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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.
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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

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