked) and Ser^{174} (O-linked) (Fig. 1A). Its calculated isoelectric point is 5.4.

DaTrypsin is a preproenzyme. The predicted site for cleavage activation is located between Thr^{119} and Val^{120}, suggesting its activating proteinase has an unusual specificity for cleaving after Thr. Proteolytic activation of DaTrypsin would yield an amino-terminal fragment consisting of residues 1–119 with a mass of 13,987 Da and a carboxyl-terminal fragment (residues 120–364, 26,744 Da). A group-1_b clip domain is located between Asp^{21} and Pro^{69}, and this structure is stabilized by three disulfide bonds formed between six conserved Cys residues (Fig. 1A). The remainder of the light chain (residues 70–119) is a linker sequence rich in hydrophilic and charged amino acids. The

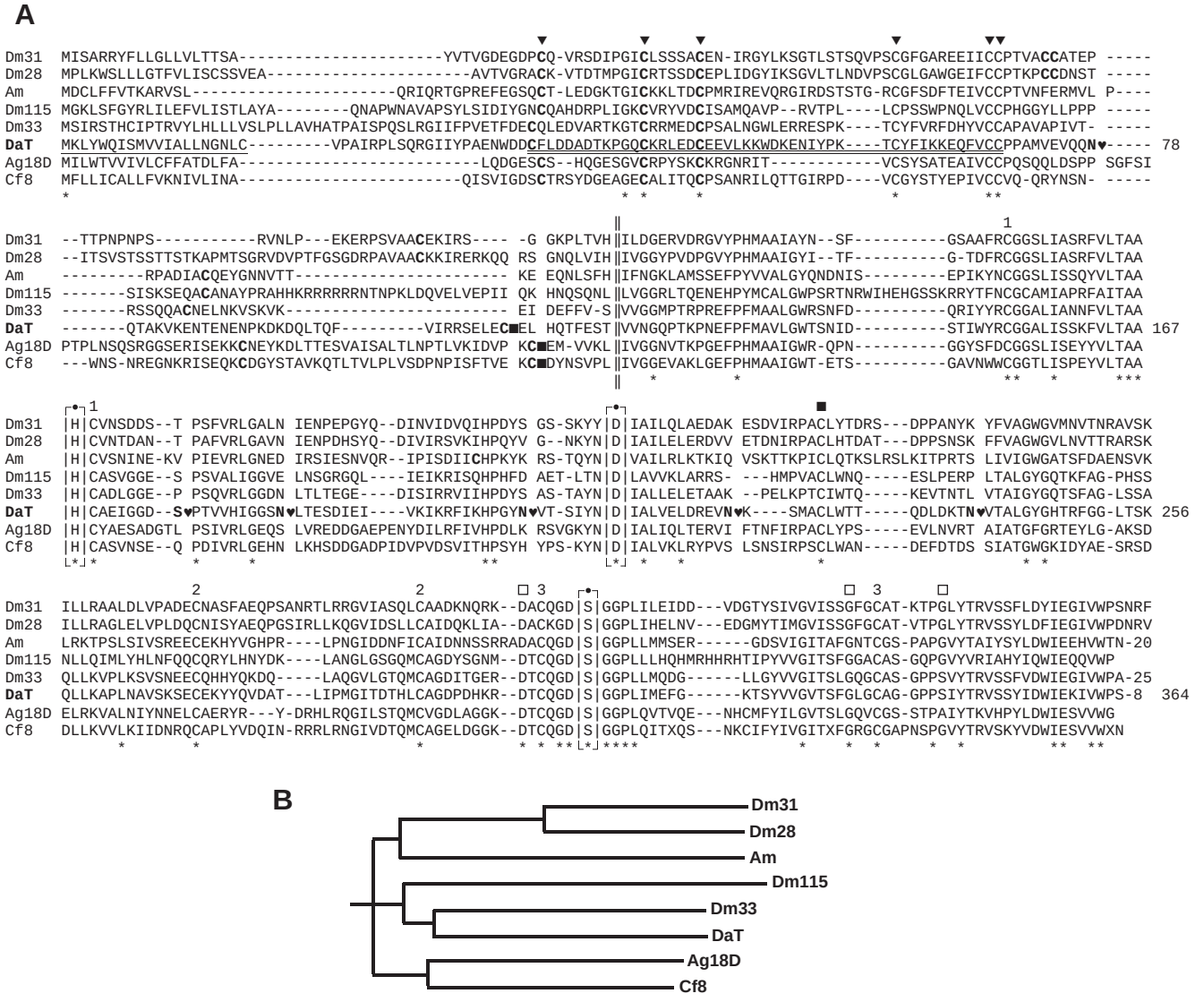

Fig. 1. Deduced amino acid sequence of *DaTrypsin* and comparison with seven group-1_b clip-domain SPs from insects. (A) DaTrypsin (DaT, AY728056), *D. melanogaster* SP28 (Dm28, CG6367), SP31 (Dm31, CG6361), SP33 (Dm33, CG2056) and SP115 (Dm115, CG11668), *A. mellifera* (Am, XP_395553), *A. gambiae* Sp18D (Ag18D, AAD38336) and *C. felis* SP8 (Cf8, AAD2184) are aligned using CLUSTAL X (Thompson et al., 1997). In DaT, the predicted signal peptide is underlined, and the clip-domain sequence is double underlined. Six potential N- or O-linked glycosylation sites are marked with "♥", and the last residue in each line in DaT is assigned a number. For comparison, completely conserved amino acid residues are indicated by "*". The proteolytic activation site is indicated with "‖". The residues of the catalytic triad are marked by "●" and boxed, whereas the determinants of the primary specificity are labeled by "□". Conserved Cys residues in the clip domains are shown by "▲", and the paired numbers above indicate the disulfide linkage in the catalytic domain. The cysteine residues involved in interdomain disulfide bonds are marked with "■". (B) A phylogenetic tree constructed by the neighbor-joining criterion.

heavy chain of DaTrypsin is typical of a SP from the chymotrypsin family, including the conserved His-Asp-Ser catalytic triad. The primary binding pocket of DaTrypsin consists of Asp^{301}, Gly^{328} and Ser^{337} that may interact with positively charged residues (i.e. Lys or Arg) in its substrate. In summary, DaTrypsin is expected to be a trypsin-like enzyme.

3.2. Sequence similarity and phylogenetic relationships

A GenBank search indicated that DaTrypsin is most similar to arthropod SPs containing a group-1_b clip domain. These include: *D. melanogaster* SP33 (CG2056), SP115 (CG11668), SP28 (CG6367 or *Persephone*) and SP31 (CG6361), *Apis mellifera* (XP395553), *A. gambiae* Sp18D (Q9Y1K5) and *Ctenocephalides felis* SP8 (AAD21841). The overall sequence similarity between different light chains is low, while the six Cys residues in the clip domains are strictly conserved (Fig. 1A). As a characteristic of group-1_a and group-1_b clip domains, a relatively short sequence (12–20 residues) is located between Cys_3 and Cys_4 in these sequences. Cleavage after Leu, His, Ser, Thr, Gln, Phe, Ile or Ala (instead of Arg/Lys in group-1_a and group-2 clip-domain SPs) is expected to lead to their activation. The observed diversity of cleavage sites at this position is consistent with the notions that group-1_b clip domains diverged earlier than those of group-1_a, and that group-2 expanded most recently (Ross et al., 2003).

An evolutionary tree constructed under the neighbor-joining criterion (Fig. 1B) shows that *DaTrypsin* is most similar in overall sequence to *D. melanogaster* SP33 (CG2056, 45% a.a. identity) and SP115 (CG11668, 32%). The catalytic domains in these proteins have a higher similarity of 37–69% (Fig. 1). Significant sequence similarity was also observed with *D. melanogaster* Snake (CG7996) and SP48 (CG3700), *Manduca sexta* HP2 (AF017664) and HP6 (AY672782), *A. gambiae* ClipC2 (EAA09203) and *Tachypleus tridentatus* Factor B (data not shown).

3.3. Expression of DaTrypsin during diapauses

When onion maggot larvae were reared at 25 °C and 16L:8D, 98% of the pupae enter SD at 2.2 days after pupariation (Ishikawa et al., 2000). Although the SD period is quite variable at 23 °C and 16L:8D, lasting between 15 and 45 days, pupae complete SD and start postdiapause development soon after the temperature decreases to 16 °C (Ishikawa et al., 2000). The expression pattern of *DaTrypsin* in the resent study is consistent with the onset and ending of SD of the previous work. The probe for *DaTrypsin* hybridized to a 1.4 kb mRNA band and that for 18S rRNA to a 1.45 kb transcript (Fig. 2A). The hybridization signals were much stronger in SD (D3, D6, D9, D12 and D15) than those in prediapause (D0.5, D1 and D2) or postdiapause (D18, D21 and D24). The relative amounts normalized to 18S rRNA levels were between 1.5 and 2.1 for SD, much higher than those for prediapause and postdiapause (<0.25) (Fig. 3A).

Almost all the pupae enter WD when larvae are maintained at 15 °C and 12L:12D (Nomura and Ishikawa, 2000). WD starts on day 4.1 and lasts for about 99 days with a postdiapause period of 23.5 days (Nomura, 2001). The expression profile of *DaTrypsin* is correlated with developmental changes associated with WD (Fig. 2B). Similar to the results under SD, *DaTrypsin* mRNA was highly abundant during WD (D29, D54, D79 and D105). The relative mRNA levels during WD, however, were higher than those in SD. Despite gradually decreasing with time from 4 at D29 to 2 at D105 (Fig. 3B), levels are still higher than those for SD (1.5–2). *DaTrypsin* transcription was detected at a low level (<0.5) in prediapause (D2, D3 and

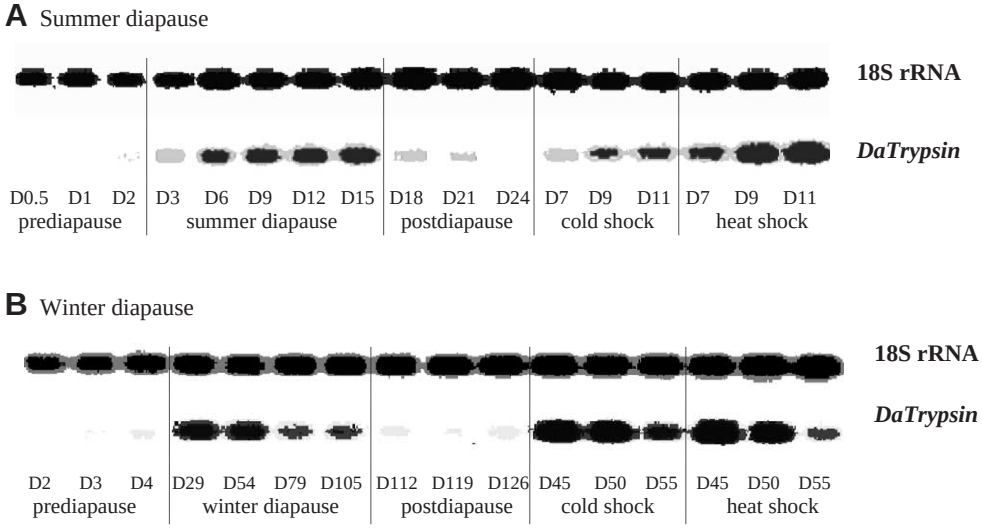

Fig. 2. Northern blot analysis of total RNA samples isolated from pupae at selected time points in SD (A) and WD (B) and under cold or heat shock. Top panel shows 1.45 kb 18S ribosomal RNA hybridized to its probe, and the bottom panel shows the 1.4 kb mRNAs hybridized to *DaTrypsin* probes.

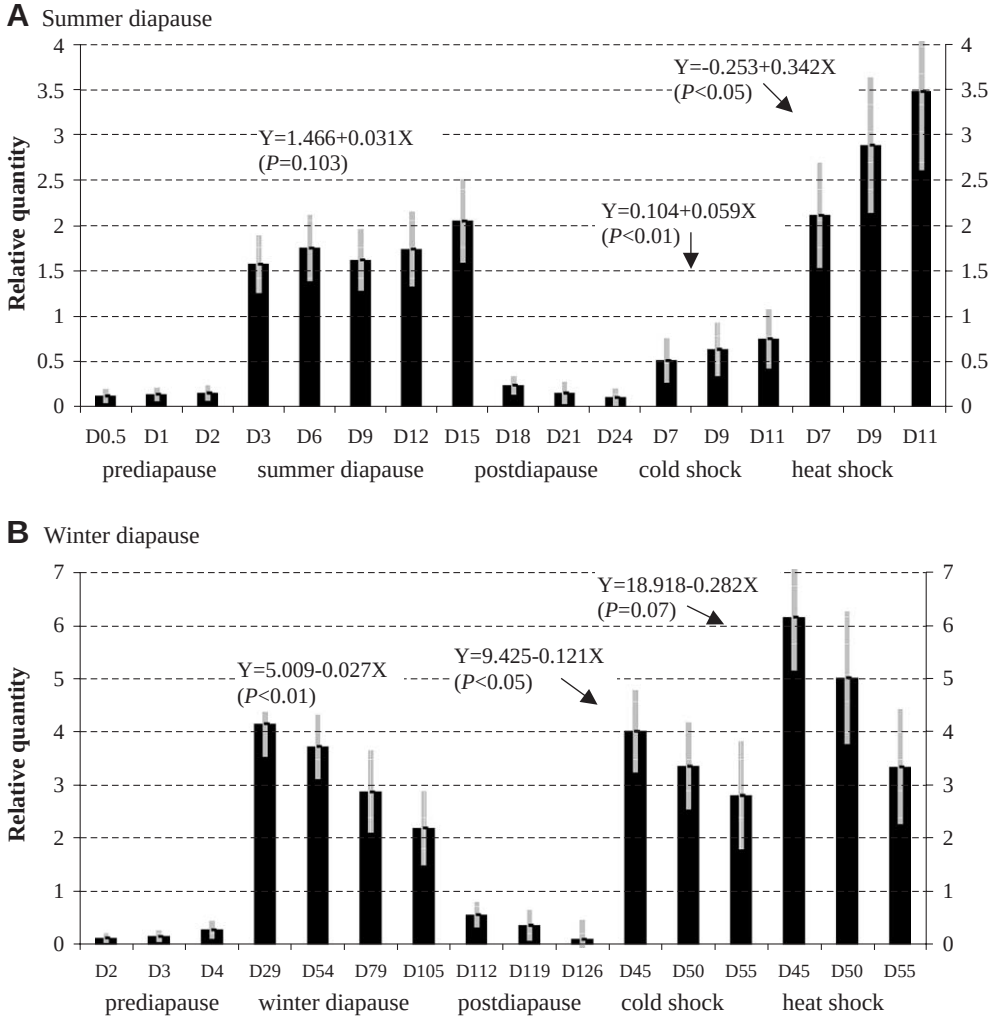

Fig. 3. Q-RT-PCR analysis of *DaTrypsin* mRNA levels in SD (A) and WD (B) and under cold or heat shock. The relative quantities indicate the levels of *DaTrypsin* transcript normalized to the internal standard 18S rRNA. The shaded bars indicate the standard deviation of four repeats. Regression equations between the quantity (*Y*) and the day (*X*) are shown above each corresponding treatment group with the *P*-value, indicating a significant slope, in brackets.

D4) and post-diapause (D112, D119 and D126), similar to non-diapausing pupae (data not shown).

3.4. Thermal stress responses during diapauses

Upon transfer of SD pupae (D5) from 25 °C and 16L:8D to −15 °C and 0L:24D there was a sharp decrease in *DaTrypsin* mRNA level in the first 2 days from 1.7 (normalized rate) at D5 (data not shown) to 0.5 at D7 (Figs. 2A and 3A). In contrast, heat shocking the pupae (at 35 °C and 16L:8D) increased *DaTrypsin* expression from 1.7 (D5) to 2.1 (D7). After that, the mRNA levels in cold- and heat-shocked pupae gradually increased with treatment duration, consistent with the pattern of unshocked SD pupae. The slope rates of *DaTrypsin* increase for heat (0.342/day)- and cold (0.059/day)-shocked pupae were 11.0 and 1.9 times higher than that for unshocked pupae (0.031/day), respectively (Fig. 3A).

After moving D40 WD pupae from 15 °C and 12L:12D to −20 °C and complete darkness, we did not detect significant change of *DaTrypsin* transcript levels in the first 5 days (relative levels of ~4.0 at both D40 and D45), but the level decreased from D45 onwards (Figs. 2B and 3B). After pupae were heat shocked (at 35 °C and 16L:8D), the relative levels of *DaTrypsin* mRNA sharply increased to 6.2 at D45 and then gradually decreased to about 3.4 at D55. In contrast to SD, *DaTrypsin* expression levels decreased with treatment duration after the first 5 days of shocks in WD pupae, consistent with the pattern of unshocked WD pupae. The slope rates of *DaTrypsin* decrease for cold (−0.121/day)- and heat (−0.282/day)-shocked pupae were 10.4 and 4.5 times faster than that for unshocked pupae (−0.027/day), respectively (Fig. 3B).

4. Discussion

A series of genes are known to be up-regulated during insect diapause. Heat-shock proteins (HSPs) are probably the most common (Denlinger et al., 2001). For example,

hsp70 expression is up-regulated throughout diapause in *S. crassipalpis*, *Leptinotarsa decemlineata*, *Ostrinia nubilalis* and five species of *Rhagoletis*, respectively (Flannagan et al., 1998; Yocum, 2001; Denlinger, 2002). A different HSP, Hsp23, is expressed in winter-diapausing pupae of *S. crassipalpis* (Yocum et al., 1998). HSPs, however, are not the only genes induced in diapause. *mEts*, a gene encoding a protein closely related to *Drosophila* and vertebrate E26 transforming sequences, was expressed exclusively in diapausing embryos of *B. mori* (Suzuki et al., 1999). In *Lymantria dispar*, alkaline phosphatase activity increases rapidly at the onset of diapause and remains high throughout diapause (Lee et al., 1998). Transcription of *pScD41* and *Samui* is up-regulated in early diapause in *S. crassipalpis* and *B. mori*, respectively (Flannagan et al., 1998; Moribe et al., 2001). In this paper, we describe a clip-domain SP gene, *DaTrypsin*, that is up-regulated throughout SD and WD. It is a trypsin-like enzyme most similar to *Drosophila* SP33 (CG2056), a clip-domain SP induced upon microbial challenge (De Gregorio et al., 2001; Irving et al., 2001).

DaTrypsin may participate in host immune responses during SD and WD. In diapausing insects, the normal development is arrested, metabolism is suppressed, and tolerance to adverse environmental condition is enhanced. These changes may involve genes specific for defense. In the leaf beetle *Gastrophysa atrocyanea*, for instance, a 7.9-kDa peptide is synthesized in the fat body and released into the hemolymph during adult diapause (Tanaka et al., 1998). It quickly disappears after diapause is terminated. The peptide was postulated to be a defense molecule in the diapausing beetle. In the spruce budworm, *Choristoneura fumiferana*, transcription of a defensin gene is up-regulated in diapausing larvae maintained at 2 °C. Upon transfer to 20 °C for diapause termination, the gene expression rapidly decreased within 2 days (Pali et al., 2001). These changes are analogous to *DaTrypsin* expression profiles before, during, and after diapauses (Figs. 2 and 3). De Gregorio et al. (2001) identified about 400 *Drosophila* immune genes that change their levels of expression after septic injury or natural fungal infection. Their protein products are involved in pathogen recognition, wound healing, iron sequestration, melanotic encapsulation or antimicrobial peptide synthesis. Among the 45 SP-related genes whose expression was up- or down-regulated upon infection (Irving et al., 2001), two genes, *SP33* (CG2056) and *SP31* (CG6361), have the closest sequence to *DaTrypsin* (45% and 29% amino acid similarity, respectively). *SP31* is also 69% similar to *SP28* (CG6367 or *Persephone*), a component of the SP pathway for antifungal responses in *Drosophila* adults (Ligoxygakis et al., 2002).

In addition to its putative role in immune defense, it is possible that *DaTrypsin* maintains the developmental status of diapausing pupae. In some lepidopteran insects, growth-blocking peptides (also known as paralytic peptides or plasmatocyte-spreading peptides) regulate several immune and developmental processes (e.g. cellular immunity and diapause) (Noguchi et al., 2003). These cytokines are produced as inactive precursors and require SPs for proteolytic activation. Although such molecules have not yet been found in dipteran insects, similar proteins may exist in *D. antiqua* that require processing by SPs such as *DaTrypsin*. This speculation is supported by the finding that spätzle is processed by *Drosophila* Easter (a clip-domain SP) (Jiang and Kanost, 2000).

While many insect species have SD and WD, most of them enter the diapauses at different developmental stages (Masaki, 1980). There are only a few documented cases in which a species enters SD and WD at the same stage (e.g. *D. antiqua* and the cabbage armyworm moth, *Mamestra brassicae*) (Kimura and Masaki, 1992; Goto and Fukushima, 1995). While Nomura and Ishikawa (2001) investigated the association of trehalose accumulation with SD and WD in *D. antiqua*, our study is the first to report changes in mRNA levels in a gene expressed at the onset of both summer and winter diapause occurring at a comparable developmental stage, the pupal stage. While *DaTrypsin* transcription was up-regulated both in SD and WD, there are clear differences in the expression levels and patterns observed during these periods (Figs. 2 and 3): The relative amount of *DaTrypsin* mRNA in WD (2–4) is twice as much as that in SD (1.5–2) and the transcript level gradually increases in D3–D15 of SD, whereas it reaches the maximum (~4) at the beginning (D29) and decreases to ~2 in the end (D105) of WD.

SD and WD allow *D. antiqua* to survive the seasonally high and low temperatures, respectively. We sought to understand, however, whether the two types of diapause produce similar responses when subjected to abnormally high or low temperature stresses. Previous work showed that while SD and WD increase pupal survival after temperature shocks, the response is not symmetrical in SD and WD pupae. Survival of summer- and winter-diapausing pupae after a 15-day exposure to −15 °C was greater than 80%, while it was less than 5% when non-diapausing pupae were tested under the same conditions (Nomura and Ishikawa, 2001). More than 80% of winter-diapausing pupae survived at −23 °C for 15 days, but survival of summer-diapausing pupae was less than 30% under those conditions. On the other hand, more than 75% of summer- and winter-diapausing pupae, but less than 10% of non-diapausing pupae, tolerated 35 °C for 15 days (Nomura and Ishikawa, 2001). To test whether the molecular/developmental responses to heat or cold stress are the same in SD and WD, we examined the pattern of *DaTrypsin* expression profiles. For SD, cold shock decreased the *DaTrypsin* mRNA levels, whereas heat shock increased its levels. The expression levels gradually increased in both treatments during the temperature shocks. For WD, cold shock had no influence on the transcript levels in the first 5 days, while heat shock sharply raised the relative levels of *DaTrypsin* mRNA. Levels then decreased progressively during treatment. In summary, *DaTrypsin* levels showed different

patterns depending on whether the temperature shocks were applied to a SD or a WD pupa. These results failed, however, to reveal a direct relationship between *DaTrypsin* transcript level and survival upon thermal stresses. Further investigation on *DaTrypsin* protein level and activation status should provide useful clues on its biological functions during diapause.

In conclusion, winter and summer diapauses seem to involve the up-regulation of the same transcript, *DaTrypsin*. The pattern of *DaTrypsin* regulation, however, is substantially different during these two developmental stages. More work will be necessary to unravel the molecular, developmental and physiological mechanisms of summer and winter diapause in order to understand their interaction with the environment where the species has evolved.

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