



Pergamon

Journal of Insect Physiology 49 (2003) 161–169

Journal
of
Insect
Physiology

www.elsevier.com/locate/jinsphys

Isolation and characterization of three diapause-associated transcripts from the Colorado potato beetle, *Leptinotarsa decemlineata*

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Received 9 September 2002; received in revised form 5 November 2002; accepted 5 November 2002

Abstract

Using suppressive subtractive hybridization, fragments of three diapause-associated transcripts (*DAT-1*, 2 and 3) were isolated from the Colorado potato beetle, *Leptinotarsa decemlineata*. Full-length clones were developed for the transcripts. *DAT-1* encodes a deduced protein 286 amino acids in length with limited identity to several proteins with leucine-rich domains. *DAT-2* encodes a deduced protein 229 amino acids in length with 27% identity, 40% similarity to the desiccation stress protein from *Tenebrio molitor*. *DAT-3* encodes a deduced protein 97 amino acids in length with identity to no known protein. *DAT-1* and 2 have similar expression patterns as determined by northern blot analysis. Trace levels of these two transcripts are first detected in 3-day-old diapause-programmed adults with a significant increase in expression on day 6. Expression of *DAT-3* begins on day 12 in diapause-programmed adults and expression levels increase until the beetles enter diapause. Expression of *DAT-1*, 2 and 3 continues at least 60 days into diapause.

Published by Elsevier Science Ltd.

Keywords: Diapause; *Leptinotarsa decemlineata*; Gene expression; Desiccation protein

1. Introduction

For an insect species to survive in any given geographic location, its life cycle must be tightly synchronized to all biotic and abiotic factors required for development and reproduction. Insects must also be able to survive predictable recurring periods of environmental stress, such as winter for temperate zone insects. Diapause is the chief physiological mechanism by which insects meet these two requirements for survival. Because of its central role enabling insect pests to survive and reproduce in a given area, an understanding of the mechanism of diapause is critical in the management of any agroecosystem (Tauber et al., 1986; van Lenteren, 1999).

The ecology and endocrinology of diapause has been thoroughly investigated, leading to a clear understanding

of these aspects of diapause (Denlinger, 1985; Tauber et al., 1986). What is missing in our knowledge of diapause is the molecular mechanism(s) regulating diapause development and its associated stress responses. The most comprehensively studied insect to date is the flesh fly *Sarcophaga crassipalpis*. Nevertheless fewer than 20 diapause-regulated genes have been investigated to date. The picture emerging from even this limited number of genes is one of great complexity (Denlinger, 2002). The complexity of the molecular regulation of diapause offers a novel avenue for improved pest management. By using diapause-regulating genes as molecular markers the diapause development of pest species may be able to be monitored under field conditions (Denlinger, 2002). The capability to accurately predict the initiation and termination of diapause would increase our effectiveness in predicting the need for and timing of control measures.

The Colorado potato beetle *Leptinotarsa decemlineata* is the major defoliator of potato (Ferro, 1985; Hare, 1990; Cloutier et al., 1996), tomato (Schalk and Stoner,

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1979), and eggplant (Cotty and Lashomb, 1982; Hamilton and Lashomb, 1996). Although diapause has been intensely studied in the Colorado potato beetle (de Kort, 1990), only three diapause-associated genes have been isolated: diapause protein 1 (de Kort et al., 1997), juvenile hormone esterase (Vermunt et al., 1999) and a 70 kDa heat shock protein (Yocum, 2001). The aim of this investigation is the isolation and characterization of additional diapause-associated genes in order to clarify the molecular mechanism(s) governing diapause development in this important agricultural pest.

2. Materials and methods

2.1. Insect rearing

L. decemlineata were reared according to Yocum (2001). To obtain nondiapausing adults the colony was maintained at 17 h light:7 h dark, 26 ± 2 °C and 65% relative humidity. Diapausing adults were obtained by rearing larvae, pupae and adults at 8 h light:16 h dark, 24 ± 2 °C and 65% relative humidity. Diapause-programmed adults were given potato leaves for 20 days, then placed in moist vermiculite and stored at 10 °C in constant darkness.

2.2. RNA isolation

Total RNA was isolated from adults by mincing a single beetle in a 2 ml RNase-free microcentrifuge tube with 0.5 ml TRIzol® reagent (Invitrogen), then adding another 0.5 ml TRIzol®. Samples were centrifuged at 12,000g for 10 min and the supernatant was transferred to a new 2 ml RNase-free microcentrifuge tube with another 0.5 ml TRIzol®. Samples were then stored at -70 °C until the RNA was purified following the manufacturer's protocol. The isolated RNA pellets were stored under absolute ethyl alcohol at -70 °C and later dissolved in either 30 µl formamide for northern blot analysis or 30 µl sterile water for mRNA isolation for library construction. mRNA was isolated using Oligo (dt)₂₅ Dynbeads (DynaL Biotech) following the manufacturer's protocol.

2.3. Suppressive subtractive hybridization

Two microgram of mRNA were isolated as described above from a 4-day-old adult nondiapausing beetle and a beetle in diapause for 14 days. Forward and reverse subtracted libraries were constructed using the Clontech PCR-Select kit (Clontech; Diatchenko et al., 1996). The forward library (diapause) was cloned into pCR2.1 (Invitrogen) and 152 clones were isolated. The clones were grown overnight in LB-ampicillin broth at 37 °C. An aliquot from each culture was used to prepare frozen

glycerol stock, and the remainder placed into 96-well plates. An aliquot from each well was transferred onto positively charged nylon membrane (Roche) using a 96 pin MULTI-PRINT applicator (V&P Scientific). The membranes were placed with the colonies side up onto LB-ampicillin agar plates and incubated at 37 °C overnight. The filters were denatured for 5 min by placing them onto filter paper soaked with 0.5 M NaOH, 1.5 M NaCl. The filters were then neutralized for 5 min by placing them on filter paper soaked with 0.5 M Tris-HCl (pH 7.4), 1.5 M NaCl. After air-drying, the filters were UV cross-linked ($12,000$ µJ/cm²). The filters were hybridized to randomly primed probes generated from 100 ng of the unsubtracted libraries' cDNA to identify clones of differentially expressed genes.

2.4. The 3' and 5'-RACE

Single strand cDNAs with adapter primer sites at either the 3' or 5' end of the cDNA were synthesized according to the manufacturer's protocol starting with 1 µg of total RNA (SMART™ RACE cDNA Amplification Kit, Clontech). The 5'-RACE was carried out according to the manufacturer's instructions using gene-specific primers based on the sequence of the original clones. The resulting 5'-RACE clones were isolated and sequenced to determine gene-specific primers for 3'-RACE, and 3'-RACE was carried out according to the manufacturer's instructions. The 3'-RACE clones were used for all sequence data presented here.

2.5. Probe labeling

2.5.1. Randomly primed labeling

Two 50 µl PCR reactions were done with each of the original clones isolated from the subtracted libraries. The reaction pairs were combined and run on a 1% TAE agarose gel. Each PCR product was isolated from its excised gel band using GENCLEAN® II (BIO 101). The clones were randomly primed with digoxigenin using Dig High Prime DNA Labeling and Detection Start Kit II (Roche). The labeled probes were stored at a concentration of 20 ng/µl at -20 °C in TE buffer (pH 8.0), and used to screen the confirmational northern blots (Fig. 1).

2.5.2. PCR labeling

Probes used in the developmental studies were labeled by PCR using the 3'-RACE clones as templates. Gene-specific primers were designed to insure that the resulting probes spanned the entire deduced open reading frame of each clone. PCR reactions were carried out in a total volume of 100 µl buffer comprising 200 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM MgCl₂, 60 pmol of each primer, 70 µM DIG-11-dUTP, 130 µM dTTP, and 200 µM dATP, dCTP, and dGTP each, approximately 20 ng of template (vector and insert) and

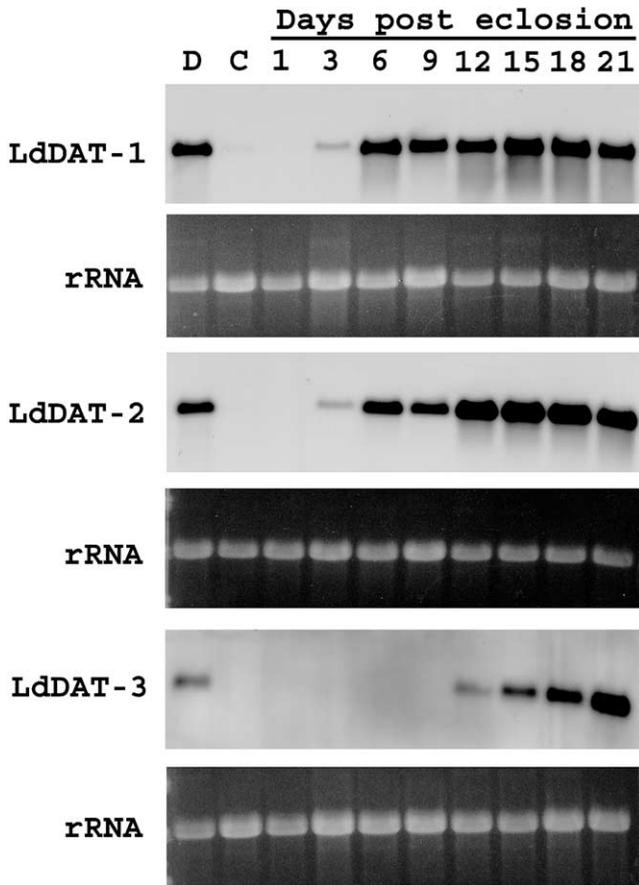


Fig. 3. Northern blot analysis of *LdDAT-1*, *-2* and *-3* expression in diapause-programmed adults of *L. decemlineata*. Total RNA (5 μ g) isolated from adults in diapause for 60 days (D), nondiapausing (C) adults, and diapause-programmed adults 1–21 days post eclosion was separated on a 1% formaldehyde-agarose gel and transferred onto a positively charged nylon membrane. The northern blots were hybridized using digoxigenin PCR labeled probes using the full-length clones as template. RNA concentrations were determined spectrophotometrically and stained with ethidium bromide to ensure equal loading. Ethidium bromide stained ribosomal RNA is shown below its respective northern blot.

Georgia on an AB 1373 DNA sequencer using dye terminator chemistry according to the manufacturer's standard protocol. The BLASTX program (Altschul et al., 1997) was used to search the GenBank sequence repository for sequence identity.

2.8. Computer analysis of deduced protein sequences

The deduced protein sequences were analyzed using statistical analysis of protein sequences (SAPS) to compare amino acid composition to the *Drosophila* amino acid usage quantile table and search for charge distribution (Vector NTI, InforMax) (Brendel et al., 1992). Protein functional analysis was performed using InterProScan (<http://www.ebi.ac.uk/interpro/scan.html>) (Apweiler et al., 2001). Protein sequences with hydrophobic regions near their N-terminus were analyzed for

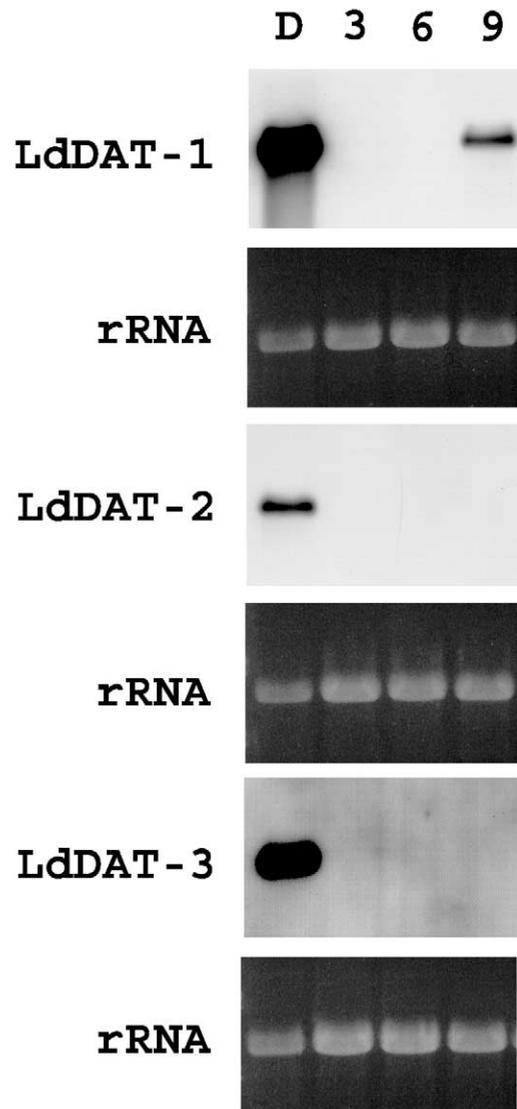


Fig. 4. Northern blot analysis of *LdDAT-1*, *-2* and *-3* expression in nondiapausing adults of *L. decemlineata*. Total RNA (5 μ g) isolated from adults in diapause for 60 days (D) and nondiapausing adults 3, 6 and 9 days post eclosion was separated on a 1% formaldehyde-agarose gel and transferred onto a positively charged nylon membrane. The northern blots were hybridized using digoxigenin PCR labeled probes using the full-length clones as template. RNA concentrations were determined spectrophotometrically and stained with ethidium bromide to ensure equal loading. Ethidium bromide stained ribosomal RNA is shown below its respective northern blot.

possible signal peptides by SIGNALP V2.0 using neutral network and hidden Markov models trained on eukaryotes (<http://www.cbs.dtu.dk/services/SignalP-2.0/>) (Nielsen et al., 1997; Nielsen and Krogh, 1998).

2.9. GenBank

The nucleotide sequences for *LdDAT-1*, *-2* and *-3* were deposited in GenBank and assigned the accession numbers AF532862, AF532863 and AF532864, respectively.

Dsp28	MNKLLIIAFALFAVISIVRCSDS----DEVLEKVKSKHLRKYLTDRREDV	50
LdDAT-2	*K*FVLL*LF***TSWAEESNE*SSEK*DF*EIAEFAKN*LN*TIIV* <u>L</u>	
Dsp28	DSGLEKLEKHCPCGVSEKLSALVSFQECDDKADD-SLTLCSAIQVYTLNC	100
LdDAT-2	MKLVK*TQEK**DIE***ER*GEEIG**IE*IELG*E*F**LLRKNGER*	
Dsp28	TAPLLKVVDDCLPDSAKGLPSLVKSIILSVADYLCKQTGESIFELGNPCL	150
LdDAT-2	<u>SKAGVEAIIAS***EES*E**AMLS*I*WV*II*QA*NS*V*E*L**F***V</u>	
Dsp28	WEDLEESNEQLDECEEKVKNFVAKKHKETIPSKTEICSTATSLRTCVQTRA	200
LdDAT-2	LNEEASKSP----ECTE**DALAS**DQL***SFV**MIPKV*S*SKKFE	
Dsp28	TTTCKNQKTKNVALGVFDALVAPCSHINEV	234
LdDAT-2	<u>SG-T*PI***AN*HFH**VEEAIKEDCDALNRT</u>	

Fig. 5. Amino acid alignment of *T. molitor* 28 kDa desiccation protein (Dps28) and the deduced desiccation-like protein from the Colorado potato beetle (LdDAT-2). Analysis (AlignX, InforMax) of the two proteins yielded a score of 27% identity, 40% similarity. Similar/chemically equivalent amino acids are underlined. (*) Identical amino acids, (-) insertion or deletion.

ACT	CCA	TTC	TAT	TGT	<u>TGA</u>	ATG	ATA	AAG	GTA	CTT	GAA	ATG	AAA	AAC	TTA	10	
																48	
	<u>L</u>	<u>Y</u>	<u>L</u>	<u>T</u>	<u>I</u>	<u>F</u>	<u>A</u>	<u>A</u>	<u>F</u>	<u>V</u>	<u>V</u>	<u>M</u>	<u>I</u>	<u>M</u>	<u>S</u>	<u>H</u>	26
CTC	TAT	TTG	ACA	ATT	TTT	GCT	GCC	TTC	GTG	GTG	ATG	ATA	ATG	TCT	CAT	96	
H	H	H	T	T	K	S	D	E	E	L	K	T	T	K	K	42	
CAT	CAT	CAC	ACC	ACA	AAA	AGT	GAT	GAA	GAA	CTT	AAG	ACT	ACA	AAG	AAG	144	
K	E	S	L	P	N	P	D	G	E	N	T	G	E	E	E	58	
AAG	GAA	TCA	CTT	CCA	AAT	CCT	GAT	GGA	GAA	AAC	ACA	GGC	GAG	GAG	GAG	192	
I	N	I	Q	I	P	D	V	E	G	S	C	S	G	Y	Q	74	
ATA	AAC	ATC	CAA	ATA	CCT	GAT	GTT	GAA	GGA	TCT	TGC	AGC	GGT	TAT	CAA	240	
C	A	N	F	C	K	K	S	G	Y	S	Y	G	Y	C	S	90	
TGT	GCA	AAC	TTC	TGC	AAA	AAG	TCT	GGG	TAC	TCA	TAC	GGA	TAC	TGT	TCA	288	
G	N	S	C	R	C	Y	*		*							97	
GGA	AAT	TCA	TGC	CGC	TGC	TAC	TGA	TCA	TAA	ATT	GAA	AAA	AAA	AGA	TCA	336	
ATA	AAT	ATA	TGA	AAT	TTG	GAA	AAA	384									
AAA	A															388	

Fig. 6. Nucleotide and deduced amino acids for LdDAT-3. Each amino acid is shown above its respective codon. The underlined segment is a hydrophobic/tentative transmembrane region as determined by SAPS. Arrow indicates tentative cleavage site of the predicted signal peptide. Amino acid numbering starts at the first methionine in the open reading frame and is given to the right of each row. Nucleotide numbering is given to the right of each row. (*) Stop codon.

3. Results

3.1. Clones

One hundred and fifty-two clones were differentially screened resulting in the identification of 60 diapause-upregulated clones. The isolated clones ranged in size from 102 to 421 bp with a mean size of 178 bp. Sequence data of the clones revealed that these clones fell into five distinct groupings. Thirty-six clones had identity to *L. decemlineata* diapause protein 1 (de Kort and Koopmanschap, 1994; accession number CAA53691). The second group with only one clone had no identity to any known gene. A third group of six clones had identity to *Tenebrio molitor* desiccation protein, Dsp28 (Graham et al., 1996; accession number ACC47527). The fourth group of five clones had no sequence identity to any known gene: sequence data revealed that they were clones of the same gene. The final group of 12 clones yielded no useful sequence data and was therefore discarded.

3.2. Confirmational northern blot analysis

A representative clone from the second, third and fourth groups of clones described above were used to generate randomly primed probes and to screen northern blots to confirm the results of the differential screening. These clones were designated as LdDAT-1, -2 or -3 respectively. The probes for *LdDAT-1*, -2 and -3 hybridized to diapause-upregulated mRNAs 1.35, 0.8 and 0.54 kb in size, respectively (Fig. 1). Low levels of *LdDAT-2* and -3 were detected in the nondiapause samples.

3.3. Characterization of three diapause-associated transcripts

3.3.1. *LdDAT-1*

All attempts to extend the sequence by 5'-RACE failed. The 3'-RACE yielded a 1272 bp clone that encodes a deduced protein 286 amino acids in length. This tentative open reading frame is bracketed by stop codons

(Fig. 2). BLASTX searches revealed that the deduced protein has low identity to a number of proteins containing leucine-rich domains. Of the possible BLASTX hits, most were significantly larger proteins and had numerous gaps indicating these hits are not related to the protein encoded by *LdDAT-1*. SAPS analysis of the amino acid usage of *LdDAT-1* compared against the *Drosophila* amino acid usage quantile table revealed that *LdDAT-1* is highly enriched for methionine and glutamic acid ($\geq 99\%$ quantile), asparagine and arginine ($\geq 95\%$ quantile), while exhibiting very low levels of proline ($\leq 5\%$ quantile), alanine, valine, and tyrosine ($\leq 1\%$ quantile). *LdDAT-1* also contains no cysteine or tryptophan (Table 1). *LdDAT-1* contains no clusters of high positive or negative charge, nor hydrophobic segments or possible transmembrane segments. InterProScan analysis tentatively identified 8–11 leucine-rich domains, indicating that *LdDAT-1* forms protein–protein complexes in vivo.

Diapause-programmed adults will normally feed for the first 2 weeks after emergence, after which their feeding decreases, they move off the plant and enter diapause. Northern blot analysis established that *LdDAT-1* was expressed at low levels by day 3 post-emergence in diapause-programmed adults with a significant increase in expression by day 6 (Fig. 3). *LdDAT-1* continued to be expressed for at least 60 days into diapause

Table 1
Percent by frequency of amino acid composition of *LdDAT-1*

Amino acid	Percent
Alanine	2.1 ^a
Cysteine	0.0
Aspartic acid	4.2
Glutamic acid	12.9 ^d
Phenylalanine	3.5
Glycine	4.5
Histidine	1.0
Isoleucine	6.3
Lysine	5.9
Leucine	12.6
Methionine	11.9 ^d
Asparagine	9.4 ^c
Proline	2.1 ^b
Glutamine	2.4
Arginine	9.1 ^c
Serine	6.3
Threonine	3.5
Valine	1.7 ^a
Tryptophan	0.0
Tyrosine	0.3 ^a

Amino acid composition of *LdDAT-1* was analyzed by SAPS using the *Drosophila* amino acid usage quantile table.

^a 1% quantile.

^b 5% quantile.

^c 95% quantile.

^d 99% quantile.

(diapause control, Fig. 3). Trace levels of *LdDAT-1* were detected in the nondiapause control (Fig. 3).

The adults used for the nondiapause controls in Fig. 5 were all within 4 days post-emergence. To ascertain whether *LdDAT-1* is expressed later in nondiapause adult development, its expression was examined in 3-, 6- and 9-day-old adults. By day 9 nondiapausing females are already laying eggs. Low levels of expression of *LdDAT-1* were detected in 9-day-old nondiapausing adults, although levels were significantly less than in the diapause control (Fig. 4).

3.3.2. *LdDAT-2*

An 825 bp clone representing *LdDAT-2* was isolated following 5'- and 3'-RACE. *LdDAT-2* encodes a deduced protein 229 amino acids in length. A BLASTX search using the full-length clone confirmed that the closest match to *LdDAT-2* is the desiccation protein from *T. molitor*, Dsp28, with an *E*-value of $4e^{-17}$ (Graham et al., 1996). Alignment of *T. molitor* desiccation protein and the *LdDAT-2* deduced protein yielded a score of 27% identity, 40% similarity (AlignX, InforMax) (Fig. 5). Comparing the amino acid composition of Dsp28 against *LdDAT-2* by SAPS using the *Drosophila* amino acid usage quantile table revealed that both of these proteins are highly enriched ($\geq 95\%$ quantile) for both cysteine and lysine. But SAPS also revealed significant differences in the amino acid composition of these two proteins. Dsp28 is methionine poor ($\leq 5\%$ quantile), whereas *LdDAT-2* is poor in glutamine ($\leq 1\%$ quantile), glycine, and arginine ($\leq 5\%$ quantile) and enriched for glutamic acid ($\geq 99\%$ quantile) (Table 2). InterProScan analysis of *LdDAT-2* found no functional domains.

The *LdDAT-2* expression pattern closely mirrors that of *LdDAT-1* (Fig. 3). Minor levels of *LdDAT-2* were detected in 3-day-old diapause-programmed adults; by day 6 there was a significant increase in its expression. As with *LdDAT-1*, *LdDAT-2* was still highly expressed in the diapause control 60 days into diapause (diapause control, Fig. 3). Trace levels of *LdDAT-2* were detected in some nondiapausing adults, but these levels were always significantly less than observed in diapausing adults (Figs. 1, 3 and 4).

3.3.3. *LdDAT-3*

A clone 388 bp in length representing *LdDAT-3* was isolated following 5'- and 3'-RACE. The longest tentative open reading frame encodes a deduced protein 97 amino acids in length (Fig. 6). This tentative open reading frame is bracketed by stop codons. BLASTX searches failed to find any gene with identity to *LdDAT-3* in GenBank or *Drosophila* genome sequence repositories. SAPS analysis demonstrated the *LdDAT-3* amino acid usage falls within the normal range of amino acid usage as compared to the *Drosophila* amino acid usage quantile table (Table 3). *LdDAT-3* contains one

Table 2

Comparison of percent by frequency of amino acid composition of LdDAT-2 and the *T. molitor* 28 kDa desiccation protein (Dsp28)

Amino acid	Percent LdDAT-2/Dsp28
Alanine	2.1/6.7
Cysteine	5.2 ^a /5.8 ^a
Aspartic acid	3.9/7.1
Glutamic acid	14.8 ^b /8.4
Phenylalanine	4.4/2.7
Glycine	2.6 ^c /3.1
Histidine	1.3/1.8
Isoleucine	7.0/4.4
Lysine	10.5 ^a /10.2 ^a
Leucine	9.6/12.0
Methionine	1.7/0.4 ^c
Asparagine	4.8/3.6
Proline	3.5/3.1
Glutamine	1.3 ^d /2.7
Arginine	2.2 ^c /2.7
Serine	9.2/8.9
Threonine	4.4/6.7
Valine	4.4/8.0
Tryptophan	0.9/0.4
Tyrosine	0.4 ^d /1.3

Amino acid composition was analyzed by SAPS using the *Drosophila* amino acid usage quantile table.

^a 95% quantile.

^b 99% quantile.

^c 5% quantile.

^d 1% quantile.

Table 3

Percent by frequency of amino acid composition of LdDAT-3

Amino acid	Percent
Alanine	3.1
Cysteine	6.2
Aspartic acid	3.1
Glutamic acid	9.3
Phenylalanine	3.1
Glycine	7.2
Histidine	4.1
Isoleucine	6.2
Lysine	9.3
Leucine	6.2
Methionine	4.1
Asparagine	6.2
Proline	3.1
Glutamine	2.1
Arginine	1.0
Serine	9.3
Threonine	6.2
Valine	4.1
Tryptophan	0.0
Tyrosine	6.2

Amino acid composition of LdDAT-3 was analyzed by SAPS using the *Drosophila* amino acid usage quantile table.

hydrophobic/tentative transmembrane segment from amino acid 10–24 as determined by SAPS analysis (Fig. 6). The SIGNALP program predicted that LdDAT-3 maybe a secreted protein due to the presence of a tentative signal peptide with the most likely cleavage site between amino acids 25 and 26. InterProScan analysis of LdDAT-3 found no functional domains.

Northern blot analysis detected *LdDAT-3* expression in day 12 diapause-programmed adults with increasing levels of expression out to day 21. *LdDAT-3* expression continued at least 60 days into diapause (diapause control, Fig. 3). As with *LdDAT-1* and -2 a low level of *LdDAT-3* expression was detected in some nondiapausing beetles but these levels were significantly less than those measured in diapausing adults (Figs. 1, 3 and 4).

4. Discussion

Overwintering insects face a number of environmental challenges to survival. Although not as thoroughly investigated as cold stress, desiccation is a serious threat to which all overwintering insects are subjected. To mitigate the naturally occurring desiccating conditions of winter, insects employ a number of different strategies to increase their desiccation tolerance (reviewed by Danks, 2000). Of particular interest here is the effect of desiccation on hemolymph protein composition. *T. molitor* larvae exposed to desiccating conditions profoundly alter their hemolymph protein profile. There is a shift from predominantly larger cysteine-labeled proteins to smaller (<46 kDa) cysteine-labeled proteins (Kroeker and Walker, 1991a). Of these desiccation upregulated proteins, the 28 kDa protein (Dsp28) has been isolated (Kroeker and Walker, 1991a) and its gene cloned (Graham et al., 1996). Dsp28 expression is also developmentally regulated by juvenile hormone (Graham et al., 1996) and induced by cold exposure (Kroeker and Walker, 1991b). Sequence analysis of LdDAT-2 in the current study indicates that it may also be a desiccation protein. What, then, is the physiological significance of a desiccation protein prior to entering diapause while the beetles are feeding and have free access to water? It is common for insects entering diapause to have lower water content than nondiapausing individuals because of increased lipid accumulation and other metabolic changes needed to survive overwintering (Danks, 2000). It is plausible that the expression of *LdDAT-2* is a natural consequence of the beetle actively storing metabolic reserves for overwintering, or it may be expressed in preparation for the predictable desiccating conditions of winter. The exact role, if any, *LdDAT-2* may be playing in desiccation tolerance is yet to be determined.

For any organism, the entrance into dormancy involves complex changes in its physiology to survive a period of decreased metabolism and possibly extreme

environmental conditions. Underlying these changes are alterations in gene expression controlling the organism's physiology and its ability to adapt to changing environmental conditions. In the flesh fly *S. crassipalpis*, there are at least six broad categories of gene expression patterns as they relate to diapause: (1) Genes whose expression is not altered by diapause, such as the 70 kDa heat shock cognate, 28S ribosomal protein (Rinehart et al., 2000), ecdysone receptor (Rinehart et al., 2001), cyclin E, p21, and p53 (Tammariello, 2001); (2) genes that are down-regulated during diapause, e.g. 90 kDa heat shock protein (Rinehart and Denlinger, 2000) and the cell cycle regulator, proliferating cell nuclear antigen (pcna) (Tammariello and Denlinger, 1998); (3) diapause upregulated genes, e.g. 23 and 70 kDa heat shock proteins (Yocum et al., 1998; Rinehart et al., 2000); (4) genes expressed early in diapause, such as pScD41 (Denlinger, 2002); (5) genes expressed late in diapause, e.g. ultraspiracle (usp) (Rinehart et al., 2001); (6) genes expressed cyclically throughout diapause, such as 60S ribosomal protein PO (Craig and Denlinger, 2000).

Although the molecular mechanism(s) of the Colorado potato beetle diapause has not been as intently studied as those in *S. crassipalpis*, diapause-associated gene expression patterns are beginning to emerge. The first category of genes, are those whose expression commences prior to diapause. These include *DP-1* (de Kort et al., 1997), juvenile hormone esterase (Vermunt et al., 1999) and *LdDAT-1* and *-2*. These four genes can be further subdivided by the onset of their expression. Juvenile hormone esterase expression increases on the second day post-emergence, whereas *DP-1*, *LdDAT-1* and *-2* are expressed starting on day 3 (de Kort et al., 1997; Vermunt et al., 1999 and Fig. 5). The second category of genes is those expressed near the onset of diapause, e.g. *LdDAT-3*. Even from this limited number of genes it is clear that as the Colorado potato beetle prepares to enter diapause there is a definite sequence of gene expression that must occur. Delineating this sequence will clarify our understanding of the physiological pathways required for the Colorado potato beetle to enter diapause.

However, our understanding of the mechanisms regulating diapause in *L. decemlineata* will be incomplete without considering the interaction of the manifold factors operating on the beetle in its natural environment. Prolonged (over 1 year) diapause has been reported in populations of the Colorado potato beetle in Europe and North America (for references see Biever and Chauvin, 1990). In Yakima, Washington, USA 30% of Colorado potato beetles under field conditions enter diapause for 2–4 years (Biever and Chauvin, 1990). A major factor determining whether the beetle will enter prolonged diapause is the soil type in which the beetle diapauses (Ushatinskaia, 1968). In addition, control of termination of diapause by environmental factors is seen in the inter-

play of temperature and soil moisture. If the soil moisture is high, beetle emergence from the soil can be accurately predicted by soil temperature. But if the soil moisture drops below a certain point, emergence stops and will not resume again until the soil is rehydrated (Tauber et al., 1994). Without doubt, the conditions a beetle experiences in the field are much more complex than those commonly used in the laboratory to induce or terminate diapause. It is clear therefore, that in order to develop a holistic picture of the molecular regulation of adult diapause in the Colorado potato beetle we must marry our molecular investigations with the rich ecophysiological literature.

Acknowledgements

I thank Dr David P. Horvath, USDA-ARS, Fargo, ND for his constructive comments on the draft of this manuscript. I also acknowledge Patsy L. Evenson, USDA-ARS, Fargo, ND for her technical assistance.

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