Eur. J. Entomol. **106**: 471–476, 2009
http://www.eje.cz/scripts/viewabstract.php?abstract=1477
ISSN 1210-5759 (print), 1802-8829 (online)

Down-regulation of gene expression between the diapause initiation and maintenance phases of the Colorado potato beetle, *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae)*

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Key words. Chrysomelidae, Coleoptera, diapause, gene expression

Abstract. The diapause initiation and maintenance phases of the Colorado potato beetle, *Leptinotarsa decemlineata*, were screened. Eight transcripts were found to be downregulated as the beetles enter the diapause maintenance phase of diapause development after day 15 postemergence. These transcripts were also expressed in early nondiapausing adults. Using BlastX, the transcripts were placed into six broad categories: regulatory (serpin), structural (apidermin), protease (serine protease), retinol binding protein (CRALBP), carbohydrate metabolism (ß-glucosidase, ß-mannosidase, and cellulose II), and unknown function.

INTRODUCTION

The Colorado potato beetle, *Leptinotarsa decemlineata* (Say), is the major defoliator of potato throughout the northern hemisphere (Gauthier et al., 1981; Ferro, 1985; Weber & Ferro, 1994; EPPO, 2006). Within the Red River Valley of North Dakota and Minnesota, USA, overwintering adults emerge in May or early June and lay eggs, normally followed by two generations each year depending on the weather. By mid-September, the adults from the second generation burrow into the soil and enter diapause. Overwintering adults will spend approximately 9 months of their lives in diapause.

Diapause is the physiological state of dormancy that enables insects to bridge periods of predictable seasonal adverse environmental conditions. Since favorable conditions necessary to enable reproduction and development are normally restricted to a narrow window of opportunity, insects spend most of their life cycle in diapause. Diapause is therefore a critical component in establishing insect phenology, and as such has a central role in insect management (Tauber & Tauber, 1973; Tauber et al., 1986). Diapause also has significant influence on other agriculturally important aspects of insect physiology. Recently, an interaction between diapause and the development of insecticide resistance in the Colorado potato beetle was discovered (Baker & Porter, 2008). Developing a more comprehensive understanding of diapause and its influence on insect life history traits will open up new avenues of pest management and offer insights into other biological processes (Denlinger, 2008).

Diapause is not a static state, but is one of progressive development with distinct phases: prediapause, diapause, and postdiapause quiescence. The diapause phase can be further subdivided into the initiation phase (IP) and maintenance phase (MP) (Košťál, 2006). Using respirometry, the IP of the Red River Valley of North Dakota and Minnesota strain of the Colorado potato beetle was determined to be the first 14 days postemergence in beetles reared under short day conditions (Yocum et al., 2009). The transition from the IP (a period of high metabolic rates and active feeding) to MP (a period of very low metabolic rates, no feeding, and very little movement) in the Colorado potato beetle is characterized by unique gene expression patterns (Yocum, 2003; Yocum et al., 2009).

To further clarify the molecular regulation of this dynamic period of transition in the life cycle of *L. decemlineata*, research was conducted with two main objectives: (1) isolate additional IP and MP differentially regulated genes and (2) examine the expression of these genes during the early gonotrophic cycle in nondiapausing adults.

MATERIAL AND METHODS

Insect rearing

L. decemlineata were reared according to Yocum et al. (2009). To obtain nondiapausing adults, all developmental stages of the beetle were reared at 16L : 8D, 24 ± 2°C, and 65% relative humidity. Diapause IP and MP adults were obtained by rearing all developmental stages of the beetle at 8L : 16D, 24 ± 2°C, and 65% relative humidity. At twenty days postemergence, diapause programmed beetles were removed from the rearing cages, placed in moist vermiculite and stored at 5°C.

Suppressive subtractive hybridization and primary screening

Suppressive subtractive hybridization was carried out as described by Yocum (2003) and Yocum et al. (2006). Total RNA was isolated from day one nondiapausing and day one and day two diapause IP beetles using TRIzol (Molecular Research

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TABLE 1. Diapause down-regulated transcripts isolated from *Leptinotarsa decemlineata* by suppressive subtractive hybridization.

Clone	Size (bp)	Predicted transcript size (kb)	Accession number	Putative identity	Score, % identity, organism, accession number	Blast
	Regulatory					
N4(1)F7	615	500	GO270916	serpin 3a	$4e^{-48}$, 66%, *Tribolium castaneum*, XP_969874	X
	Structural					
N4(1)B5	614	1200	GO270910	apidermin 1	$1e^{-14}$, 55%, *Apis mellifera*, XP_625247	X
	Protease			serine		
N4(2)C2	702	1400	GO270919	protease	$4e^{-41}$, 41%, *Tenebrio molitor*, ABC88734	X
	Retinol binding					
P1(2)B11	504	900	GO270863	CRALBP	$6e^{-51}$, 68%, *Nasonia vitripennis*, XP_001606183	X
	Carbohydrate metabolism					
N2(1)C4	664	2000	GO270895	ß-glucosidase	$4e^{-63}$, 52%, *Tribolium castaneum*, XP_972437	X
N3(2)G5	667	2100	GO270907	ß-mannosidase, lyso-	e^{-58}, 62%, *Tribolium castaneum*, XP_974359	X
N6(1)G8	278	1500	GO270944	somal cellulase II	$6e^{-33}$, 70%, *Apriona germari*, AAR22385	X
	Unknown function					
P3(1)F3	581	600	GO271611			

[a]The Blast program was used to search GenBank sequence repository for possible sequence identities. A three step search strategy was employed: (1) A BlastX search of the non-redundant protein sequences (nr) was done. (2) For those sequences failing to yield a significant hit (e^{-05}), the BlastX search was repeated with the low complexity region filter turned off. (3) For the sequence failing the two BlastX searches, a BlastN search of the nucleotide collection (nr/nt) database was conducted.

Center, Cincinnati, OH, USA) (Yocum, 2001). Suppressive subtractive hybridization was carried out using the BD Clontech PCR-Select kit (BD Biosciences, Mountain View, CA, USA). The colony filters were screened with complex probes generated by the PCR DIG Probe Synthesis kit (Roche, Indianapolis, IN, USA) using the subtracted nondiapause and early IP libraries as template and the nested PCR primers 1 and 2R from the BD Clontech PCR-Select kit. Northern blots were screened with probes of individual clones of interest using the same kits and nested PCR primers as above.

Northern blot analysis

Northern blot analysis was carried out according to Yocum et al. (2009) with at least three replications. Five micrograms of total RNA per sample (whole body from an individual beetle) were separated on a 1% denaturing agarose gel. All samples contained ethidium bromide, and after electrophoresis, a photograph of the gel was taken to compare the intensity of the rRNA bands to insure equivalent loading of samples. Prehybridization and hybridization were carried out in DIG Easy Hyb hybridization buffer (Roche). The DIG High Prime DNA Labeling and Detection Starter Kit II (Roche) was used to detect the digoxigenin-labeled probes. Following the hybridization and detection, membranes were stripped in buffer (50% molecular grade formamide, 5% SDS, and 50 mM Tris-HCl, pH 7.5). Membranes were stripped only three times; after the third stripping, the membranes were probed with a control gene to insure equivalent loading and transfer of RNA onto the membranes.

Bioinformatics

The Blast program was used to search the GenBank sequence repository for possible sequence identities (Altschul et al., 1997). A three-stage analysis was conducted: (1) First a BlastX search of the non-redundant protein sequences (nr) was carried out. (2) If no significant hit was returned, the BlastX search was rerun with the low complexity region filter turned off. (3) Finally, if the first two BlastX searches failed to yield a possible identity, a BlastN search of the nucleotide collection (nr/nt) database was carried out. All the sequences were deposited in GenBank and assigned accession numbers (Table 1).

RESULTS

Clones

In an unpublished study, suppressive subtractive hybridization failed to isolate genes that were differentially regulated between the early diapause IP and nondiapausing Colorado potato beetle. Yocum et al. (2009) have shown that the transition between the diapause IP and MP is characterized by down-regulation of select genes. Therefore, genes isolated in the unpublished investigation were subjected to more extensive developmental studies, revealing that eight genes were down-regulated as the beetles enter the MP of diapause.

Blast results

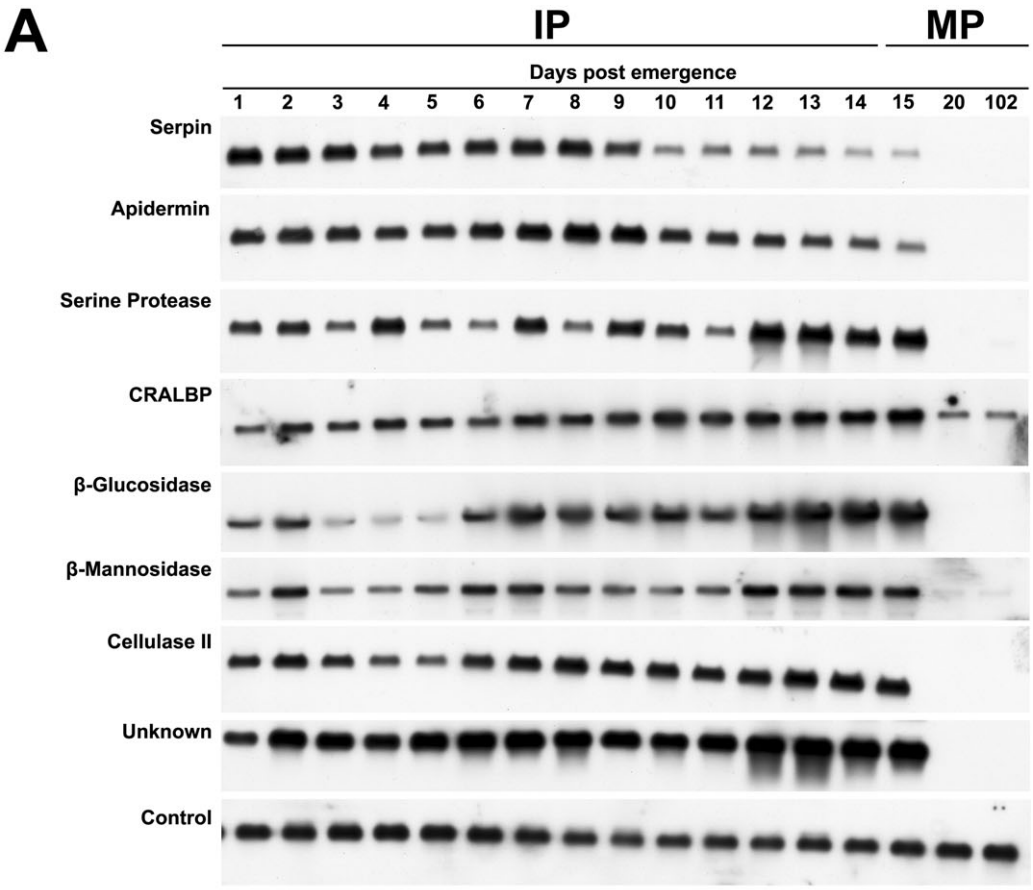
A BlastX search of the GenBank database yielded tentative identification for seven of the eight down-regulated genes: clone N4(1)F3 (serpin), clone N4(1)B5 (apidermin), clone N4(2)C2 (serine protease), clone P1(2)B11, (cellular retinal-binding protein, CRALBP), clone N2(1)C4 (ß-glucosidase), clone N3(2)G5 (ß-mannosidase), and clone N6(1)G8 (cellulase II) (Table 1).

Northern blot screening of the diapause initiation and maintenance phase

Transcript expression patterns were examined in days 1 to 14 IP and days 15, 20, and 102 postemergence MP beetles. Eight of the transcripts were differentially regulated between the IP and MP of diapause. These transcripts had very similar expression patterns, being highly expressed during the diapause IP and down-regulated or below the level of detection in the days 20 and 102 postemergence MP samples (Fig. 1A).

Northern blot screening of early nondiapausing adults

To determine if the MP down-regulated transcripts are also expressed in young nondiapausing adults, day 1, 3, 5,



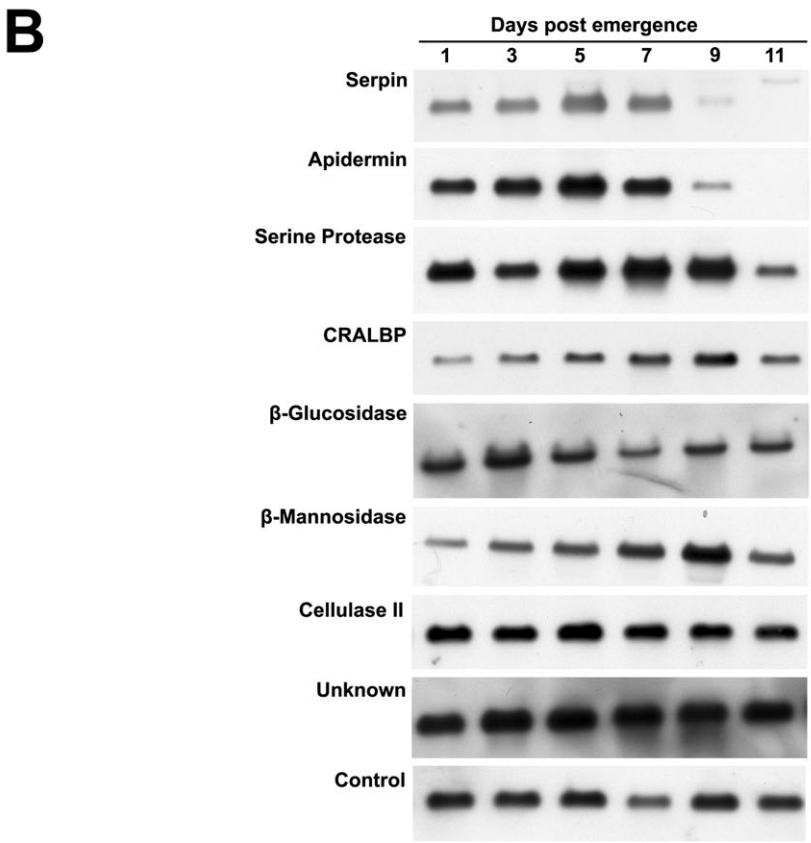


Fig. 1. Northern blot analysis of gene expression for Colorado potato beetle adults during the diapause initiation (IP) and maintenance (MP) phases of diapause (A) and during early nondiapausing adults (B). Total RNA (5 μg) was separated on a 1% formaldehyde-agarose gel and screened using digoxigenin PCR labeled probes. Blots were rescreened using a control gene (ribosomal protein L18a); a representative blot is presented in the bottom panel.

7, 9, and 11 nondiapausing adults were screened. This eleven day period covers a time of rapid increase in the beetles' ovipositioning rate (Peferoen et al., 1981). All the MP down-regulated genes were expressed during the first eleven days postemergence in the nondiapausing adults. In nondiapausing adults the expression of the serine and apidermin genes were not detectable by day eleven, whereas in IP adults the expression persisted until day 15 (Fig 1A and B).

DISCUSSION

Eight transcripts were isolated and shown to be down-regulated between the IP and MP of adult diapause in the Colorado potato beetle. These clones were subdivided into six putative functional groups: regulatory (serpin), structural (apidermin), protease (serine protease), retinol binding protein (CRALBP), carbohydrate metabolism (ß-glucosidase, ß-mannosidase and cellulase II), and unknown function.

Transcripts of two interacting gene families [serine proteases, N4(2)C2; and serpins, N4(1)F7], were isolated and shown to be below the level of detection between day 15 and 20 postemergence. The serpin gene expression was down-regulated by day 9 postemergence in nondiapausing beetles and not detectable by day 11, whereas, the serine protease was expressed at high levels through day 11 in nondiapausing beetles (Fig 1B). Serine proteases (e.g., chymotrypsin, trypsin, elastase and others) are involved in a large array of physiological functions such as development, homeostasis, digestion, and stress response mechanisms (Verhagen et al., 2002; Antao & Malcata, 2005; Hachem et al., 2006; Li et al., 2008). Activity of these proteases is regulated by a diverse family of inhibitors called serpins (serine protease inhibitors) (Silverman et al., 2001; Law et al., 2006; Roberts & Hejaard, 2008). Loss of these critical protease inhibitors results in disease or death of the organism (De Gregorio et al., 2002; Luke et al., 2007; Gooptu & Lomas, 2008; Schefrer et al., 2008). What possible role serpins may play in the adult diapause of *L. decemlineata* is unclear, but the persistence of its expression four days longer in the diapause-programmed beetles than the nondiapausing beetles suggests that it indeed plays some role. As for the serine protease, the most likely function is digestion, since its decrease in expression correlates with the cessation of feeding as the beetles enter diapause MP.

The cuticle is a dynamic structure that responds to external factors such as insecticides and desiccation (Zhang et al., 2008). In response to photoperiodism, the expression of four glycine-rich cuticle protein genes from the Colorado potato beetle have been shown to persist four days longer in diapause IP adult beetles than in early nondiapausing adults (Yocum et al., 2009). One transcript [N4(1)B5] isolated in this study is similar to apidermin 1, a member of a newly described cuticle protein family from the honey bee *Apis mellifera* (Kucharski et al., 2007). Apidermin 1 is 122 amino acids in length and is constructed primarily of the amino acids alanine, glycine, leucine, proline, and valine (AGLPV) (100 amino acids out of 122), making it highly hydrophobic. Apidermin 1 contains two AAPA motifs which are found in cuticular proteins (Willis et al., 2005). The predicted partial protein of N4(1)B5 is 122 bp in length, and like apidermin 1 is extremely hydrophobic, being rich in AGLPV (99 amino acids out of 122). The expression pattern of *L. decemlineata* apidermin gene in early nondiapausing beetles and during the diapause IP mirrored that of four glycine-rich cuticle proteins (Yocum et al., 2009) in that they are expressed four days longer postemergence in diapause IP beetles than they are in nondiapausing beetles. This expression pattern strengthens the case that the protein composition of diapausing beetle cuticle is distinct from that of nondiapausing beetles.

A BlastX search using clone P1(2)B11 revealed significant similarities to the cellular retinal-binding protein (CRALBP) from the parasitic wasp *Nasonia vitripennis*. Retinoid-binding proteins are involved in the transport of the highly hydrophobic retinoids and in regulating the titer and activity of the active forms of the retinoids. CRALBP is essential in the processing of trans-retinol to 11-cis-retinal (vitamin A) which binds to opsin to form the active photopigment rhodopsin (Noy, 2000). Though there is evidence that CRALBP and vitamin A are involved in insect seasonality (Veerman et al., 1985; Overmeer et al., 1989; van Houten & Veerman, 1990; Claret & Volkoff, 1992; Bosse & Veerman, 1996; Gao et al., 1999), *L. decemlineata* remains responsive to photoperiodism independent of diapause history (Tauber et al., 1988). Therefore, the decrease in the level of expression in CRALBP observed in this study would appear not to be affecting the ability of *L. decemlineata* to respond to photoperiodism.

Three enzymes involved in carbohydrate metabolism, two ß-glycosidases (ß-glucosidase and ß-mannosidase) and a cellulase, were isolated and shown to be down-regulated in the diapause MP. ß-glycosidases are enzymes that are involved in the cleavage of the ß-1, 4-linked monosaccharide residues of glycosides (Terra & Ferreira, 1994). ß-glucosidase and ß-mannosidase activity have been detected in insect midguts (Ferreira et al., 1998; Scrivener et al., 1997), while ß-glucosidases are also present in the colleterial glands (Koeppe et al., 1985) and in the hemolymph (Franzl et al., 1989) of various insect species. Although once thought to be restricted to microorganisms, endogenous cellulase transcripts have been isolated in a diverse range of animal species (review, Watanabe & Tokuda, 2001). Even though the phylogenetic distribution of endogenous cellulases is currently unclear, we believe this is the first report of a putative cellulase in *L. decemlineata*. Although the two ß-glycosidases are known to be involved in the intracellular regulation of glycoside titers as well as digestion, their down-regulation as well as the cellulose II gene as the beetles enter the nonfeeding stage of diapause MP (Fig. 1A) would suggest that their primary role is digestive. The downregulation of digestive enzymes during diapause MP in *L. decemlineata* has been observed previously (Yocum et al., 2009). Knowledge of the expression

pattern of key digestive and metabolic genes can provide keen insights into diapause physiology and behavior. Based on the expression patterns of a trypsin, a chymotrypsin, and a fatty-acid synthase transcript, Robich & Denlinger (2005) ruled out the possibility of blood feeding by the mosquito *Culex pipiens* L. on warm autumn days. Drawing on the expression data and other observations, these authors also hypothesized that field overwintering *C. pipiens* are behaviorally and physiologically capable of sugar feeding. This elegant manuscript clearly demonstrates that the power of gene expression studies goes beyond simply understanding regulatory mechanisms to that of being able to predict field behavior.

In summary, from previous results (Yocum et al., 2009) and results presented here, we observed the following for the Red River Valley of North Dakota and Minnesota strain of the Colorado potato beetle. (1) Diapause-programmed beetles make the transition from IP to MP phase of diapause 13–20 days postemergence. (2) The expression of the examined cuticle protein transcripts persists longer in the IP beetles than in the early nondiapausing adults. (3) The expression pattern of one transcript [N6(1)D5] was similar in both IP and nondiapausing beetles, being down-regulated to trace levels by day 11 postemergence (data not shown). (4) Although to date a number of different functional categories of genes have been isolated that are down-regulated during the transition between the diapause IP and MP, most of these genes appear to be involved in digestion either directly or indirectly.

ACKNOWLEDGEMENTS. We thank Lisa B. Yocum for her editing of the various drafts of this manuscript.

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Received May 18, 2009; revised and accepted July 20, 2009