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A specific glycerol kinase induces rapid cold hardening of the diamondback moth, *Plutella xylostella*

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ABSTRACT

Insects in temperate zones survive low temperatures by migrating or tolerating the cold. The diamondback moth, *Plutella xylostella*, is a serious insect pest on cabbage and other cruciferous crops worldwide. We showed that *P. xylostella* became cold-tolerant by expressing rapid cold hardiness (RCH) in response to a brief exposure to moderately low temperature (4 °C) for 7 h along with glycerol accumulation in hemolymph. Glycerol played a crucial role in the cold-hardening process because exogenously supplying glycerol significantly increased the cold tolerance of *P. xylostella* larvae without cold acclimation. To determine the genetic factor(s) responsible for RCH and the increase of glycerol, four glycerol kinases (GKs), and glycerol-3-phosphate dehydrogenase (PxGPDH) were predicted from the whole *P. xylostella* genome and analyzed for their function associated with glycerol biosynthesis. All predicted genes were expressed, but differed in their expression during different developmental stages and in different tissues. Expression of the predicted genes was individually suppressed by RNA interference (RNAi) using double-stranded RNAs specific to target genes. RNAi of PxGPDH expression significantly suppressed RCH and glycerol accumulation. Only PxGK1 among the four GKs was responsible for RCH and glycerol accumulation. Furthermore, PxGK1 expression was significantly enhanced during RCH. These results indicate that a specific GK, the terminal enzyme to produce glycerol, is specifically inducible during RCH to accumulate the main cryoprotectant.

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1. Introduction

Insects are the most abundant terrestrial animals particularly in temperate climate zones because they have successfully adapted to low temperatures during winter. Although poikilothermic insects can physiologically adapt to subzero temperatures, low temperature remains a physical barrier for insects to expand their habitats (Denlinger and Lee, 2010). Cold injuries due to low temperature are divided into freezing and nonfreezing injures. Freezing injuries include physical damage of biological membranes and chemical damage by osmolality shock and anoxia, whereas nonfreezing injuries include short-term (minutes to hours) direct chilling injury (=cold shock damage) and long-term (days to months) indirect chilling injury due to damage to biological membrane integrity (Lee and Denlinger, 1985).

Insects have developed major survival strategies to avoid these cold injuries due to low temperatures. The first strategy, migration, is complete avoidance of the temperature range that poses a threat to survival. If an insect cannot migrate, they must stay and deal with the cold temperatures using a second strategy of cold hardiness. Cold hardiness is the capacity of an insect to survive under low temperature and is classified into two categories of freeze-tolerant and freeze-susceptible types (Baust and Rojas, 1985; Zachariassen, 1985; Storey and Storey, 1998; Holmstrup et al., 2002). Freeze-tolerant insects can bear the formation of extracellular ice crystals due to ice-nucleating proteins that raise the supercooling point (SCP; the temperature at which spontaneous ice nucleation occurs, also known as the nucleation or crystallization temperature) of body fluids and serve as catalysts for nucleation of ice to be restricted only in extracellular areas, while low-molecular-mass polyhydroxyl alcohols (polyols) are mainly localized within cells not to be frozen (Lee and Denlinger, 1991; Wilson et al., 2003). In contrast, freeze-susceptible insects produce hemolymph cryoprotectants, such as polyols and sugars that allow them to supercool and remain in a liquid state without ice crystal formation (Salt, 1961; Sømme, 1982; Neven et al., 1986; Nickell et al., 2013).

Low temperature acclimation occurs when insects are first exposed to nonlethal low temperatures, which trigger the accumulation of cryoprotectants (Denlinger and Lee, 1998). Rapid cold hardening (RCH) is accomplished by a brief exposure (within

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minutes to hours) to a moderately low temperature and plays an important role in enhancing survival to lethally low temperature (Chen et al., 1987; Lee et al., 1987; Coulson and Bale, 1992; Kelty and Lee, 1999). Membrane remodeling to increase fluidity (Michaud and Denlinger, 2004; Overgaard et al., 2005) or chemical change in hemolymph composition to increase polyols has been proposed to be associated with RCH (Michaud and Denlinger, 2007).

Polyols belong to a cryoprotectant group synthesized by insects as biochemical adaptation to survive in low temperatures (Storey and Storey, 1991). Both freeze-susceptible and freeze-tolerant species accumulate polyol cryoprotectants. Polyols permit colligative suppression of SCP to prevent body-freezing in freeze-avoiding species, whereas polyols offer a protective barrier against intracellular freezing by restricting ice formation in extracellular compartments in freeze-tolerant species (Storey and Storey, 2012). Various polyols have been reported as cryoprotectants in insects. During RCH, the flesh fly, *Sarcophaga crassipalpis* and the beet armyworm, *Spodoptera exigua*, increase titers of glycerol as a major polyol in their hemolymph (Michaud and Denlinger, 2007; Park and Kim, 2013). The link between RCH and glycerol production suggests a cascade of sensors and effectors in response to RCH conditions. Cold sensing may be centrally coordinated via brain control to initiate glycerol production (Yoder et al., 2006). Alternatively, calcium influx in all tissues exposed to low temperature may autonomously induce RCH (Teets et al., 2013). Nevertheless, the cold signal activates effector(s) to produce glycerol. However, little is understood about the genetic aspects of effector(s) during RCH.

The diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), is a major pest of cabbage and other cruciferous crops worldwide (Talekar and Shelton, 1993; Zalucki et al., 2012; Furlong et al., 2013). The annual cost for managing *P. xylostella* is estimated to be more than one billion US dollars due to their short life cycle and high fecundity (Kim et al., 1999, 2013; Zalucki et al., 2012; Tian et al., 2013). In temperate climate regions, *P. xylostella* overwinter if its host is available (Gu, 2009), suggesting potential for cold tolerance probably through RCH. However, little is known about the cryoprotectant(s) and genetic factors in *P. xylostella*.

In this study, we determined the potential of *P. xylostella* to induce RCH during all developmental stages because they have no designated overwintering stage. We also measured changes in polyol and sugar contents in the hemolymph during RCH. We hypothesized that glycerol is the main RCH factor in *P. xylostella*. To determine the genetic factor that increases glycerol content during RCH, we analyzed cold tolerance after individual RNA interferences of two kinds of genes, glycerol kinases (GKs) and glycerol-3-phosphate dehydrogenase (GPDH), associated with glycerol biosynthesis.

2. Materials and methods

2.1. Insect rearing

P. xylostella larvae were collected from a field population infesting cabbage in Andong, Korea. The larvae were reared on fresh cabbage leaves at 25 °C, a 16:8 h (light:dark) photoperiod, and relative humidity (RH) of 60 ± 5%. Adults were fed 10% sucrose solution.

2.2. Genomic approach to search GK and GPDH genes associated with glycerol biosynthesis

A whole genome sequence of *P. xylostella* was obtained from the DBM-DB, Diamondback Moth Genome Database (www.fafu.edu.cn/xce). Domain structures of glycerol kinase (*PxGK*) and glycerol-3-phosphate dehydrogenase (*PxGPDH*) were probed using SMART (Simple Modular Architecture Research Tool) program server (http://smart.embl-heidelberg.de/smart/set_mode.cgi). Introns of the predicted *PxGK*s and *PxGPDH* were determined in their numbers and locations using ISREC ProfileScan program server (http://myhits.isb-sib.ch/cgi-bin/motif_scan).

2.3. Semi-quantitative and quantitative RT-PCR (qRT-PCR)

To analyze mRNA expression levels of *PxGK* and *PxGPDH*, total RNAs were extracted from whole bodies of fourth instar *P. xylostella* larvae with Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instruction. After digestion of genomic DNA contaminants with a RNase-free DNase (Bioneer, Seoul, Korea), 1.5 μg RNA from the total RNA extract was incubated at 70 °C for 3 min and then used for constructing cDNA using RT-mix kit (Intron, Seoul, Korea). The synthesized single-stranded cDNA was used as a template for PCR amplification with 35 cycles under conditions of 1 min at 95 °C for denaturation, 10 s at 50 °C for annealing, and 40 s at 72 °C for extension with gene-specific primers for *PxGK*s and *PxGPDH* (Table S1).

The mRNA expression levels of the *PxGK* genes were assessed by qRT-PCR. qRT-PCR was performed with an Applied Biosystems 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) using SYBR® Green Realtime PCR master mix (Toyobo, Osaka, Japan) according to the manufacturer's instructions with the gene-specific primers described above. After a hot start at 94 °C for 10 min, qRT-PCR was performed with 40 cycles of 1 min at 95 °C, 20 s at 50 °C, and 40 s at 72 °C. β-Actin gene was used as a control (Table S1) because its expression was not significantly varied during temperature treatment (Choi et al., 2013). Each condition was independently replicated three times. Relative mRNA levels were quantified using the comparative C_T ($\Delta\Delta C_T$) method (Livak and Schmittgen, 2001).

2.4. RNA interference (RNAi)

Template DNA was amplified with gene-specific primers (Table S1) that contained the T7 RNA polymerase promoter sequence for double stranded-RNA specific to GKs (dsGKs) or specific to GPDH (dsGPDH). PCR products were used to prepare dsRNA using the MEGA Script RNAi kit according to the manufacturer's instruction (Ambion, Austin, TX, USA). The synthesized RNAs were annealed at 37 °C for 4 h and then left at 70 °C for 5 min. The dsRNAs were mixed with Metafectene PRO transfection reagent (Biontex, Plannegg, Germany) in a 1:1 (v:v) ratio after a 20 min incubation at 25 °C. Cabbage (1 × 1 cm) was used as a carrier for the dsRNA feeding assay by applying 4 μL of the dsRNA (150 ng) solution on a fresh cabbage fragment. Third instar larvae starved for 6 h were allowed to feed for 8 h on the cabbage treated with dsRNA at 25 °C and thereafter the larvae were supplied with fresh cabbage leaves to continue development. Knockdown of *PxGK* or *PxGPDH* gene expression was evaluated by RT-PCR at selected periods up to 96 h post feeding dsRNA. The CpBV-ORF302 viral gene was used as a negative dsRNA control (Park and Kim, 2010).

2.5. RCH bioassay

All *P. xylostella* developmental stages were analyzed in RCH treatments. Test individuals were divided into three groups: control (exposed to 4 °C for 7 h at 55 ± 15% RH), cold-shock (directly transferred to −10 °C for 1 h), and RCH (exposed to 4 °C for 7 h prior to −10 °C for 1 h). Effect of pre-exposure periods (0, 1, 2, 4, 6, 7, and 8 h) on RCH (exposed to 4 °C) was analyzed in fourth instar *P. xylostella* larvae. Test individuals in each treatment group were placed in a Petri dish (10 × 15 mm). After a cold shock treatment, the survival rates of all developmental stages were

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