Journal of Experimental Biology

RNA interference of glycerol biosynthesis suppresses rapid cold hardening of the beet armyworm, *Spodoptera exigua*

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Running title: RNA interference of glycerol production

SUMMARY

The beet armyworm, *Spodoptera exigua*, is a freeze-susceptible species that overwinters in temperate zones without diapause. The rapid cold hardening (RCH) and supercooling capacity usually play crucial roles in the survival during overwintering period. This study determined a cryoprotectant as an RCH factor of *S. exigua*. A pre-exposure of *S. exigua* larvae to 4°C significantly increased survival at -10°C in all developmental stages from egg to adult. The RCH was dependent on the duration of the pre-exposure period. The RCH also significantly enhanced the supercooling capacity. Cryoprotectant analysis using HPLC showed that the pre-exposure treatment allowed the larvae to accumulate glycerol in the hemolymph. Two genes, *glycerol-3-phosphate dehydrogenase* (*GPDH*) and *glycerol kinase* (*GK*), were identified as being associated with glycerol biosynthesis, and were cloned in *S. exigua* larvae. Both *GPDH* and *GK* were expressed in all developmental stages of *S. exigua*. Either RNA interference (RNAi) of *GPDH* or *GK* significantly inhibited glycerol accumulation in the hemolymph of *S. exigua*. Larvae treated with RNAi of *GPDH* or *GK* gene expression exhibited a significant decrease in RCH capacity. The glycerol accumulation in response to 4°C appeared to be under the control of humoral signal, because a ligation experiment prevented glycerol accumulation in the other half of the body. This study indicated that glycerol is a RCH factor of *S. exigua* and is induced to be synthesized in response to low temperature via humoral mediation.

Key words: rapid cold hardiness, glycerol, RNA interference, GPDH, GK, *Spodoptera exigua*

INTRODUCTION

Low temperature is a physical barrier preventing insects from expanding their habitats, due to their poikilothermic nature (Lee and Denlinger, 1991). Damage by low temperature can be divided into cold and freezing injuries (Kelly et al., 1996). Cold injury occurs above freezing temperature, and is induced by damage of membrane fluidity or proteins (Salt, 1961; Michaud and Denlinger, 2004). Freezing injury occurs directly by growth of ice crystals to break cell membranes or by dehydration due to ice formation (Salt, 1961). Thus, depending on survival strategies at low temperatures, insects or other invertebrates are classified into freeze-avoiding, freeze-tolerant or vitrification (a cryoprotective dehydration) types (Storey and Storey, 1988; Holmstrup et al., 2002).

Rapid cold hardening (RCH) is accomplished by a brief exposure to a certain low temperature above freezing point, and plays a crucial role in enhancing survival to low temperatures in all types of cold-survival strategies (Lee et al., 1987). Membrane remodeling to increase fluidity (Overgaard et al., 2005) and chemical change in hemolymph composition to increase polyols (Michaud and Denlinger, 2007) are associated with RCH. More recently, Li and Denlinger (2008) demonstrated a complex response of RCH, which was accompanied by changes in energy metabolism and cytoskeletal factors through analysis of brain proteomics, in response to a low temperature.

Polyols are a cryoprotectant group synthesized by insects and other organisms (Storey and Storey, 1991). Both freeze-avoiding and freeze-tolerant species accumulate polyol cryoprotectants: in freeze-avoiding species, polyols permit the colligative depression of supercooling point (SCP) to prevent body freezing, whereas in freeze-tolerant species polyols offer a protective barrier against intracellular freezing, by restricting ice formation in extracellular compartments (Storey and Storey, 2012). Different insects accumulate different polyols (Doucet et al., 2012). In response to a brief cold exposure, the flesh fly, *Sarcophaga crassipalpis*, increase glycerol and sorbitol, as well as free amino acids of alanine and glutamine (Michaud and Denlinger, 2007).

The beet armyworm, *Spodoptera exigua*, migrates long distances to locate suitable breeding places, and continuously intimidates crops in the temperate region (Mikkola, 1970; Zheng et al., 2012). The supercooling ability of *S. exigua* allows them to overwinter in some restricted zones

(Kim and Kim, 1997). Moreover, its RCH helps their survival at low temperature during winter in temperate zones, in which unidentified protein and glycerol have been proposed to be the factors of RCH (Song et al., 1997; Kim and Song, 2000).

This study tested a hypothesis that glycerol is the main RCH factor in *S. exigua*. To test the hypothesis, the carbohydrates and polyols in the hemolymph were quantitatively analyzed by HPLC. To prove the role of glycerol in RCH, we used RNA interference (RNAi) of two genes associated with glycerol biosynthesis, and subsequently analyzed the intensity of RCH and glycerol accumulation.

MATERIALS AND METHODS

Insect rearing

The beet armyworm, *S. exigua*, larvae were collected from a field population infesting welsh onion in Andong, Korea. The larvae were reared on an artificial diet (Goh et al., 1990) at 25°C, a photoperiod of 16:8 (L:D) h, and RH 60 ± 5%. Adults were fed 10% sucrose solution. All experiments were carried out on one-day-old fifth instar larvae.

RCH bioassay

All developmental stages of *S. exigua* were analyzed in RCH treatments. Test individuals were divided into three groups: control (exposed to 4°C for 6 h), cold-shock (directly transferred to -10°C for 1 h), and RCH (exposed to 4°C for 6 h prior to -10°C for 1 h). For each treatment group, test individuals were placed in a Petri dish (10 × 15 mm). After cold treatment, the survival rates of all developmental stages were determined after 2 h recovery at 25°C. Autonomous movement of individuals was the criteria for being categorized as alive. Egg survival was determined by hatching at 25°C recovery condition. Pupal survival was determined by adult emergence at 25°C recovery condition. Each treatment was replicated three times. Each replication used 10 individuals.

SCP measurement

SCPs were measured according to the method of Kim and Kim (1997), using a thermocouple, BTM-4208SD (LT Lutron, Taipei, Taiwan) to detect the release of the latent heat of fusion as

body water freezed. The specimens were affixed to the thermocouple with a plastic tape. A thermocouple with an attached specimen was placed in a styrofoam box (30 × 30 × 15 cm), and the box was placed into a freezer at -70°C. The cooling rate was measured as 1°C/min.

Polyol analysis using HPLC

Hemolymph from fifth instar larvae of *S. exigua* was collected into 1.5 mL tubes containing a pinch of anticoagulant powder, phenylthiourea (PTU, Sigma-Aldrich Korea, Seoul, Korea), and diluted with the distilled and deionized water. After centrifugation at 400 x *g* for 5 min, the supernatant plasma was cleaned with Sep-Pak C18 cartridge (Walters Associates, Inc., Milford, MA, USA) which was prewashed with distilled and deionized water. The plasma samples were further cleaned by passing through a 0.22 μm syringe filter (Pall Corporation, Ann Arbor, MI, USA). Analysis of the composition and amounts of polyols were carried out using an ion exchange HPLC (BioLC, Dionex, Sunnyvale, CA, USA) equipped with a guard column (CarboPac MA1, 4× 50 mm, Dionex) and a main column (CarboPac MA1, 4× 250 mm, Dionex). 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.

Sequence determination of *glycerol-3-phosphate dehydrogenase 1* (*SeGPDH1*) and *glycerol kinase 1* (*SeGK1*) of *S. exigua*

Three transcriptomes of *S. exigua* were used to determine both *SeGPDH1* and *SeGK1* open reading frames (ORFs). Hemocyte and gut transcriptomes were generated by 454 pyrosequencing (Hwang et al., 2013). A mixed transcriptome of *S. exigua* was generated using different tissues under different stress conditions (Pascualb et al., 2012). Partial 5' sequence (Fig. S1) of *SeGPDH1* was obtained from gut transcriptome (contig01119). Partial 5' and 3' sequences (Fig. S1) of *SeGK1* were obtained from hemocyte transcriptome (contig05715) and gut transcriptome (GHYDHZH02C854U, GHYDHZH02DD3DD, GHYDHZH02DM7OZ). These partial sequences were connected by contig sequences from the mixed transcriptome (SeUC41023TC01, SeUC41023TC05).

RT-PCR

To analyze glycerol synthesis by SeGPDH1 and SeGK1, total RNAs were extracted from the whole body of 5th instar larval stages of *S. exigua* with Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. One μg of RNA from the total RNA extract was incubated at 70°C for 3 min, and was then used for constructing cDNA using an RT-mix kit (Intron, Seoul, Korea). The synthesized single stranded cDNA was used as a template for PCR amplification with gene-specific primers for *SeGPDH1* (5'-TGC GTC TGA GGT GGC TGA AG-3' and 5'-ACA GAG GGA ACT TGT TCT CC-3') and *SeGK1* (5'-TAC CTG CCT TCA GTG GTT TG-3' and 5'-ATC GGT CGA CGG CCA TTC TC-3') with 35 cycles under conditions of 1 min at 94°C for denaturation, 10 s at 50°C for annealing, and 40 s at 72°C for extension.

RNA interference (RNAi)

Template DNA was amplified with the following primers that contained T7 RNA polymerase promoter sequence for dsRNA^{SeGPDH1} (5'-TAA TAC GAC TCA CTA TAG GGA GAG TGC GTC TGA GGT GGC TGA AG-3' and 5'-TAA TAC GAC TCA CTA TAG GGA GAG ACA GAG GGA ACT TGT TCT CC-3') and for dsRNA^{SeGK1} (5'-TAA TAC GAC TCA CTA TAG GGA GTA CCT GCC TTC AGT GGT TTG-3' and 5'-TAA TAC GAC TCA CTA TAG GGA GAT CGG TCG ACG GCC ATC TTC-3'). The PCR product was used for preparation of dsRNA using the MEGA Script RNAi kit according to the manufacturer's instructions (Ambion, TX, USA). The synthesized RNAs were annealed at 37°C for 4 h and then left at 70°C for 5 min. Injection of dsRNA^{SeGPDH1} or dsRNA^{SeGK1}was carried out with Metafectene PRO transfection reagent (Biontex, Plannegg, Germany) in a 1:1 volume ratio after incubation for 20 min at 25°C. For dsRNA injection into larval hemocoel, four μL of the dsRNA (150 ng) solution was injected into each fifth instar larva with a Hamilton microsyringe. After 24 h, another injection with the same dose of dsRNA was performed. Knockdown of *SeGK1* or *SeGPDH1* gene expression was evaluated by RT-PCR at selected periods up to 72 h post injection. A viral gene, *CpBV-ORF302*, was used as a negative dsRNA control (Park and Kim, 2010).

Quantitative real-time PCR (qRT-PCR)

qRT-PCR was performed on a Bioneer Exicycle^{TM} using SYBR green chemistry and real-time fluorescence measurements with primers of *SeGK1* or *SeGPDH1*. Template cDNAs were constructed as described above. The reaction mixture (20 µL) consisted of 1 × Greenstar TM PCR master mix, 10 mM $MgCl_2$, each 0.5 µM of forward and reverse primers, and 90 ng of cDNA. The PCR condition began with activation of Hot-start Taq DNA polymerase by heat treatment at 95 °C for 10 min and was followed by 40 cycles of 15 s at 95 °C, 30 s at 50 °C, and 40 s at 72 °C with a final extension for 10 min at 72 °C. β-Actin was used as an internal control in each sample for equivalent of template and was amplified with actin primer as mentioned above. Fluorescence values were measured and amplification plots were generated in real time by an Exicycler TM program. Quantitative analysis followed a comparative C_T ($\Delta\Delta C_T$) method (Livak and Schimittgen, 2001).

Ligation analysis of RCH

Fifth instar larvae of *S. exigua* were ligated between second and third abdominal segments by a thread (Fig. 6A). Ligation at the middle of the whole body divided it into two parts as anterior part ('AP') and posterior part ('PP'). Ligated larvae were exposure at 4°C for 6 h as RCH treatment, and controls were exposure at 25°C for 6 h. After RCH, hemolymph was bled and extracted from each AP or PP into a tube, which contained a pinch of PTU. The collected hemolymph was analyzed by HPLC after cleaning, as described above.

Data analysis

All bioassays were performed in three independent replicates. Means and variances of treatments were analyzed in one-way ANOVA by PROC GLM of SAS program (SAS Institute, 1989). The means were compared by the least squared difference (LSD) test at Type I error = 0.05.

RESULTS

Effect of RCH on survival of all developmental stages of *S. exigua* to cold shock

All developmental stages of *S. exigua* were highly susceptible to 1 h exposure to -10^{o}C (Fig. 1). However, a pretreatment with 4^{o}C for 6 h significantly increased the survival in all

194 developmental stages (Fig. 1A). This RCH effect was dependent on the pre-exposure period (Fig.
195 1B). At the fifth instar, the effective RCH required at least 4 h pre-exposure to 4^{o}C and exhibited
196 a maximal level at more than 6 h pre-exposure. Subsequent RCH experiments used pre-exposure
197 at 4^{o}C for 6 h.
198
199 **Enhancing supercooling capacity by RCH**
200 Supercooling is required for overwintering of *S. exigua* to avoid body fluid freezing (Kim and
201 Kim, 1997). The effect of RCH on SCP was investigated in larval stages (Table 1). Both third
202 and fifth instar larvae exhibited SCPs at temperatures below -10^{o}C. RCH treatment did not
203 increase the supercooling capacity in the third instar, but significantly lowered SCPs of the fifth
204 instar larvae.
205
206 **Glycerol content in plasma increases in response to RCH**
207 The fifth instar larvae exhibited a significant increase of survival at -10^{o}C for 1 h and enhanced
208 supercooling capacity in response to RCH treatment. Thus, the cryoprotectant(s) were
209 investigated in the fifth instar after RCH treatment (Fig. 2). Nine carbohydrates and polyols were
210 separated using HPLC (Fig. 2A). When plasma samples were analyzed in this HPLC system,
211 more than 10 peaks were detected. Among these peaks, six peaks were matched to the retention
212 times of the standards (Fig. 2B). Without RCH treatment, the main peak was trehalose. Based on
213 the retention times, glycerol, sorbitol, mannitol, glucose, and galactose were detected. RCH
214 treatment altered the control chromatogram. Increasing the RCH treatment period resulted in an
215 apparent increase of glycerol content. When two polols and trehalose were quantitatively
216 analyzed (Table 2), glycerol content significantly increased from 0.7 mM to 4.0 mM. Sorbitol,
217 another polyol, did not show significant change, maintaining 0.5-0.8 mM. Trehalose content
218 exhibited somewhat fluctuation, but did not reflect any linear association with RCH by
219 maintaining 5.2 - 11.1 mM.
220
221 **Expression profile of *GPDH* and *GK* in *S. exigua***
222 To explain the increase of glycerol content, enzymes associated with glycerol biosynthesis were
223 predicted (Fig. 3A). From glycolysis intermediates, dihydroxyacetone-3-phosphate (DHAP) was

chosen as a precursor of glycerol biosynthesis. DHAP would be changed into glycerol with catalytic activities of glyceraldehyde-3-phosphate dehydrogenase (GPDH) and glycerol kinase (GK). Three different transcriptomes of *S. exigua* were used to determine full ORFs of *GPDH* (*SeGPDH1*) and *GK* (*SeGK1*) of *S. exigua*. The resulting cDNA sequences were deposited to GenBank with accession numbers of KF170736 for SeGPDH1 and KF170737 for SeGK1. ORFs of *SeGPDH1* and *SeGK1* encode 356 and 514 amino acid residues, respectively. The amino acid sequences predicted from *S. exigua* shared high sequence homologies with GPDH and GK sequences of *Bombyx mori*, respectively (data not chown). Gene expressions of *SeGPDH1* and *SeGK1*, which were predicted to be involved in the synthesis of glycerol as an important cryoprotectant in insects under severe cold temperatures, were analyzed by RT-PCR in different developmental stages and tissues from fifth instar larvae of *S. exigua*. Both *SeGPDH1* and *SeGK1* genes were expressed in all developmental stages, with slight expression at egg stage and in different tissues, such as gut, fat body, hemocyte, nerve, and salivary gland from fifth instar larvae (Fig. 3B).

RNAi of *SeGK1* and *SeGPDH1* gene expression reduced RCH effect

Since *SeGPDH1* and *SeGK1* genes were expressed in *S. exigua*, the significance of those genes was tested by assessing the survival of the RNAi-treated larvae to cold shock. Using a specific dsRNA against *SeGPDH1* or *SeGK1*, their physiological functions in subsequent developments were assessed. The RNAi used dsRNA specific to *SeGPDH1* (dsRNA^{SeGPDH1}) or *SeGK1* (dsRNA^{SeGK1}). When the dsRNA ^{SeGPDH1} or dsRNA^{SeGK1} was injected to the fifth instar larvae, *SeGPDH1* and *SeGK1* expression levels were significantly decreased from 24 h post-dsRNA injection, and their knockdown in expression levels was maintained until 72 h for both *SeGPDH1* and *SeGK1* (Fig. 4A). Specific suppressions of two gene expressions were confirmed by qRT-PCR (Fig. 4B). Larvae at 48 h post-dsRNA injection did not increase their survival after RCH treatment (Fig. 4C). There was no significant difference in survival between RCH and control treatments after either RNAi of *SeGPDH1* or *SeGK1*.

RNAi of *SeGPDH1* or *SeGK1* gene expression reduced the synthesis of glycerol after RCH

To explain the loss of RCH in RNAi-treated larvae, their plasma contents of glycerol and other cryoprotectants were analyzed (Fig. 5). Larvae at 48 h post-dsRNA injection did not increase glycerol content after RCH treatment. Control larvae exhibited about 360 ng/μL of glycerol content, but the RNAi-treated larvae in both genes had a basal level (60-70 ng/μL) of glycerol (Fig. 5). However, *SeGPDH1* and *SeGK1* gene silencing did not influence on the change of other cryoprotectants, such as sorbitol and trehalose.

Humoral mediation of RCH

A specific change of glycerol, but not other cryoprotectants, raised the possibility of humoral mediation of glycerol biosynthesis. To test this hypothesis, a ligation was performed to separate the hemocoel into two parts ('AP' and 'PP', Fig. 6A). When the glycerol contents were compared in the two parts, only AP plasma significantly increased glycerol content (Fig. 6B). However, there were no differences between two parts in other cryoprotectants.

DISCUSSION

Cold hardiness and diapause are essential components of winter survival for most insects in temperate zones (Denlinger, 1991). Especially, cold hardiness provides tolerance to low temperatures, while diapause allows the overwintering insects to maintain vital bodily functions in harsh environment without feeding. Cold tolerance can be achieved by freeze tolerance withstanding internal ice formation. However, the large majority of terrestrial insects is freeze-susceptible, and should avoid the formation of internal ice formation by enhancing supercooling capacity through the massive production of polyols or other forms of cryoprotectants (Storey and Storey, 2012). *S. exigua* is classified into a freeze-susceptible insect and possesses a supercooling capacity (Kim and Kim, 1997). In this current study, the survival of *S. exigua* at low temperatures was analyzed in RCH, because this species does not have diapause development to be prepared to overcome any upcoming harsh environment. In our previous study, Song et al. (1997) showed that fifth instar *S. exigua* significantly increased survival after 2 h exposure to 5^{o}C. In this current study, *S. exigua* exhibited a significant RCH in all developmental stages, from egg to adult. This RCH is especially crucial in non-diapausing state, to overcome fatal cold shocks by quickly enhancing cold tolerance (Lee et al., 1987). Moreover, along with a deep

supercooling capacity (Kim and Kim, 1997), the RCH of all developmental stages would be significant in *S. exigua* to overwinter in temperate zones, because all developmental stages can be exposed to the low temperatures. On the other hand, the RCH of *Drosophila melanogaster* was decreased with aging in adult stages (Czajka and Lee, 1990). Thus, we analyzed 1-2 day old young adults in this current RCH assay.

The RCH was accompanied by a significant increase of glycerol titers in hemolymph of *S. exigua*. The glycerol titer was dependent on the exposure period at low temperature. Other polyols (sorbitol and mannitol) were also detected and fluctuated, but did not show any correlated increase with exposure period to the low temperature. The hemolymph polyol analysis showed that trehalose was detected as the major blood sugar, at a concentration of 7.52 mM concentration in hemolymph, and showed a slight decrease with exposure to low temperature (5.21 mM after 6 h at 4^{o}C). In general, trehalose titers in insect hemolymph are quite high as a main blood sugar, but diverse among insects from < 0.1 mM to 133 mM (Kramer et al., 1978). Trehalose has been also used as a cryoprotectant in several insects. In codling moth, *Cydia pomonella*, diapause development stimulates a threefold increase of trehalose in its body content, which plays crucial roles in survival at low temperatures by enhancing the supercooling capacity and cold tolerance (Khan et al., 2007). In *S. exigua*, trehalose synthesis and degradation enzymes are reported. Trehalose-6-phosphate synthase catalyzes a critical step for trehalose biosynthesis, and is cloned and expressed in fat body (Tang et al., 2010). Trehalase catalyzes the disaccharide into two glucose monomers. Two types (soluble and membrane-bound) of trehalases are encoded in the *S. exigua* genome, in which a membrane-bound trehalase is characterized in midgut and fat body (Tang et al., 2008). A slight decrease of trehalose titer in the hemolymph of *S. exigua* in this current study may be induced from a shutdown of trehalose biosynthesis from fat body as well as from the continuous usage by peripheral tissues. In the German cockroach, *Blatella germanica*, hypertrehalosemic hormone (HTH) is responsible for the release of trehalose from fat body (Huang and Lee, 2011). In *B. mori*, adipokinetic hormone behaves like HTH to increase trehalose titer (Oda et al., 2000). Under cool temperatures, glycogen catabolism directs a massive production of polyols, in which the synthesis and release of trehalose is likely blocked by turning-off the HTH-like endocrine signal.

The increase of glycerol content was associated with an increase of cold tolerance. Glycerol is by far the most common cryoprotectant due to its high solubility, nontoxicity, and compatibility with biological macromolecules (Yoder et al., 2006). A well-known evidence of its cryoprotectant property is found in the overwintering last instar of the goldenrod gall moth, *Epiblema scudderiana*, which accumulates almost 2 M in midwinter, and supercools to -38^{o}C (Kelleher et al., 1987). Glycerol can be biosynthesized using two biosynthetic pathways via glycolysis and the pentose phosphate cycle (Storey and Storey, 2012). One pathway is the formation from glyceraldehyde-3-phosphate (GAP) (Joanisse and Storey, 1994; Muise and Storey, 1997). GAP is dephosphorylated by glyceraldehyde-3-phosphatase to produce glyceraldehyde, which is then reduced by polyol dehydrogenase (PDH) with a reducing power of NADPH+H^{+}. Another pathway is the glycerol formation from DHAP via GPDH and glycerol-3-phosphatase or GK. ESTs of some lepidopteran species, including *B. mori*, *Manduca sexta*, *Plutella xylostella*, and *S. exigua*, do not contain PDH and glycerol-3-phosphatase. Thus, we tested the glycerol synthesis pathway using GPDH and GK. Both genes were identified in this study. Their expressions were suppressed by specific dsRNAs. These RNAi treatments prevented glycerol accumulation in response to pre-exposure to a low temperature, and inhibited RCH. GPDH is an NAD^{+}-dependent cytosolic enzyme which is present in the tissues of all eukaryotic organisms (Bewley and Cook, 1990). It plays a central role in intermediary metabolism (Park et al., 2001) and especially in the operation of α-glycerophosphate cycle in insect flight muscles (O'Brien and MacIntyre, 1972). In *D. melanogaster*, three isoforms of GPDH are formed by alternative splicing of its eight exons, and exhibit tissue and developmental specificity in their expressions (Wright and Shaw, 1969; Rechsteiner, 1970; von Kalm et al., 1989). In this current study, GPDH exhibited ubiquitous expression in different developmental stages and tissues of *S. exigua*. Isoforms of GPDH remain unknown in *S. exigua*. Considering that the primers analyzed for RT-PCR are located at the 5' region, which is common to all GPDH isozymes in *D. melanogaster*, our expression analysis may be mixed with all expressions of endogenous isozyme genes of *S. exigua*. The RNAi of GPDH must have suppressed all isozyme expressions if *S. exigua* has isoforms. GK catalyzes glycerol phosphorylation for utilization of glycerol (Martinez Agosto and McCabe, 2006; Stanczak et al., 2007). In diapausing *Hyalophora cecropia* eggs, which accumulate glycerol, GK plays a role in diapause termination to convert glycerol to

glycerol-3-phosphate for other intermediary metabolism (Wyatt, 1975). This current study suggested that GK catalyzes the reverse reaction of dephosphorylation of glycerol-3-phosphate to produce glycerol, because RNAi of GK significantly prevented glycerol accumulation in the 4^{o}C pretreatment. In *B. mori*, at least three GK isozymes are reported, in which only one isoform, GK3, appears to be associated with the utilization of glycerol (Kihara et al., 2009). Here, we speculate that GK isomerases in *S. exigua* may have different substrate affinities, and some GK isozymes, including GK, which were analyzed in this study, may have a higher affinity to glycerol-3-phosphate to catalyze its dephosphorylation. Further analysis of GK isozymes is necessary to clarify this speculation.

RCH of *S. exigua* was mediated with humoral factor(s) because the ligation experiment limited the glycerol production in the brain. This result recapitulates a similar experiment performed in RCH assessment of *S. bullata* (Yoder et al., 2006). Li and Denlinger (2008) then analyzed the brain protein profiles after RCH and found that several factors, such as ATP synthase, a small heat shock protein, and a tropomyosin, are highly up-regulated. However, it remains unknown which type of humoral factor(s) mediate the RCH.

This study showed that glycerol is a central cryoprotectant in RCH in *S. exigua*, based on HPLC analysis of glycerol titers in response to pre-exposure to low temperature. This observation was further supported by RNAi of two critical genes associated with glycerol biosynthesis. In addition, this study proposes a novel glycerol production pathway using GPDH and GK during RCH.

ACKNOWLEDGEMENTS

We appreciate the kind donation of cDNA sequences by Dr. Salva Herrero, University of Valencia, Spain) affording us the whole ORF sequences of SeGK1 and SeGPDH1.

COMPETING INTERESTS

No competing interest declared.

FUNDING

This study was carried out with the support of the Cooperative Research Program for Agriculture Science & Technology Development (PJ00941601), of the Rural Development Administration, Republic of Korea.

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Table 1. Changes in supercooling points (SCPs) of *Spodoptera exigua* after a rapid cold hardening (RCH) treatment (4°C for 6 h)

Stage	RCH treatment	N	SCP (°C)[1]
Third instar	No	10	-13.7 ± 0.3[a]
	Yes	10	-14.0 ± 0.4[a]
Fifth instar	No	10	-10.1 ± 0.2[b]
	Yes	10	-14.0 ± 0.2[a]

[1]Different letters following standard deviations indicate significant difference between means in each instar, at Type I error = 0.05 (LSD test).

Table 2. Change in polyol contents in hemolymph of fifth instar *Spodoptera exigua* in response to exposure to 4°C

Exposure period (h)	Glycerol		Sorbitol		Trehalose	
	ng/µL	mM[1]	ng/µL	mM[1]	ng/µL	mM[1]
0	64.7 ± 23.0	0.7 ± 0.2 [a]	145.6 ± 66.5	0.8 ± 0.4 [a]	2845.7 ± 1484.7	7.5 ± 3.9 [ab]
2	35.1 ± 10.5	0.4 ± 0.1 [a]	147.1 ± 72.0	0.8 ± 0.4 [a]	4178.7 ± 928.7	11.1 ± 2.5 [b]
4	114.3 ± 18.5	1.2 ± 0.2 [a]	100.6 ± 50.9	0.6 ± 0.3 [a]	2442.5 ± 683.1	6.5 ± 1.8 [ab]
6	372.7 ± 122.5	4.0 ± 1.3 [b]	83.8 ± 24.7	0.5 ± 0.1 [a]	1969.4 ± 1024.3	5.2 ± 2.7 [a]

[1]Different letters following standard deviations indicate significant difference among means in each polyol and carbohydrate, at Type I error = 0.05 (LSD test).

Figure captions

Fig. 1. Rapid cold hardening (RCH) of *Spodoptera exigua*. Cold tolerance was analyzed by exposure to -10^{o}C for 1 h and expressed as survival rate. (A) RCH induced in all developmental stages by pre-exposure to 4^{o}C for 6 h. 'E', 'L1-L5', 'P', and 'A' represent egg, first-fifth instar larva, pupa, and adult stages, respectively. Each development was replicated three times with 10 individuals per replication. '*' and '**' indicate significant differences between RCH, and no RCH treatments at Type I error = 0.05 and 0.01, respectively. (B) Effect of pre-exposure period on RCH in L5. Each treatment was replicated three times with 10 larvae per replication. Different letters indicate significant differences among means at Type I error = 0.05 (LSD test).

Fig. 2. Increase of glycerol titers in hemolymph of fifth instar *Spodoptera exigua* in response to exposure to 4^{o}C. (A) An HPLC chromatogram separating nine standard carbohydrate and polyols: glycerol (1), sorbitol (2), trehalose (3), mannitol (4), mannose (5), glucose (6), galactose (7), fructose (8), and sucrose (9). Eluent used 400 mM NaOH at a flow rate of 0.4 mL per min. (B) Chromatograms of hemolymph extracted from larvae treated with different exposure periods (0-6 h) at 4^{o}C.

Fig. 3. Gene prediction associated with glycerol biosynthesis *of Spodoptera exigua*. (A) A hypothetical pathway of glycerol biosynthesis. Glucose is catabolized to dihydroxyacetone-3-phosphate (DHAP), which is then reduced into glycerol-3-phosphate (G3P) to form glycerol. (B) Expression profiles of two genes associated with glycerol biosynthesis: glycerol-3-phosphate dehydrogenase (GPDH) and glycerol kinase (GK). Expression of GPDH and GK in different developmental stages. E', 'L1-L5', 'P', and 'A' represent egg, first-fifth instar larva, pupa, and adult stages, respectively. Expression of GPDH and GK in different tissues of fifth instar larvae. Tissues analyzed include gut, fat body ('FB'), hemocyte ('HC'), nerve ('NEV'), and salivary gland ('SG'). Expression of β-actin confirms an integrity of cDNA preparation.

Fig. 4. Influence on rapid cold hardiness (RCH) of fifth instar larvae after RNA interference (RNAi) of two genes associated with glycerol biosynthesis: glycerol-3-phosphate dehydrogenase

(GPDH) and glycerol kinase (GK). RNAi was performed by injection (100 ng per larva) of dsRNAs specific to the genes to fifth instar *Spodoptera exigua*. dsCON represents dsRNA specific to a viral gene, CpBV-ORF302. (A) Suppression of GPDH and GK expressions after their specific dsRNA injection. (B) qRT-PCR to monitor changes of mRNA levels of *SeGPDH1* or *SeGK1* gene after RNAi. β-Actin was used an internal control. Each treatment was replicated three times. Different letters above standard deviation bars indicate significant difference among means at Type I error = 0.05 (LSD test). (C) Suppression of cold tolerance after RNAi treatment of either GPDH or GK. Each treatment was replicated three times with 10 individuals per replication. '**' indicates significant difference between RCH and no RCH treatments at Type I error = 0.05 (LSD test). 'NS' represent no significant difference.

Fig. 5. Suppression of glycerol titers in hemolymph by RNA interference (RNAi) of two genes associated with glycerol biosynthesis: glycerol-3-phosphate dehydrogenase (GPDH) and glycerol kinase (GK). RNAi was performed by injection (100 ng per larva) of dsRNAs specific to the genes to fifth instar *Spodoptera exigua*. dsCON represents dsRNA specific to a viral gene, CpBV-ORF302. (A) Chromatograms of hemolymph extracted from larvae treated with their specific dsRNA injection after exposure at 4^{o}C for 6 h: glycerol (1), sorbitol (2), trehalose (3). (B) Suppression of GPDH and GK expressions after their specific dsRNA injection. Different letters above standard deviation bars indicate significant differences among means at Type I error = 0.05 (LSD test).

Fig. 6. Humoral control of rapid cold hardiness (RCH) in *Spodoptera exigua*. (A) Ligation of a fifth instar larva to seperate anterior part ('AP') and posterior part ('PP'). Ligation was performed between second and third abdominal segments. The ligated larvae were exposed to 4^{o}C for 6 h, and hemolymph samples were obtained from each body part. (B) Comparison of three cryoprotectant titers in each body part with RCH. Different letters above standard deviation bars indicate significant differences among means at Type I error = 0.05 (LSD test).

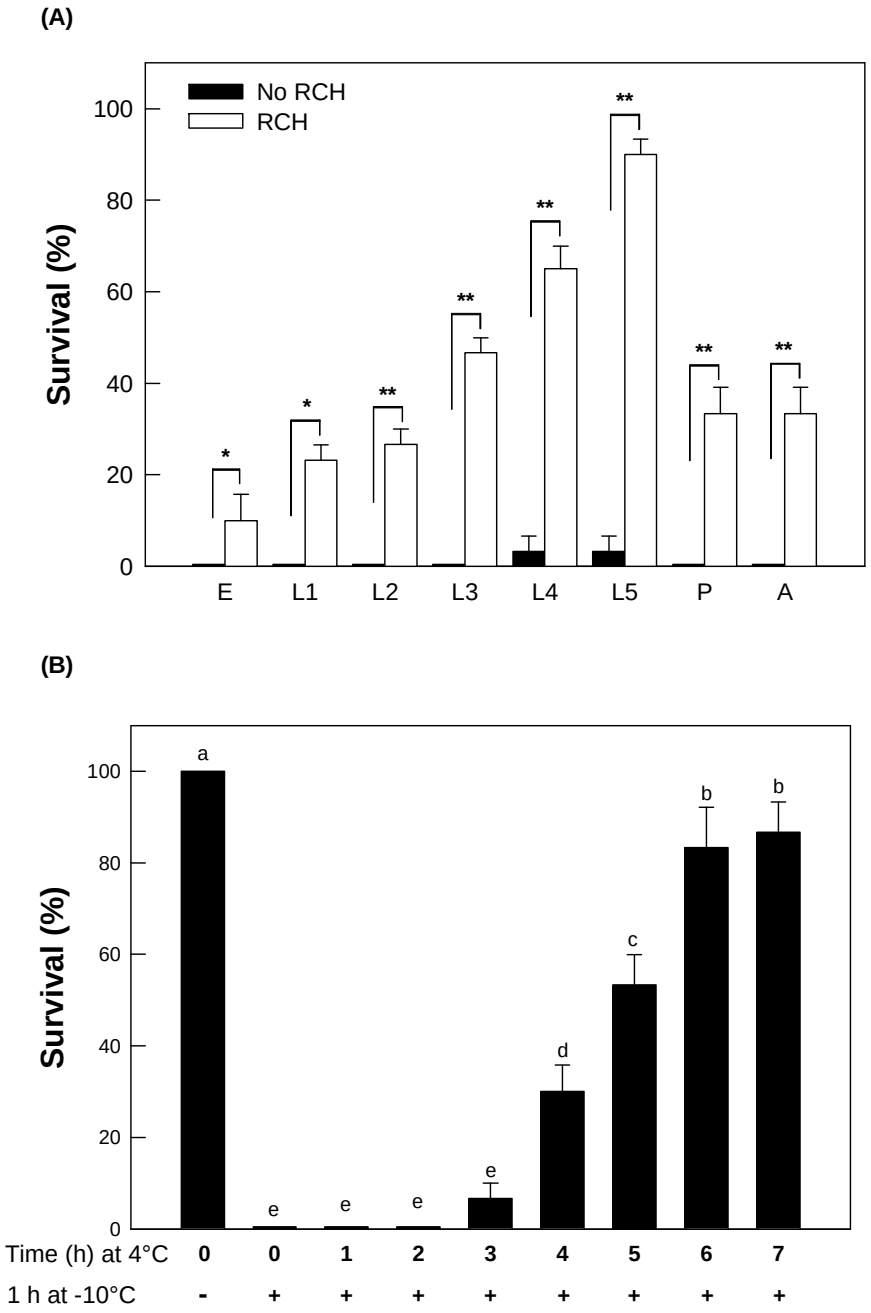


Figure 1

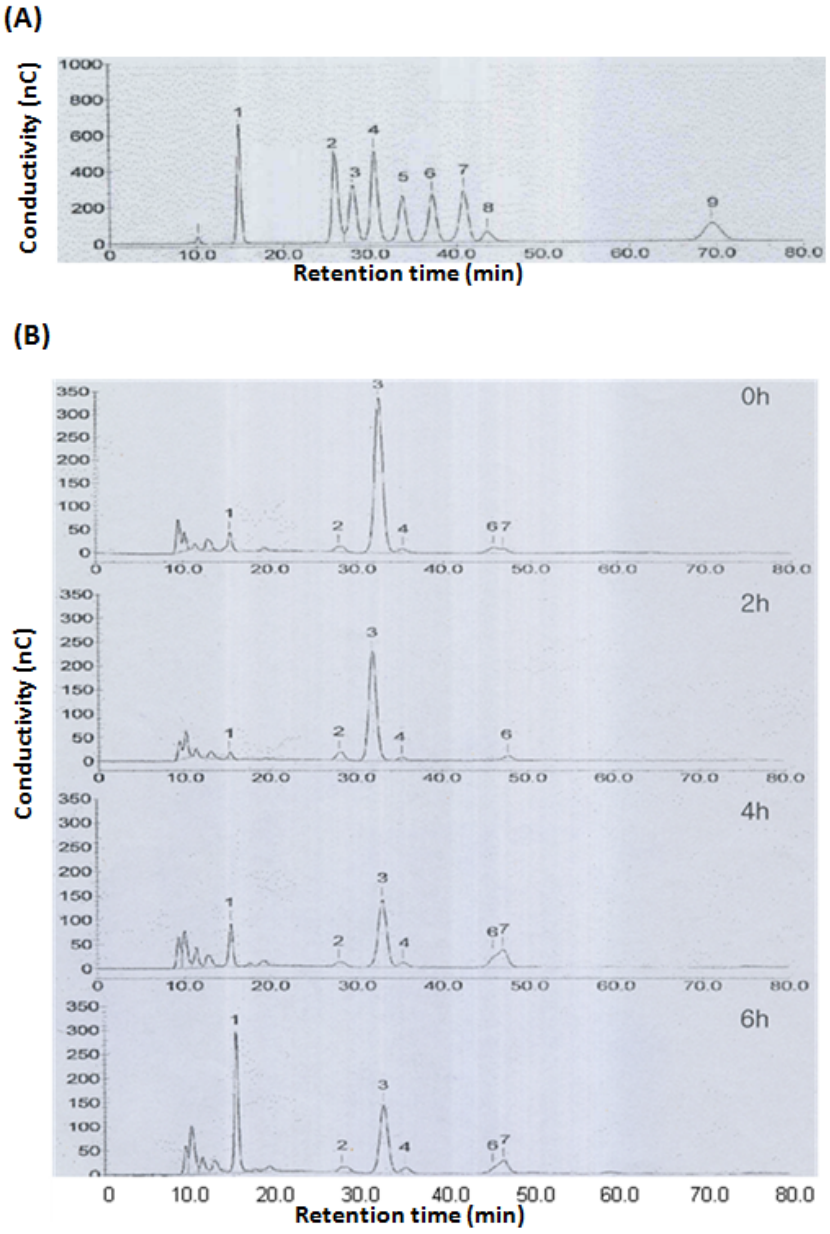


Figure 2

(A)

(B)

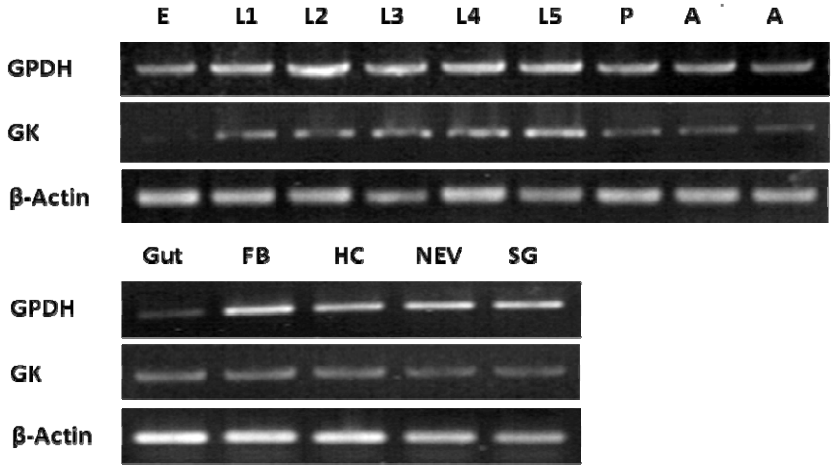
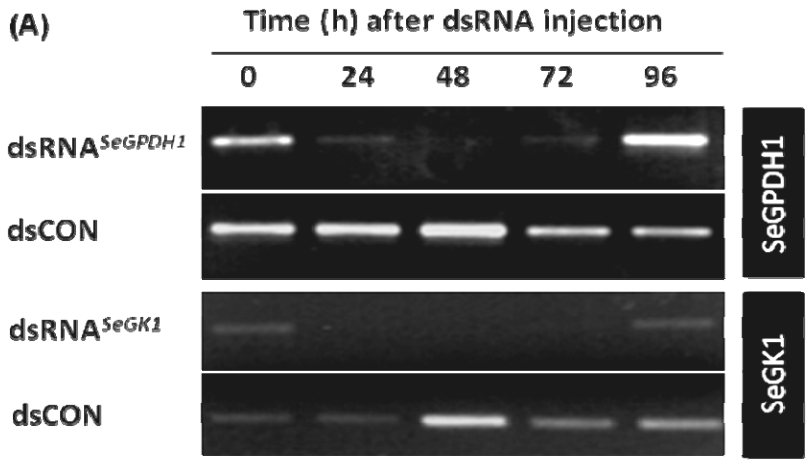


Figure 3



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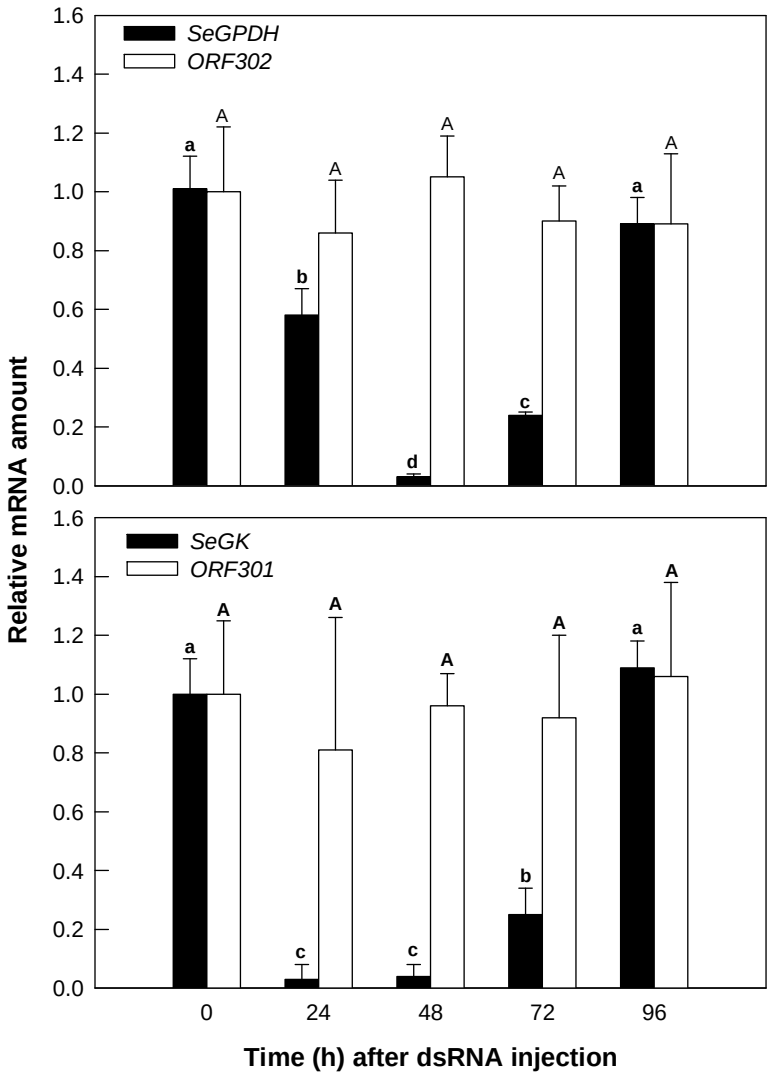

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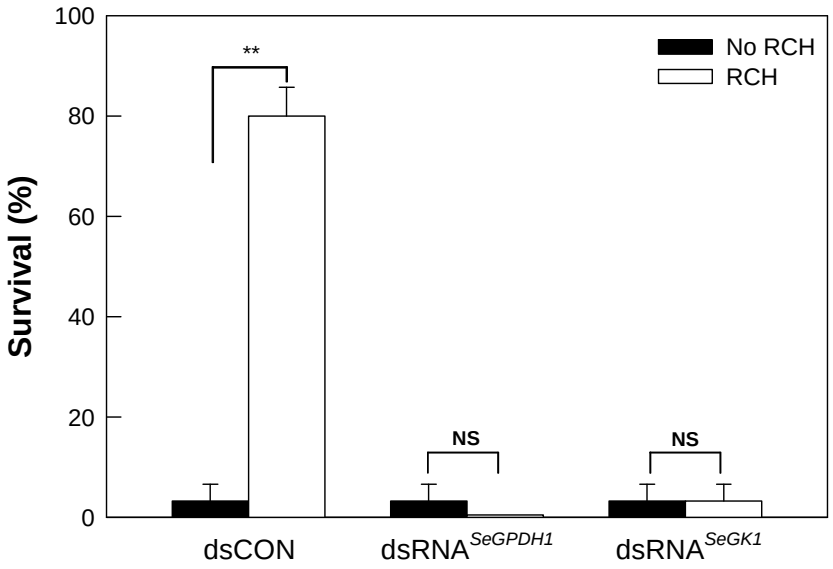

Figure 4

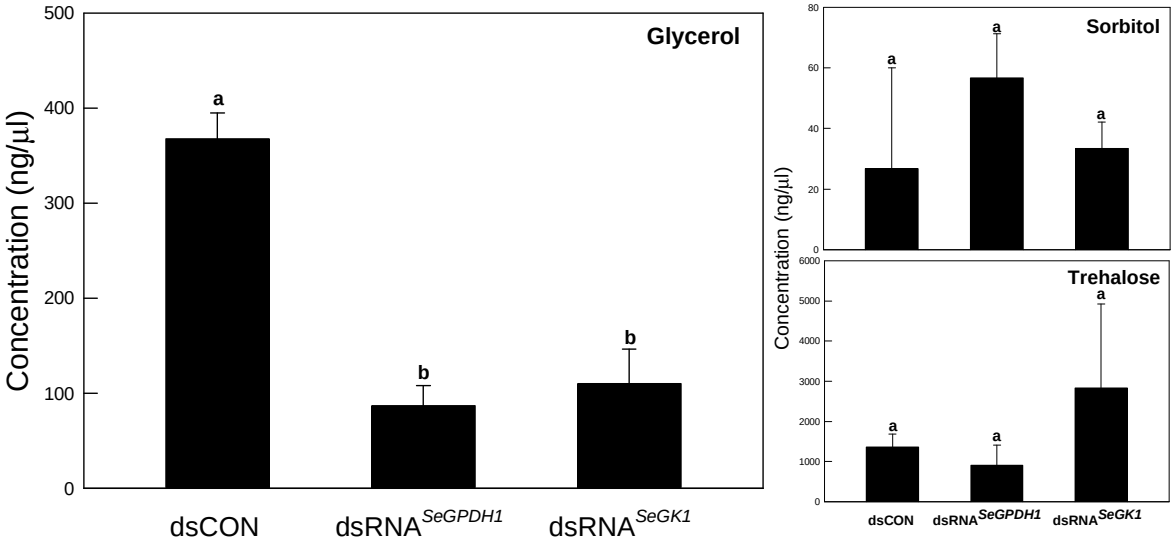

Figure 5

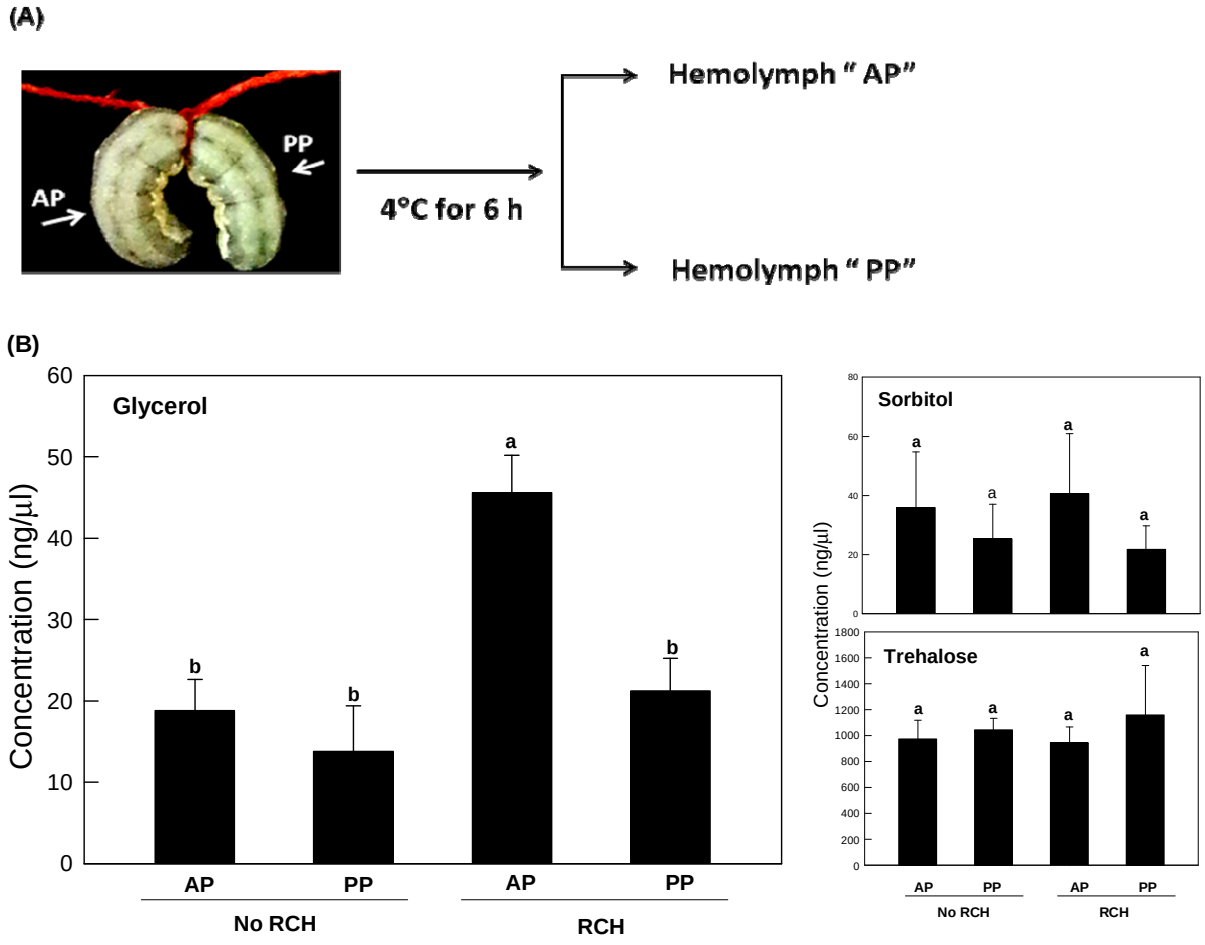

Figure 6