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Identification of rapid cold hardening-related genes in the tobacco budworm, *Helicoverpa assulta*

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ABSTRACT

A rapid cold hardening (RCH) and supercooling capacity usually play crucial roles in the survival of the tobacco budworm, *Helicoverpa assulta*, which is a freeze-tolerant species during the overwintering period. Cryoprotectants such as polyols or sugars are known to affect RCH and maintain fluid status of hemolymph improving the survival rate. This study is performed to identify cryoprotectants as a RCH factor in *H. assulta*. Pre-exposure of *H. assulta* larvae to 4 °C significantly increased survival at −10 °C in all developmental stages from egg to adult. RCH was dependent on the duration of the pre-exposure period and also significantly enhanced the supercooling capacity. Analysis of cryoprotectants from the hemolymph revealed that the distinguished changes of polyols were found in the peaks of glycerol and trehalose. As cold treatment was prolonged, the amount of trehalose was continuously increased. In contrast, glycerol showed the highest level at 4 h treatment, and was gradually declined. To understand the pathway of trehalose biosynthesis during the pre-exposure treatment, expression level of genes related to trehalose biosynthesis were analyzed from fat body transcriptome of the 4th instar larvae of *H. assulta*. Based on the expression level of transcripts, the expression of trehalose phosphate synthase (TPS) harboring TPS and trehalose phosphate phosphatase domain contributes to the accumulation of trehalose in the hemolymph, suggesting that trehalose is responsible for overcoming RCH in *H. assulta*.

Introduction

The physical environment such as exposure to low temperature threatens the survival of insects during the winter. To overcome the physical limits such as temperature and freezing, insects have developed the effective mechanism like diapause and anti-freezing protein as the survival strategies. In particular, as shown in insect pest development and economic damages to crops, cold hardiness is very important to the agriculture and forestry and subsequently affects geographical distribution due to the rapid world-wide climate changes (Morin and Comrie, 2010; Bale and Hayward, 2010; Rose and Wall, 2011).

Cold hardiness to overcome low temperature can be divided into two types: freeze avoidance and freeze tolerance (Storey and Storey, 1988; Clark and Worland, 2008). Freeze avoidance involves deep supercooling of fluid in the body of overwintering insects. Anti-freezing protein, high concentration of cryoprotectants and partial dehydration of body fluid contribute to freezing avoidance (Storey and Storey, 1988). Mechanism of freeze tolerance is to regulate ice formation triggered by ice nucleate agents or proteins in extracellular space (Ramlov and Westh, 1993; Block et al., 1998). Cryoprotectants in freezing tolerance play a role protection of lipid bilayer of the cell membrane (Storey and Storey, 1991; Zachariassen and Kristiansen, 2000; Duman, 2001).

Cryoprotectants belonging to carbohydrates are used for the protective mechanism of insect cold hardiness when an insect is exposed subzero temperature (Denlinger and Lee, 2010; Storey and Storey, 1991; Zachariassen and Kristiansen, 2000; Graham et al., 2007; Clark and Worland, 2008; Doucet et al., 2009). Due to the induction of rapid cold hardiness, the insects exposed simply to a certain low temperature above freezing point prior to exposing near freezing point exhibited higher survival rate than that of the insects exposed near freezing point directly (Lee et al., 1987; Park and Kim, 2013).

The oriental tobacco budworm *Helicoverpa assulta* (Lepidoptera: Noctuidae) is a main insect pest damaging hot peppers, and causes over 50% deduction without proper control application (Yang et al., 2004). *H. assulta* is also known as a species which overwinters in the form of pupa in the soil, such as *Helicoverpa zea*, *Heliothis virescence*, and *H. armigera*, and emerges after June in the next year (Han and Lee, 1998; Stadelbacher and Martin, 1980; Shimizu et al., 2006). Therefore, *H. assulta* can be regarded as freeze-tolerant species. However, the factors contributing to overcoming rapid cold hardness has not been explained while the ecological pattern of overwintering *H. assulta* was well known.

In this study, we examined if pre-exposure of cold treatment to *H. assulta* larvae contributed to the survival and change of supercooling point. Also, quantitative changes of carbohydrates or polyols in the hemolymphs which may contribute to RCH was analyzed by HPLC. Gene expressions related to polyol production in the fat body were examined

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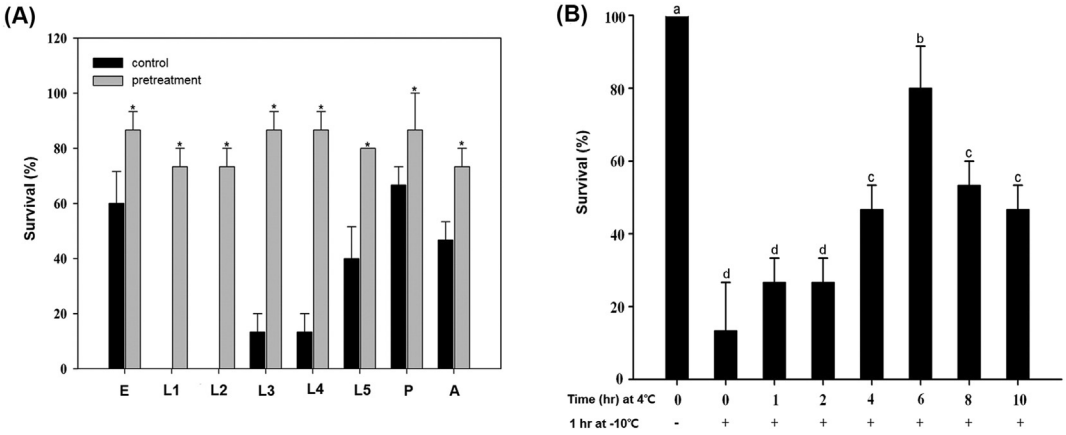

Fig. 1. Rapid cold hardening (RCH) of *Helicoverpa assulta*. Cold tolerance was analyzed by exposure to −10 °C for 1 h and expressed as survival rate. (A) RCH was induced in all developmental stages by pre-exposure to 4 °C for 6 h. E, egg; L1–L5, first–fifth instar larva; P, pupa; and A, adult. Tests at each developmental stage were replicated three times with 10 individuals per replication. The asterisk indicates the statistical difference between treatment and control. (B) Effect of pre-exposure period on RCH in 4th instar larvae. Each treatment was replicated three times with 10 larvae per replication. The different letter indicates the statistical difference between treatment and control.

based on the transcription analysis and the key enzyme for trehalose biosynthesis, trehalose phosphate synthase, was identified.

Materials and methods

Insect rearing

The tobacco budworm, *Helicoverpa assulta*, larvae were obtained from Dr. S.J. Ahn (Rural Development Administration, Korea). The larvae were fed on an artificial diet at 27 °C, relative humidity 50 ± 5% and a photoperiod (L:D = 16:8). Adults were maintained in a cage (dimension: 30 × 30 × 30 cm) and fed on 10% sucrose solution.

Supercooling point (SCP) measurement

SCPs of 3rd and 4th instar larvae of *H. assulta* were measured following the previous report (Park and Kim, 2013) with a thermodetector (Tecpel Co., Ltd., Taiwan) to detect the temperature when the latent heat of freezing body was released. The larvae were fixed on a petri dish with an adherent tape. The wire of the thermodetector was attached to the larvae and they were put into the styrofoam container which was placed into a deep freezer (Daihan Science Inc., Korea) at −80 °C.

Rapid cold hardening (RCH) assay

All developmental stages of *H. assulta* were examined in RCH treatment. The condition of RCH was exposure at 4 °C for 6 h prior to transferring to −10 °C for 1 h. For cold tolerance, the larvae directly exposed to −10 °C without cold pretreatment were used as control. Eggs were examined if they hatch at 25 °C. Pupal survival was determined by emergence. After 4th instar larvae were differently exposed to cold pretreatment (4 °C for 1 to 10 h), their survival rate were examined. Each treatment was replicated three times with 5 larvae per experiment.

Table 1
Supercooling point (SCP) changes of *Helicoverpa assulta* after rapid cold hardening (RCH) treatment.

Stage	RCH treatment*	N	SCP (°C)
3rd instar	No	5	−13.58 ± 0.82[a]
	Yes	5	−14.82 ± 0.82[a]
4th instar	No	5	−10.46 ± 0.16[a]
	Yes	5	−13.10 ± 0.70[b]

Different character indicates statistical difference between two groups.
* RCH treatment was incubation of 6 h at 4 °C.

Identification of polyols from hemolymph of H. assulta

Hemolymph from 4th instar larvae of *H. assulta* was pretreated with cold condition at 4 °C for 0 to 24 h and collected into 1.5 mL tube containing anticoagulant powder, phenylthiourea (Sigma-Aldrich Korea, Seoul, Korea). One-fifth of collected hemolymph was diluted with the distilled ddH_2O and the supernatant was collected and cleaned up with Sep-Pak C18 cartridge (Walters Associates, Inc., Milford, MA, USA) and a 0.22 μm syringe filter (Pall Corporation, Ann Arbor, MI, USA) after centrifugation at 400 ×*g* for 5 min. The composition and amounts of polyols were analyzed with an ion exchange column (CarboPac MA1, 4 × 250 mm, Dionex) via HPLC (BioLC, Dionex, Sunnyvale, CA, USA). A sample was injected with a 25 μL volume. Elution buffer was 400 mM NaOH at a constant rate of 0.4 mL/min. The separated samples were detected by an electrochemical detector (ED40, Dionex) in a pulse amperometry mode. The standard solution contains 9 materials, glycerol, sorbitol, trehalose, mannitol, mannose, glucose, galactose, fructose, and sucrose.

Transcriptome of fat body in 4th instar larvae.

Total RNAs of the fat body from two groups of the 4th instar larvae, one was grown at 27 °C and the other at 3 to 10 °C, were isolated using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. DNase (Life Technologies, NY, USA) was treated to digest putatively remaining DNAs. RNA was further purified by using the RNeasy MiniElute Clean up Kits (Qiagen, Valencia, CA, USA). The RNA quality and yield were assessed by 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Messenger RNAs were purified with poly-T oligo-attached magnetic beads and fragmented into small pieces using divalent cations under elevated temperature. The cleaved RNA fragments were copied into first strand cDNA using reverse transcriptase and random primers. After the end repair and ligation of adaptors, the products were cleaned up with an AMPure beads (Beckman Coulter Genomics, Danvers, MA, USA) to create the final cDNA library. The library was sequenced on the Illumina HiSeq™ 2000 (Illumina, Inc., San Diego, CA, USA) using paired end technology in 1/2 lane. The average read length of 101 bp was generated as raw data. These contigs were obtained by de novo assembly of RNA-seq short reads and gene annotation were performed with BlastX (http://blast.ncbi.nlm.nih.gov).

Statistical analysis

SigmaStat ver. 3.5 (Systat software Inc., USA) was used for ANOVA and *t*-test to detect statistical difference.