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RESEARCH ARTICLE

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

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SUMMARY

The beet armyworm, *Spodoptera exigua*, is a freeze-susceptible species that overwinters in temperate zones without diapause. A rapid cold hardening (RCH) and supercooling capacity usually play crucial roles in survival during the overwintering period. This study identified a cryoprotectant as a RCH factor of *S. exigua*. Pre-exposure of *S. exigua* 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. 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 from *S. exigua* larvae. Both *GPDH* and *GK* were expressed in all developmental stages of *S. exigua*. RNA interference (RNAi) of either *GPDH* or *GK* significantly inhibited glycerol accumulation in the hemolymph of *S. exigua*. Larvae treated with RNAi for *GPDH* or *GK* exhibited a significant decrease in RCH capacity. The glycerol accumulation in response to 4°C appeared to be under the control of a humoral signal, because a ligation experiment prevented glycerol accumulation in the other half of the body. This study indicates that glycerol is a RCH factor of *S. exigua* and its synthesis is in response to low temperature *via* humoral mediation.

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Key words: cold tolerance, glycerol, RNA interference, GPDH, GK, *Spodoptera exigua*.

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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 (Kelty et al., 1996). Cold injury occurs above freezing temperature, and is induced by impairment of membrane fluidity or proteins (Salt, 1961; Michaud and Denlinger, 2004). Freezing injury occurs directly by the growth of ice crystals, which 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 at low temperatures in all types of cold-survival strategies (Lee et al., 1987). Membrane remodeling to increase fluidity (Overgaard et al., 2005) and chemical changes in hemolymph composition to increase polyols (Michaud and Denlinger, 2007) are associated with RCH. More recently, Li and Denlinger 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 (Li and Denlinger, 2008).

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., 2009). In response to a brief cold exposure, the flesh fly, *Sarcophaga crassipalpis*, increases glycerol and sorbitol levels, as well as free amino acids of alanine and glutamine (Michaud and Denlinger, 2007).

The beet armyworm, *Spodoptera exigua* (Hübner 1808), 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 it to overwinter in some restricted zones (Kim and Kim, 1997). Moreover, RCH helps it to survive at low temperature during winter in temperate zones, and an unidentified protein(s) and glycerol have been proposed to be the factors involved (Song et al., 1997; Kim and Song, 2000).

This study tested the hypothesis that glycerol is the main RCH factor in *S. exigua*. To do so, the carbohydrates and polyols in the hemolymph were quantitatively analyzed by HPLC. To examine 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

Beet armyworm (*S. exigua*) larvae were collected from a field population infesting welsh onions in Andong, Korea. The larvae

were reared on an artificial diet (Goh et al., 1990) at 25°C, a photoperiod of 16 h:8 h (light:dark) and a relative humidity of 60±5%. Adults were fed 10% sucrose solution. All experiments were carried out on 1 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 criterion for being categorized as alive. Egg survival was determined by hatching in the 25°C recovery condition. Pupal survival was determined by adult emergence in the 25°C recovery condition. Each treatment was replicated three times. Each replication used 10 individuals.

SCP measurement

SCPs were measured according to a previous method (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 froze. The specimens were fixed to the thermocouple with a plastic tape; they were then put 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^{-1}.

Polyol analysis using HPLC

Hemolymph from fifth instar *S. exigua* larvae was collected into 1.5 ml tubes containing a small amount of anticoagulant powder [phenylthiourea (PTU), Sigma-Aldrich Korea, Seoul, Korea), and diluted with distilled and deionized water. After centrifugation at 400 ***g*** for 5 min, the supernatant plasma was cleaned with Sep-Pak C18 cartridge (Walters Associates, 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 amount of polyols was 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 mol l^{-1} NaOH at a constant rate of 0.4 ml min^{-1}. The separated samples were detected by an electrochemical detector (ED40, Dionex) in pulse amperometry mode.

Sequence determination of *glycerol-3-phosphate dehydrogenase 1* and *glycerol kinase 1*

Three transcriptomes of *S. exigua* were used to determine both *glycerol-3-phosphate dehydrogenase 1* (*SeGPDH1*) and *glycerol kinase 1* (*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 (Pascual et al., 2012). A partial 5′ sequence (supplementary material Fig. S1) of *SeGPDH1* was obtained from a gut transcriptome (contig01119). Partial 5′ and 3′ sequences (supplementary material Fig. S1) of *SeGK1* were obtained from a hemocyte transcriptome (contig05715) and a 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 RNA was extracted from the whole body of fifth instar larval stages of *S. exigua* with Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. A 1 μg sample 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.

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, 4 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

Quantitative real-time PCR (qRT-PCR) was performed on a Bioneer Exicycle using SYBR green chemistry and real-time fluorescence measurements with primers for *SeGK1* or *SeGPDH1*. Template cDNAs were constructed as described above. The reaction mixture (20 μl) consisted of 1× Greenstar PCR master mix, 10 mmol l^{-1} $MgCl_2$, 0.5 μmol l^{-1} each of forward and reverse primers, and 90 ng 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 an equivalent amount 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 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 the second and third abdominal segments by a thread. Ligation at the middle of the body divided it into two parts: anterior part (AP) and posterior part (PP). Ligated larvae were exposed to 4°C for 6 h as the RCH treatment, and controls were exposed at 25°C for 6 h. After RCH,

hemolymph was extracted from each AP or PP into a tube, which contained a small amount of PTU. The collected hemolymph was cleaned and analyzed by HPLC, as described above.

Data analysis

All bioassays were performed in three independent replicates. Means and variances of treatments were analyzed by one-way ANOVA using PROC GLM (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 cold-shock survival of *S. exigua* at all developmental stages

All developmental stages of *S. exigua* were highly susceptible to 1 h exposure to −10°C (Fig. 1). However, pretreatment at 4°C for 6 h significantly increased survival for all developmental stages (Fig. 1A). This RCH effect was dependent on the pre-exposure period (Fig. 1B). For the fifth instar, the effective RCH required at least 4 h pre-exposure to 4°C and exhibited a maximal level at more than 6 h pre-exposure. Subsequent RCH experiments used pre-exposure at 4°C for 6 h.

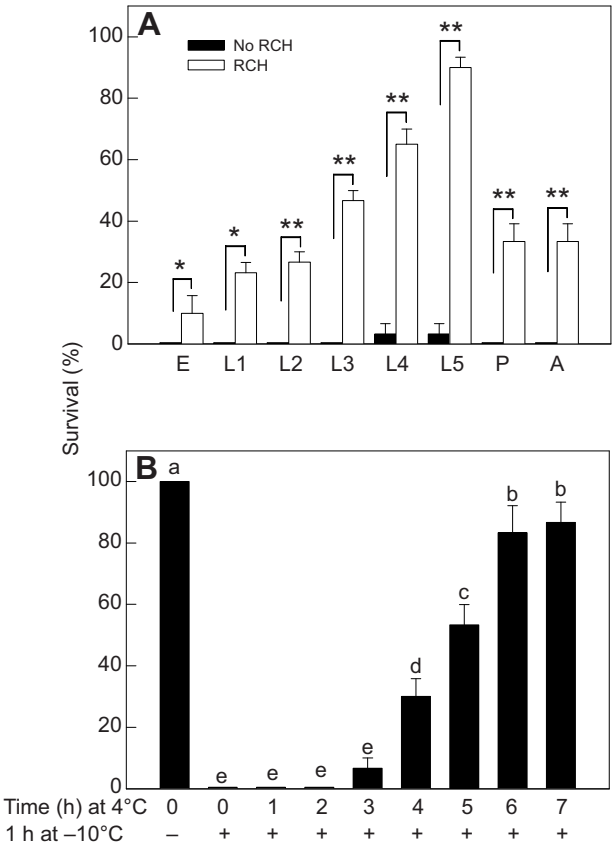

Fig. 1. Rapid cold hardening (RCH) of *Spodoptera exigua*. Cold tolerance was analyzed by exposure to −10°C for 1 h and expressed as survival rate. (A) RCH was induced in all developmental stages by pre-exposure to 4°C for 6 h. E, egg; L1–L5, first–fifth instar larva; P, pupa; and A, adult. Tests at each developmental stage were replicated three times with 10 individuals per replication. Significant differences between RCH and no RCH treatments are indicated (Type I error=*0.05 and **0.01, respectively). (B) Effect of pre-exposure period on RCH in fifth instar larvae. 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).

Table 1. Changes in supercooling points of *Spodoptera exigua* after rapid cold hardening treatment

Stage	RCH treatment	*N*	SCP (°C)
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]

SCP, supercooling point: RCH, rapid cold hardening.
The RCH treatment was exposure to 4°C for 6 h.
Different superscript letters indicate significant differences between means in each instar (Type I error=0.05, LSD test; see Materials and methods).

Enhanced supercooling capacity by RCH

Supercooling is required for overwintering *S. exigua* to avoid freezing of body fluid (Kim and Kim, 1997). The effect of RCH on SCP was investigated in two larval stages (Table 1). Both third and fifth instar larvae exhibited SCPs at temperatures below −10°C. RCH treatment did not increase the supercooling capacity of the third instar, but significantly lowered SCP in fifth instar larvae.

Glycerol content in plasma increases in response to RCH

The fifth instar larvae exhibited a significant increase of survival at −10°C for 1 h and enhanced supercooling capacity in response to RCH treatment. Thus, the cryoprotectant(s) was investigated in fifth instar larvae after RCH treatment (Fig. 2). Nine carbohydrates and polyols were separated using HPLC (Fig. 2A). When plasma samples were analyzed in this HPLC system, more than 10 peaks were detected. Among these peaks, six peaks were matched to the retention times of the standards (Fig. 2B). Without RCH treatment, the main peak was trehalose. Based on the retention times, glycerol, sorbitol, mannitol, glucose and galactose were detected. RCH treatment altered the control chromatogram. Increasing the RCH treatment period resulted in an apparent increase of glycerol content. When two polyols and trehalose were quantitatively analyzed (Table 2), glycerol content significantly increased from 0.7 to 4.0 mmol l^{-1}. Sorbitol, another polyol, did not show a significant change, maintaining 0.5–0.8 mmol l^{-1}. Trehalose content somewhat fluctuated, but did not reflect any linear association with RCH, maintaining 5.2–11.1 mmol l^{-1}.

Expression profile of *GPDH* and *GK* in *S. exigua*

To explain the increase of glycerol content, we tried to determine the enzymes associated with glycerol biosynthesis (Fig. 3A). From glycolysis intermediates, we chose to look at dihydroxyacetone-3-phosphate (DHAP) as a precursor of glycerol biosynthesis. DHAP is converted to glycerol through the catalytic activity of GPDH and GK. Therefore, GPDH and GK genes were predicted to be involved in the synthesis of glycerol as an important cryoprotectant in insects under extremely low temperatures. 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 in GenBank with accession numbers 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* share high sequence homologies with GPDH and GK sequences of *Bombyx mori* (data not shown). The expression of the *SeGPDH1* and *SeGK1* genes was analyzed by RT-PCR at different developmental stages and in different tissues from fifth instar larvae of *S. exigua*. Both *SeGPDH1* and *SeGK1* genes were expressed in all developmental stages, with lower expression at the egg stage, and in different tissues, such as

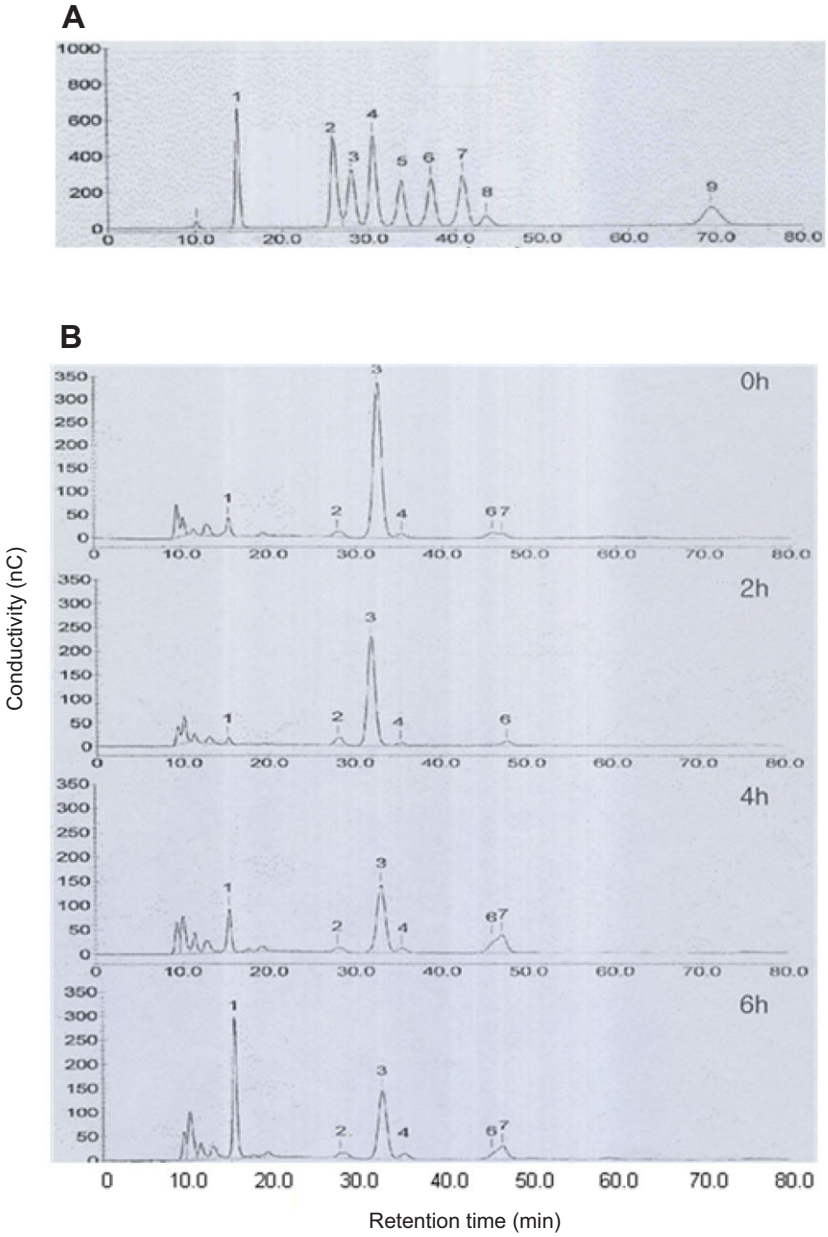

Fig. 2. Increase of glycerol titer in hemolymph of fifth instar *S. exigua* in response to exposure to 4°C. (A) An HPLC chromatogram separating nine standard carbohydrates and polyols: 1, glycerol; 2, sorbitol; 3, trehalose; 4, mannitol; 5, mannose; 6, glucose; 7, galactose; 8, fructose; and 9, sucrose. The eluent was 400 mmol l^{-1} NaOH at a flow rate of 0.4 ml min^{-1}. (B) Chromatograms of hemolymph extracted from larvae exposed to 4°C for different periods (0–6 h).

gut, fat body, hemocyte, nerve and salivary gland from fifth instar larvae (Fig. 3B).

RNAi of *SeGK1* and *SeGPDH1* reduces RCH effect

As *SeGPDH1* and *SeGK1* genes were expressed in *S. exigua*, the significance of these genes was tested by assessing the survival of RNAi-treated larvae in response to cold shock. RNAi was carried out using specific dsRNA against *SeGPDH1* ($dsRNA^{SeGPDH1}$) or *SeGK1* ($dsRNA^{SeGK1}$) to assess their physiological function in subsequent development. When $dsRNA^{SeGPDH1}$ or $dsRNA^{SeGK1}$ was injected into fifth instar larvae, *SeGPDH1* and *SeGK1* expression levels were significantly decreased from 24 h post-injection, and this knockdown was maintained until 72 h for both *SeGPDH1* and *SeGK1* (Fig. 4A). Specific suppression of gene expression was confirmed by qRT-PCR (Fig. 4B). Larvae at 48 h post-dsRNA injection did not increase their survival after RCH treatment

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

Exposure period (h)	Glycerol		Sorbitol		Trehalose	
	(ng μl^{-1})	(mmol l^{-1})	(ng μl^{-1})	(mmol l^{-1})	(ng μl^{-1})	(mmol l^{-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[a,b]
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[a,b]
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]

Different superscript letters indicate significant differences between means for each polyol and carbohydrate (Type I error=0.05, LSD test).

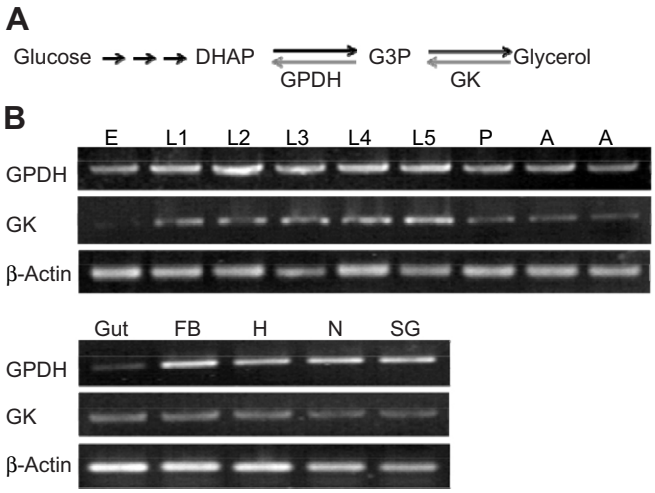

Fig. 3. Gene prediction associated with glycerol biosynthesis *of S. exigua*. (A) A hypothetical pathway of glycerol biosynthesis. Glucose is catabolized to dihydroxyacetone-3-phosphate (DHAP), which is then reduced to glycerol-3-phosphate (G3P) to form glycerol. (B) Expression profiles of two genes associated with glycerol biosynthesis, *glycerol-3-phosphate dehydrogenase* (*GPDH*) and *glycerol kinase* (*GK*), at different developmental stages (top). E, egg; L1–L5, first–fifth instar larva; P, pupa; and A, adult. Expression of *GPDH* and *GK* in different tissues of fifth instar larvae is also shown (bottom). Tissues analyzed include gut, fat body (FB), hemocyte (H), nerve (N) and salivary gland (SG). Expression of β-actin confirms the integrity of the cDNA.

(Fig. 4C). There was no significant difference in survival between RCH and control (no RCH) treatment after RNAi of either *SeGPDH1* or *SeGK1*.

RNAi of *SeGPDH1* or *SeGK1* reduces glycerol synthesis after RCH

To explain the loss of RCH in RNAi-treated larvae, their plasma content of glycerol and other cryoprotectants was analyzed (Fig. 5). Larvae at 48 h post-dsRNA injection did not increase glycerol content after RCH treatment. Control larvae (injected with dsRNA to a viral gene) exhibited about 360 ng μl^{-1} glycerol, but RNAi-treated larvae for both genes had a basal level (60–70 ng μl^{-1}) of glycerol (Fig. 5A). However, *SeGPDH1* and *SeGK1* gene silencing did not influence changes in the level of other cryoprotectants, such as sorbitol and trehalose (Fig. 5B,C).

Humoral mediation of RCH

The 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 showed a significant increase in glycerol content (Fig. 6B). However, there were no differences in other cryoprotectants between the two parts (Fig. 6C,D).

DISCUSSION

Cold hardiness and diapause are essential components of winter survival for most insects in temperate zones (Denlinger, 1991); cold hardiness provides tolerance to low temperatures, while diapause allows the overwintering insects to maintain vital bodily functions in harsh environments without feeding. Cold tolerance can be achieved by freeze tolerance, through the resistance of internal ice

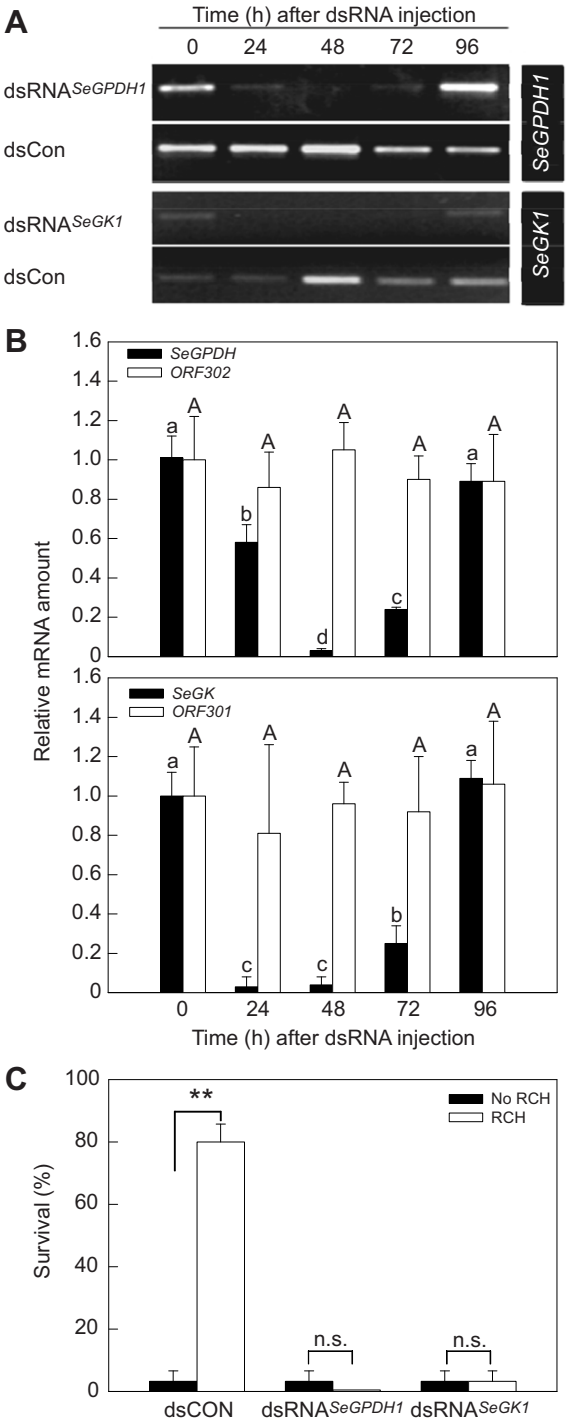

Fig. 4. Influence of RNA interference (RNAi) of two genes associated with glycerol biosynthesis, *GPDH* and *GK*, on RCH of fifth instar *S. exigua* larvae. RNAi was performed by injection (100 ng per larva) of dsRNA specific to these genes into fifth instar larvae. dsCon represents dsRNA specific to a viral gene, *CpBV-ORF302* (control). (A) Suppression of *GPDH* and *GK* expression after specific dsRNA injection. (B) Quantitative real-time PCR (qRT-PCR) to monitor changes in mRNA levels of *SeGPDH1* and *SeGK1* after RNAi. β-Actin was used as an internal control. Each treatment was replicated three times. Different letters indicate significant differences among means (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. **Significant difference between RCH and no RCH treatments (Type I error=0.05, LSD test). n.s., no significant difference.

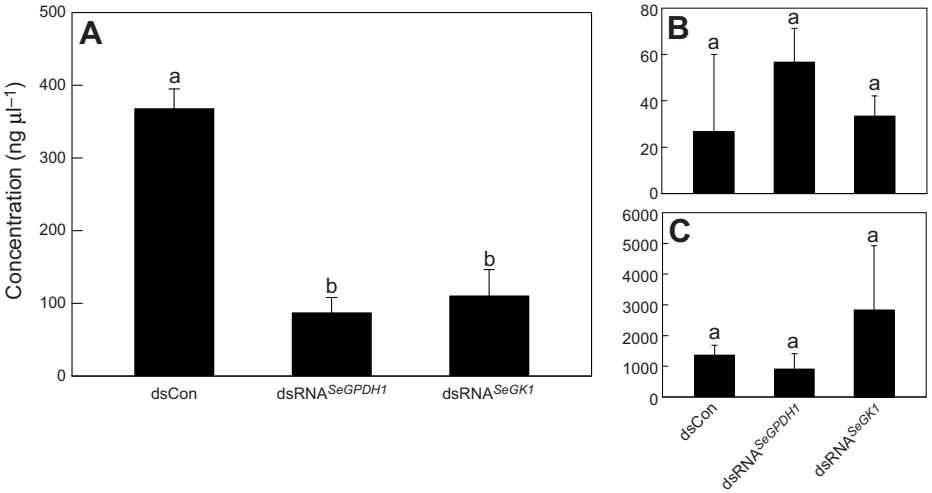

Fig. 5. Suppression of (A) glycerol, (B) sorbitol and (C) trehalose titer in hemolymph by RNAi of *GPDH* and *GK*. RNAi was performed by injection (100 ng per larva) of dsRNA specific to the genes into fifth instar larvae. dsCon represents injection with dsRNA specific to a viral gene, *CpBV-ORF302*. Different letters indicate significant differences among means (Type I error=0.05, LSD test).

formation. However, the large majority of terrestrial insects are freeze susceptible, and therefore should avoid the formation of internal ice by enhancing supercooling capacity through the massive production of polyols or other forms of cryoprotectants (Storey and Storey, 2012). *Spodoptera exigua* is classified as a freeze-susceptible insect and possesses a supercooling capacity (Kim and Kim, 1997). In the current study, the survival of *S. exigua* at low temperatures was analyzed by RCH, because this species does not have the capacity to enter diapause to overcome any upcoming harsh environment. In our previous study (Song et al., 1997), we showed that fifth instar *S. exigua* significantly increased survival after 2 h exposure to 5°C. In the current study, *S. exigua* exhibited a significant RCH in all developmental stages, from egg to adult. This RCH is especially crucial in the non-diapausing state to overcome fatal cold shock 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 play a significant role in overwintering of *S. exigua* in temperate zones, because all developmental stages can be exposed to the low temperatures. In contrast, 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 the current RCH assay.

RCH was accompanied by a significant increase of glycerol titers in the 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 their levels 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 mmol l^{-1} in hemolymph, and showed a slight decrease with exposure to low temperature (5.21 mmol l^{-1} after 6 h at 4°C). In general, trehalose titers in insect hemolymph are quite high as it is the main blood sugar, but are diverse among insects, ranging from <0.1 to 133 mmol l^{-1} (Kramer et al., 1978). Trehalose is also used as a cryoprotectant in several insects. In codling moth, *Cydia pomonella*, diapause development stimulates a threefold increase of the body content of trehalose, which plays a crucial role in survival at low temperatures by enhancing supercooling capacity and cold tolerance (Khani et al., 2007). Trehalose synthesis and degradation enzymes have been reported in *S. exigua*. Trehalose-6-phosphate

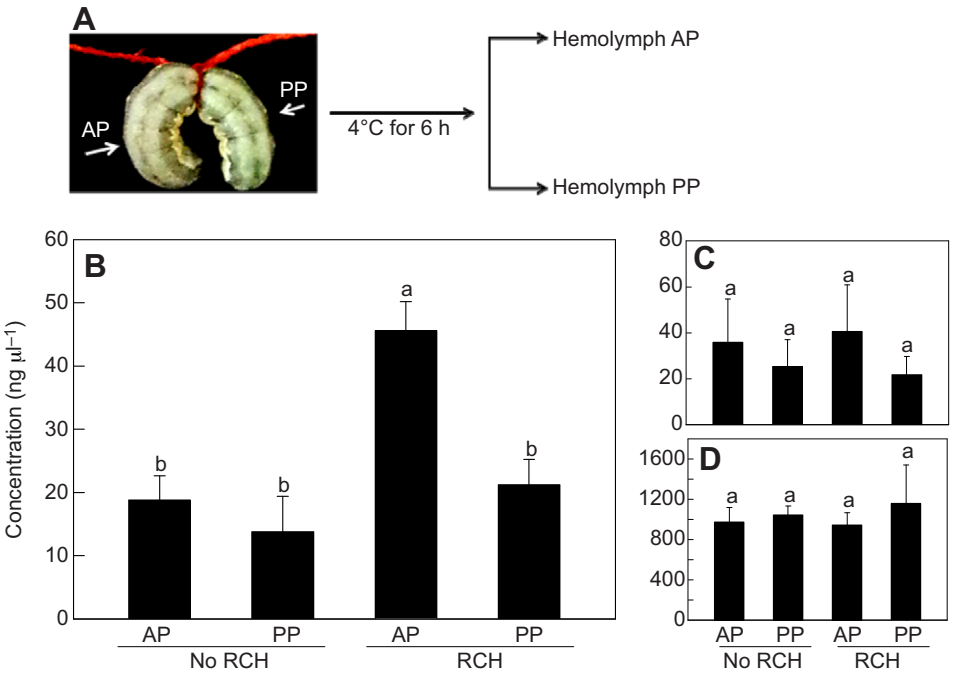

Fig. 6. Humoral control of RCH in *S. exigua*. (A) Ligation of a fifth instar larva between the second and third abdominal segments to create separate anterior (AP) and posterior parts (PP). The ligated larvae were exposed to 4°C for 6 h, and hemolymph samples were obtained from each body part. (B–D) Comparison of (B) glycerol, (C) sorbitol and (D) trehalose titer in each body part with RCH. Different letters indicate significant differences among means (Type I error=0.05, LSD test).

synthase catalyzes a critical step for trehalose biosynthesis, and is cloned from the fat body and its expression has been confirmed there (Tang et al., 2010). Trehalase catalyzes the conversion of the disaccharide trehalose into two glucose monomers. Two types of trehalases (soluble and membrane bound) are encoded in the *S. exigua* genome; the membrane-bound trehalase has been characterized in the midgut and fat body (Tang et al., 2008). The slight decrease of trehalose titer in the hemolymph of *S. exigua* in the current study may have been induced by the shutdown of trehalose biosynthesis in fat body as well as by the continuous usage by peripheral tissues. In the German cockroach, *Blatella germanica*, hypertrehalosemic hormone (HTH) is responsible for the release of trehalose from the 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 the turning off of 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 as a result of its high solubility, non-toxicity, and compatibility with biological macromolecules (Yoder et al., 2006). A well-known example of its cryoprotectant property is found in the overwintering last instar of the goldenrod gall moth, *Epiblema scudderiana*, which accumulates almost 2 mol l^{-1} glycerol in midwinter, and supercools to −38°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 for glycerol formation is that 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 expression was suppressed by specific dsRNAs. This RNAi treatment prevented glycerol accumulation in response to pre-exposure to a low temperature, and inhibited RCH. GPDH is an NAD^+-dependent cytosolic enzyme that 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 the 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 these exhibit tissue and developmental specificity in their expression (Wright and Shaw, 1969; Rechsteiner, 1970; von Kalm et al., 1989). In the 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 contain a mixture of all endogenous isozyme genes expressed in *S. exigua*. The RNAi of *GPDH* must have suppressed all isozyme expression 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 reduced glycerol accumulation in the 4°C pretreatment. In *B. mori*, at least three GK isozymes have been 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 isozymes in *S. exigua* may have different substrate affinities, and some *GK* isozymes, including *SeGK1*, which were analyzed in this study, may have a higher affinity for glycerol-3-phosphate to catalyze its dephosphorylation. Further analysis of *GK* isozymes is necessary to clarify this speculation.

RCH of *S. exigua* was mediated *via* a humoral factor(s) because the ligation experiment limited glycerol production in the brain. This result recapitulates a similar experiment to assess RCH of *S. bullata* (Yoder et al., 2006). Li and Denlinger subsequently 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 upregulated (Li and Denlinger, 2008). However, it remains unknown which type of humoral factor(s) mediates RCH.

In summary, this study has shown that glycerol is a central cryoprotectant in RCH in *S. exigua*, based on HPLC analysis of glycerol titers in response to pre-exposure to a 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.

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AUTHOR CONTRIBUTIONS

Y.P. performed the experiment and prepared the draft manuscript. Y.K. designed the experiment and revised the manuscript for publication.

COMPETING INTERESTS

No competing interests declared.

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[contig05715]

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[contig01119]

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[GHYDHZH02C854U]

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Fig. S1. Contigs and singletons used to construct the full ORF sequences of *SeGPDH1* and *SeGK1*.