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Physiological roles of trehalose in *Leptinotarsa* larvae revealed by RNA interference of trehalose-6-phosphate synthase and trehalase genes

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ABSTRACT

Trehalose is proposed to serve multiple physiological roles in insects. However, its importance remains largely unconfirmed. In the present paper, we knocked down either a trehalose biosynthesis gene (trehalose-6-phosphate synthase, *LdTPS*) or each of three degradation genes (soluble trehalases *LdTRE1a*, *LdTRE1b* or membrane-bound *LdTRE2*) in *Leptinotarsa decemlineata* by RNA interference (RNAi). Knockdown of *LdTPS* decreased trehalose content and caused larval and pupal lethality. The *LdTPS* RNAi survivors consumed a greater amount of foliage, obtained a heavier body mass, accumulated more glycogen, lipid and proline, and had a smaller amount of chitin compared with the controls. Ingestion of trehalose but not glucose rescued the food consumption increase and larval mass rise, increased survivorship, and recovered glycogen, lipid and chitin to the normal levels. In contrast, silencing of *LdTRE1a* increased trehalose content and resulted in larval and pupal lethality. The surviving *LdTRE1a* RNAi hypomorphs fed a smaller quantity of food, had a lighter body weight, depleted lipid and several glucogenic amino acids, and contained a smaller amount of chitin. Neither trehalose nor glucose ingestion rescued these *LdTRE1a* RNAi defects. Silencing of *LdTRE1b* caused little effects. Knockdown of *LdTRE2* caused larval death, increased trehalose contents in several tissues and diminished glycogen in the brain-corpora cardiaca-corpora allata complex (BCC). Feeding glucose but not trehalose partially rescued the high mortality rate and recovered glycogen content in the BCC. It seems that trehalose is involved in feeding regulation, sugar absorption, brain energy supply and chitin biosynthesis in *L. decemlineata* larvae.

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1. Introduction

Trehalose (α-D-glucopyranosyl-(1,1)-α-D-glucopyranoside), a non-reducing disaccharide formed by an α1-α1 bond between two glucose molecules, is widespread in invertebrates, plants, fungi and bacteria, but appears to be absent in vertebrates (Shukla et al., 2015; Tang et al., 2011). In general, trehalose is the major hemolymph sugar in insects, typically between 5 and 100 mM, depending on environmental condition, physiological state and nutrition (Thompson, 2003).

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Trehalose is synthesized mainly in insect fat body, a principal organ of intermediary metabolism and nutrition storage equivalent to vertebrate liver and adipose tissue, under the catalyzation of trehalose-6-phosphate synthase (TPS, EC 2.4.1.15) and trehalose-6-phosphate phosphatase (TPP, EC 3.1.3.12) (Candy and Kilby, 1959, 1961; Kern et al., 2012). Insect TPSs include a conserved TPS domain and a TPP domain, it has been suggested that TPS enzymes in *Drosophila melanogaster* and *Helicoverpa armigera* may act not only as TPS, but also as TPP (Chen and Haddad, 2004; Xu et al., 2009). Trehalose is then released into hemolymph, and absorbed by trehalose-utilizing cells, where it is hydrolyzed into two molecules of glucose by trehalase (TRE, EC 3.2.1.28) (Mitsumasu et al., 2010). Two types of TREs have been documented in insects: soluble TRE1s and membrane-bound TRE2s (Mitsumasu et al., 2005; Mori et al., 2009; Takiguchi et al., 1992).

Trehalose is proposed to serve multiple physiological roles in insects (Shukla et al., 2015). Up to now, however, the physiological

importance of trehalose remains largely unconfirmed (Matsuda et al., 2015). Firstly, conversion of glucose to trehalose is believed to facilitate carbohydrate absorption. 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 may be aided by the immediate conversion of glucose to trehalose in the fat body surrounding the gut (Chapman, 1998). Consistently, wide searches and functional analyses of heterelogously expressed proteins predict that insect species, such as *Acyrthosiphon pisum* and *Nilaparvata lugens*, only have facilitative sugar transporters for sugar homeostasis (Kikuta et al., 2010; Price and Gatehouse, 2014; Price et al., 2010), although two pathways are known in the mammalian: the passive, paracellular absorption and the active transport mediating by Na^+/or K^+/glucose cotransporters (Chen et al., 2015; Deng and Yan, 2016). An interesting question is: Can hemolymph glucose be converted to glycogen or other chemicals to facilitate monosaccharide absorption?

Secondly, trehalose is an energy source that meets the demands of flight muscles and other energy-consuming organs in many insects (Becker et al., 1996; Candy and Kilby, 1961). However, Coleopterans and some Dipterans metabolize amino acids, especially proline, in these energy-consuming organs, although the energy is ultimately derived from lipid (Chapman, 1998; Thompson, 2003). In many energy-consuming tissues of the Colorado potato beetle *Leptinotarsa decemlineata* (Say), for instance, proline is partially degraded to generate alanine, catalyzed by proline dehydrogenase (*Ld*ProDH), pyrroline-5-carboxylate dehydrogenase (*Ld*P5CDH) and alanine aminotransferase (*Ld*ALT). Alanine, in turn, is released to the hemolymph and transported to the fat body, where it is used to resynthesize proline, under the catalyzation of *Ld*ALT, Δ1-pyrroline-5-carboxylate synthetase (*Ld*P5CS) and Δ1-pyrroline-5-carboxylate reductase (*Ld*P5CR). Newly biosynthesized proline is then released from the fat body to the hemolymph, transported to and absorbed by the energy-consuming tissues (Wan et al., 2014a, 2014b, 2015a, b). Is trehalose an energy source in specific tissues in Coleopterans such as *L. decemlineata*?

Thirdly, as a major blood sugar in insects, trehalose is considered as a major substrate for chitin synthesis. However, the immediate precursor for chitin synthesis is glucose. Glucose briefly enters glycolysis to generate fructose-6-phosphate. Fructose-6-phosphate is subsequently diverted towards the hexosamine pathway to produce uridine diphosphate N-acetyl glucosamine, which will be ultimately polymerized into chitin (Doucet and Retnakaran, 2012). In L. *decemlineata*, four chitin biosynthesis genes (*LdUAP1* and *LdUAP2* encoding uridine diphosphate N-acetylglucosamine pyrophosphorylase, *LdChSA* and *LdChSB* encoding chitin synthase) have been identified (Shi et al., 2016a, 2016b). To which extent is trehalose involved in chitin biosynthesis in *L. decemlineata*?

Lastly, trehalose is suggested as a component of a feedback mechanism regulating feeding behavior and nutrient intake, where blood metabolite levels including trehalose act through modulation of taste receptor responses and through the central nervous system to influence food selection and consumption (Thompson, 2003). Does trehalose content in the hemolymph affect the feeding in *L. decemlineata*?

In addition, in some insect species such as *Polypedilum vanderplanki*, trehalose is involved in anhydrobiosis (protection against desiccation) (Mitsumasu et al., 2010). To address the physiological roles of trehalose in *L. decemlineata*, we knocked down either a trehalose synthesis gene *LdTPS* or each of three degradation genes (*LdTRE1a*, *LdTRE1b* or *LdTRE2*) in the larvae, and compared several phenotypes and metabolism changes in carbohydrates, lipids, amino acids and chitin. Our results confirmed that trehalose is involved in the four physiological functions, to different extents, in *L. decemlineata*.

2. Materials and methods

2.1. Experimental animal

The L. *decemlineata* beetles were kept in an insectary according to a previously described method (Shi et al., 2013), with potato foliage at vegetative growth or young tuber stages in order to assure sufficient nutrition. At this feeding protocol, the larvae progressed through four distinct instars, with approximate periods of the first-, second-, third-, and fourth-instar stages of 2, 2, 2 and 4 days, respectively. Upon reaching full size, the fourth larval instars stopped feeding, dropped to the ground, burrowed to the soil and entered the prepupae stage. The prepupae spent an approximately 4 days to pupate. The pupae lasted about 6 days and the adults emerged.

2.2. Molecular cloning

The fragments of putative *LdTPS*, *LdTRE1a*, *LdTRE1b*, *LdTRE2* and the representative glucose and lipid metabolism genes were obtained from the genome (https://www.hgsc.bcm.edu/arthropods/colorado-potato-beetle-genome-project) and transcriptome data (Shi et al., 2013). The correctness of the sequences was substantiated by polymerase chain reaction (PCR) using primers in Table S4. The full-length cDNAs were obtained by 5′- and/or 3′-RACE, using SMARTer RACE kit (Takara Bio.), with specific primers listed in Table S1. All of the sequenced cDNAs were submitted to GenBank (accession numbers were listed in Table S2).

2.3. Preparation of dsRNAs

The same method as previously described (Zhou et al., 2013) was used to express ds*TPS*-1, ds*TPS*-2, ds*TRE1a*-1, ds*TRE1a*-2, ds*TRE1b*-1, ds*TRE1b*-2, ds*TRE2*-1, ds*TRE2*-2 and ds*egfp*, derived from a 325 bp and a 500 bp fragments of *LdTPS*, a 426 bp and a 414 bp fragments of *LdTRE1a*, a 432 bp and a 305 bp fragments of *LdTRE1b*, a 485 bp and a 381 bp fragments of *LdTRE2*, and a 414 bp fragment of enhanced green fluorescent protein gene. The nine dsRNAs were individually expressed with specific primers in Table S4, using *Escherichia coli* HT115 (DE3) competent cells lacking RNase III. Individual colonies were inoculated, and induced to express dsRNA by addition of isopropyl β-D-1-thiogalactopyranoside to a final concentration of 0.1 mM. The expressed dsRNA was extracted and confirmed by electrophoresis on 1% agarose gel (data not shown). Bacteria cells were centrifuged at 5000 × g for 10 min, and resuspended in 0.05 M PBS (the concentration of phosphate, pH 7.4) at the ratio of 1:1. The bacterial suspension with dsRNA concentration of about 0.5 μg/μL was used for bioassay.

2.4. Bioassays

The same bioassay method as previously reported was used (Fu et al., 2015). The newly-ecdysed second- and fourth-instar larvae were allowed to feed foliage immersed with bacterial suspension containing each of the two dsRNAs of *LdTPS* (ds*TPS*-1 and ds*TPS*-2), *LdTRE1a* (ds*TRE1a*-1 and ds*TRE1a*-2), *LdTRE1b* (ds*TRE1b*-1 and ds*TRE1b*-2) or *LdTRE2* (ds*TRE2*-1 and ds*TRE2*-2) (replaced with freshly treated ones each day) for 3 days. The PBS- and ds*egfp*-dipped foliage were used as controls. The larvae were then transferred to untreated foliage if necessary.

The consumed foliage areas per repeat per day were measured on day 3 after the initiation of the bioassays. The larvae were weighed twice during trial period. The growth of the survivors was observed at 4-h intervals. Instars were identified by head capsule width, the appearance of exuviae, the black color of the pronotum,

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