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Transcription changes of a putative *trehalose-6-phosphate synthase* gene in response to hormone stimulation in *Leptinotarsa decemlineata* (Say)

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ABSTRACT

Trehalose metabolism is critical for production of ATP, provision of carbon source, and facilitation of carbohydrate absorption. Trehalose-6-phosphate synthase (TPS) is a rate-limiting enzyme in trehalose biosynthesis. In the present paper, a *TPS* gene (*LdTPS*) was cloned in *Leptinotarsa decemlineata*. At young larval instars, the expression levels of *LdTPS* were high just before and right after ecdysis, and were low at the mid instar stages. In the fourth-instar larvae, two peaks occurred at 24 h after ecdysis and at the wandering stage. *In vitro* midgut culture and an *in vivo* bioassay revealed that 20E stimulated *LdTPS* transcription. Conversely, a reduction of 20E by RNA interference (RNAi) of a prothoracicotropic hormone receptor gene *LdTorso* and an ecdysteroidogenesis gene *LdSHD* repressed *LdTPS* expression. Moreover, disruption of 20E signaling by knockdown of *LdEcR*, *LdE75* and *LdFTZ*-F1 reduced *LdTPS* transcript levels. Similarly, *in vitro* culture and an *in vivo* bioassay showed that exogenous JH or JH analog methoprene and pyriproxyfen activated *LdTPS* expression. An increase in endogenous JH by RNAi of an allatostatin gene *LdAS-C* enhanced the transcription. In contrast, a decrease in JH by silencing of a JH biosynthesis gene *LdJHAMT* downregulated *LdTPS* expression. Moreover, knockdown of *LdILP2* repressed *LdTPS* transcription. Therefore, *LdTPS* transcription is tuned by 20E, JH and ILP signaling pathways in *L. decemlineata*.

Introduction

Trehalose (α-D-glucopyranosyl-(1,1)-α-D-glucopyranoside), a non-reducing disaccharide formed by an α1-α1 bond between two glucose molecules, represents the main hemolymph sugar in most insects (Chapman, 1998). The main site of trehalose synthesis is the insect fat body. The initial reaction is the transfer of α-glucose from uridinediphosphate-glucose to carbon 1 of glucose-6-phosphate, forming trehalose-6-phosphate by trehalose-6-phosphate synthase (TPS, EC 2.4.1.15). The product is subjected to hydrolytic dephosphorylation by trehalose-6-phosphate phosphatase (TPP, EC 3.1.3.12) to yield trehalose (Candy and Kilby, 1959; Candy and Kilby, 1961; Kern et al., 2012).

Trehalose biosynthesis is believed to facilitate carbohydrate absorption by insect guts (Wyatt and Kale, 1957; Candy and Kilby, 1961; Becker et al., 1996; Chapman, 1998). In insects, carbohydrates are absorbed mainly as monosaccharides by a passive process depending on diffusion from a high concentration in the gut to a low one in the hemolymph. This is facilitated by the immediate conversion of glucose, fructose and mannose to trehalose in the fat body surrounding the gut (Chapman, 1998).

Moreover, trehalose is also used for the metabolic production of adenosine triphosphate (ATP), the energy currency in organisms, and as carbon sources. It is a substrate for chitin biosynthesis. Chitin is the major component of insect cuticle, linings of salivary gland, foregut, hindgut and tracheae, and peritrophic membrane of midgut (Cohen, 2010; Merzendorfer, 2011; Shi et al., 2016).

In insects and other Arthropodans, sclerotized cuticle is virtually inextensible. For a remarked increase in size, the cuticle must be shed and replaced periodically, a process commonly known as ecdysis or molting. Therefore, insect requires a great amount of trehalose for chitin biosynthesis just before and right after molting of each instar. During early and mid instar stage, insect larvae feed a large quantities of food. Absorbed monosaccharides have to rapidly be converted to trehalose to facilitate further absorption (Chapman, 1998). For economical reasons, it appears plausible that, just before and right after the ecdysis when trehalose biosynthesis is active, transcription of relative genes should be upregulated concomitantly. TPS is a rate-limiting enzyme in trehalose synthesis. Therefore, its expression should be tightly regulated by 20-hydroxyecdysone (20E), juvenile hormone (JH) and/or insulin-like peptides (ILPs), three hormones that play critical roles in larvae development and nutrition homeostasis (Liu et al., 2015; Zhang et al., 2015; Zhu et al., 2015).

In *Leptinotarsa decemlineata* (Say), a notorious defoliator of potato, several cytochrome P450 monooxygenases such as Spook, Phantom, Disembodied, Shadow, and Shade (SHD) are involved in

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the biosynthesis of ecdysone in the prothoracic glands (PGs), and the production of 20E in the peripheral tissues (Kong et al., 2014). Ecdysteroidogenesis by PGs is regulated by a brain-derived peptide, prothoracicotropic hormone (PTTH) (Smith and Rybczynski, 2012; Johnson et al., 2013), through binding to its receptor Torso, a receptor tyrosine kinase in *Drosophila melanogaster* (Grillo et al., 2012; Johnson et al., 2013) and *L. decemlineata* (Zhu et al., 2015). The activated Torso triggers the canonical mitogen activated protein kinase (MAPK) pathway, and stimulates ecdysteroidogenesis (Caldwell et al., 2005; McBrayer et al., 2007; Rewitz et al., 2009; Grillo et al., 2012; Zhu et al., 2015). 20E signal, acting through its heterodimeric receptor ecdysone receptor (EcR)/ultraspiracle (USP), initiates specific gene expression cascades. Among the activated genes, *ecdysone-induced protein 75* (*E75*), *hormone receptor 3* (*HR3*) and *fushi tarazu factor* 1 (*FTZ*-F1) are confirmed to mediate the 20E signaling (Ogura et al., 2005; Liu et al., 2014; Guo et al., 2015b, 2016).

JH is produced in the corpora allata (*CA*). JH acid methyltransferase (JHAMT) participates in JH biosynthesis (Fu et al., 2016a). An allatostatin AS-C, tunes JH biosynthesis in *L. decemlineata* (Meng et al., 2015). JH binds to it receptor heterodimer Methoprene-tolerant (MET)/Taiman (Tai), and triggers the expression of JH-responsive genes such as *Krüppel homolog* 1 (*LdKr-h1*) (Meng et al., 2015; Fu et al., 2016a).

In insects, the ILP signaling cascade is initiated by binding of an ILP to its receptor (InR) (Fernandez et al., 1995; Chen et al., 1996). The ILP-InR complex subsequently activates downstream components such as phosphoinositide 3-kinase (PI3K), AKT, and target of rapamycin (TOR) (Puig and Tjian, 2006; Hietakangas and Cohen, 2009; Walsh and Smith, 2011). Feeding stimulates the secretion of ILP into the circulatory system. It activates ILP signaling pathway, and leads to uptake of nutrients and synthesis of protein and lipid (Prentki et al., 2013).

To better understand the hormone regulation of *TPS* gene, in this report, we identified *LdTPS* gene and tested its expression throughout the larval stage. We then determined the regulation of *LdTPS* by ILP, 20E, and JH signaling pathways. Our results revealed that endogenous hormones tuned the expression of *LdTPS* to coordinate the trehalose biosynthesis during larval development in *L. decemlineata*.

Materials and methods

Insects and chemicals

L. decemlineata beetles were kept in an insectary according to a previously described method (Zhou et al., 2013), and were supplied with potato foliage at vegetative growth or young tuber stages in order to assure sufficient nutrition. At this feeding protocol, *L. decemlineata* larvae progressed through four distinct instars, with the approximate periods of 2, 2, 2 and 4 days, respectively.

Ecdysteroid 20-hydroxyecdysone (20E) (Sigma-Aldrich, USA), juvenile hormone (JH) (Sigma-Aldrich, USA), JH analog methoprene (isopropyl (2E,4E,7S)-11-methoxy-3,7,11-trimethyldodeca-2,4-dienoate) (Shanghai Kewelchem Company, Shanghai, China) and pyriproxyfen (2-(1-methyl-2-(4-phenoxyphenoxy) ethoxy) pyridine) (Ivy Fine Chemicals Corporation, USA) were purified by a reverse phase high performance liquid chromatography (RP-HPLC) or an HPLC before experiments.

Cloning of the LdTPS cDNA

The *L. decemlineata* transcriptome (Shi et al., 2013) and genome data (https://www.hgsc.bcm.edu/arthropods/colorado-potato-beetle-genome-project) were searched with TBLASTN using TPS amino acid sequence of *T. castaneum* as the query. This resulted in the identification of a cDNA that we have named as *LdTPS*. A second set of searches was done using *Ld*TPS amino acid sequence in an attempt to identify additional genes encoding proteins related to TPS.

The correctness of *LdTPS* sequence was substantiated by polymerase chain reaction (PCR) using primers in Table 1. This was followed by 5′- and 3′-RACE to complete the sequence, with SMARTer RACE cDNA amplification kit (Takara Bio., Dalian, China) and SMARTer RACE kit (Takara Bio.). The antisense/sense gene-specific and the nested primers corresponding to the 5′-end and 3′-end of the sequences were listed in Table 1. The full-length *LdTPS* was submitted to GenBank (KU756283).

Analysis of the protein domain was performed using SMART (http://smart.Embl-heidelberg.de/) and Pfam (http://pfam.xfam.org/).

In vitro midgut culture

Day 1 third-instar larval midguts were dissected in saline. The midguts together with their peritrophic membranes were ligated tightly using cotton thread at the anterior and posterior ends, and then cut to the ligatures. The isolated midguts were rinsed four times with medium, and then independently cultured in EX-CELL® 405 Serum-Free Medium for Insect Cells (Sigma-Aldrich, USA) at 25 °C. Twelve individual midguts were cultured in culture medium (control, CK), 10^{-4} M cycloheximide (Chx) (Sigma-Aldrich, USA), 10^{-6} M 20E, 10^{-4} M Chx + 10^{-6} M 20E, or 10^{-6} M methoprene. Each group repeated three times. Samples for mRNA expression analysis were selected randomly at 0, 6, 12 and 24 h after incubation.

Table 1
Primers used in RT-PCR, RACE, ORF verification, dsRNA synthesis, and qPCR.

Fragement name	Forward primer	Reverse primer
RT-PCR		
LdTPS	GGTGGAGGACTATGGGTA	GTTTCTTCCGCTGACAAT
RACE		
LdTPS 5′-GSP		GCATACCTTGTAGGATTTCG
LdTPS 5′-NGSP		GGGCGTTATGTCTGATGG
LdTPS 3′-GSP	ATGAACCTGGTGGCTAAA	
LdTPS 3′-NGSP	ATGGAACTCTGGCACCTA	
ORF verification		
LdTPS	AATGGGTTTGTGGAGAAA	AAATGGTATCGGGAATGA
dsRNA synthesis		
ds*Torso*	CTAAATTCAAACCCCTTC	TCCCGTTACATTGCTACT
ds*SHD*	CTCTTCCTCGGTTATTCTTGCC	ATGCAAACCAGTTCAGGCC
ds*EcR*	GATCTATCCCCTCCCAGCAG	TCGTTCCGTTTGACAGCG
ds*E75*	TCCCAAGAGAGAAAAAGCG	GGCAAGCAAGGAGAATCC
ds*FTZ*-F1	TCTGCTGAGTTTGGGGTT	GTGCTTGTGGTAGAGGTGTT
ds*AS-C*	GGCATCTGGGAGAAAATAG	GAAGTAGCAGGCTCTGAATCT
ds*JHAMT*	GGACAAGCCCGACTTATACTC	GCGAACTCCACCTCATCAA
ds*ILP2*	GTCCTCCTCGTCATCCTT	GTAACCTGTCGTGTCCCA
ds*egfp*	AAGTTCAGCGTGTCCG	CACCTTGATGCCGTTC
qPCR		
q*LdTPS*	GAGTCTGGATGCTGCTAGTT	CGGGAGAGAGCGAAGATTT
q*LdTorso*	CCAAAGTGCAGACTCCTCAA	CTTTGTTGCTCCCTCTTTCC
q*LdSHD*	GGCCTGAACTGGTTTGCAT	GGCAAGAATAACCGAGGAAGAG
q*LdEcR*	GAATGAGGGCAGAGTGTGTG	TCGTAGTGCTATTGGGCTTG
q*LdE75*	CCAACTCCAGATGCAGCTTA	AATGATTTCGCCAACATTGA
q*LdFTZ*-F1	GGCTAATCAGGCCTCCAG	CATGGTTTGCTGGCAACTAC
q*LdAS-C*	ATGCAAGGCTTTCTCCATCT	TTGATTGTTGTCCGGGTCTA
q*LdJHAMT*	GGAAGTGGAGATGGCAAGTT	CTACCAACGAGTTTCCCGAT
q*LdILP2*	GTCCTCCTCGTCATCCTTGT	CCTGGGTTCTTGTCGTGTTA
q*LdRP4*	AAAGAAACGAGCATTGCCCTTCCG	TTGTCGCTGACACTGTAGGGTTGA
q*LdRP18*	TAGAATCCTCAAAGCAGGTGGCGA	AGCTGGACCAAAGTGTTTCACTGC
q*LdARF*1	CGGTGCTGGTAAAACGACAA	TGACCTCCCAAATCCCAAAC
q*LdARF4*	GTGCTCGTGAACCATGTGAA	AACCTCCAATCCCTCGTGAA