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# Identification of the genes which related cold (low temperature) stress in *Bombyx mori*

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## Abstract

Stress may be defined as any modification of environmental parameters that leads to a response by biological organisms. Stresses that affect biolpgical structures may be nonthermal, such as ultraviolet radiation, pH, or salinity, or thermal. Temperture is one of the major stresses that all living organism face. The major effects of cold(low emperature) are decrease of membrane fluidity and the stabilization of secondary structures of RNA and DNA in the cells, which may effect the efficiency of translation, transcription, and DNA replication. In this study, we focus on discovering the genes that are expressed by the cold(low temperature) stress in the silkworm. In cold(low temperature) stress test, we found 100% survive from cold stress at 0°C up to 12h and –5°C up to 2h, and then, survive rate was rapidly decrease in silkworm. Thereafter two whole genes have selected by SSH(Suppression subtractive hybridization). (GenBank accession : GQ149511, GQ338156)

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## Introduction

It has been argued that class Insecta contains the largest number of species on earth. Among them, the orders Coleoptera (i.e., beetles) and Lepidoptera (i.e., butterflies and moths) show outstanding functional diversity. Insects have a remarkable ability to adjust their physiology in response to daily cycles of temperature change (A. Li and D. L. Denlinger., 2008).

Several studies have uncovered transcriptional responses to reduced temperatures in species that do not show homeostasis at lowered temperatures. Brief exposure to a moderately low temperature promotes survival at an even more severe low temperature: a response referred to as rapid cold hardening (RCH) by Lee *et al* .(1987). These structures have evolved independently in a variety of organisms that may experience supercooling of their body fluids, including many species of spiders and insects (Zachariassen and Husby, 1982; Duman *et al*., 2004; E. Kristiansen *et al*. 2011). Normal functioning in the tissues of insect at lowered temperatures is likely to require a suite of adaptations extending beyond those required for cold(low temperature) resistance.

In the relatively poikilothermal insect, lowering the temperature to a moderate range resulted in the up-regulation of a variety of genes; in each case, these included chaperone genes, which underscore the challenge of maintaining functional

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integrity at reduced temperatures, tissue or temperature regimen (Raymond, J.A and DeVries, A.L., 1977; Takumi K and Yukio I., 2009)

These studies reveal the stress response to low temperature and suggest some avenues by which insect may lessen the limitations of cold(low temperature) on membranes, gene expression and metabolic processes. However, in each case, the species studied are incompletely adapted to cold(low temperatures), tolerating only moderately reduced temperatures and/ or showing a stress response. Thus, screening for genes that are up- or down-regulated in cold(low temperature) silkworm compared with normal silkworm, may offer the possibility of revealing other genes that are regulated seasonally . (Yoriko M. *et al*., 2001; Herve C. *et al*., 2007; Robert C. R. *et al*., 2008; N. Guz *et al*., 2014).

In the present study, suppression subtractive hybridization was employed to identify genes in liver of silkworm that are differentially regulated between cold(low temperature) and warm(normal temperature)(Joseph P. R. *et al*. 2010). In order to increase the understanding of cold(low temperature) acclimation in silkworm, a PCR-select cDNA subtraction method, also known as suppression subtractive hybridization (SSH), was selected to profile genes whose expression increases upon low temperature treatment. In comparison to other techniques, like as DNA microarray technology, this method has the advantage that it allows to find novel genes. We identified a group of novel genes induced by cold(low temperature) stress where the majority of genes shared similarity on the amino acid level with known proteins in other plant species. In addition, further characterization showed that a selected group of genes was induced by abiotic stresses other then cold(low temperature) and by signalling molecules.

## Materials and Methods

### Insect rearing

Silkworm B. mori lavers were obtained from the KNU (Kyungpook National University). Larvae were reared on fresh mulberry leaves, and pupae were incubated at 25$^{o}$C.

### Cold(low temperature) stress and determining the cold coma recovery times

The effects of low temperatures on the survival of B.mori larvae were examined for both male and female larvae. The fifth instars larvae used in this study were JAM123 strain, JAM123 strain. Groups of 2 larvae (20 larvae of each sex, at each temperature, for each date) were placed into wrap. Wrap containing larvae were placed into a programmable test chamber and cooled 0$^{o}$C, -5$^{o}$C. The larvae were held at these temperatures for 24 h then warmed to 25$^{o}$C. Thereafter, the numbers of live and dead larvae were counted. The larvae showing no movement were judged to be dead. A non-parametric test (Mann–-Whitney U Test and Kruskal–-Wallis Test) were used to test for differences in the proportionate mortality of each treatment (Clark MS and Worland MR, 2008).

### SDS-PAGE

Equal amounts of proteins samples (10 μg) were analyzed on SDS-PAGE using 5–12.5% gels according to Laemmli (1970) using Tris-Glycine-SDS as running buffer and run for about 2 h at 100 V (Mini-Protean Tetra Cell 165–8001; Bio-Rad, USA). After staining with Coomassie Brilliant Blue (Serva)

### mRNA isolation and suppressive subtractive hybridization library construction

RNA was prepared from fat bodies (about 100 mg) of 5.5-day-old laver (JAM123) with approximately 10 volumes of TRIzol® reagents (Invitrogen, Cergy Pontoise, France) according to manufacturer's recommendation. mRNA was synthesized according to the method of FastTrack¤ 2.0 mRNA Isolation Kit(Invitrogen, Cergy Pontoise, France). The cDNA subtraction was performed by using a cDNA Subtraction kit (Clontech, Mountain View, CA, USA). ‘‘Tester’’ was the cold stressed fatbody at 0$^{o}$C for 8 h and ‘‘driver’’ was the sample from normal fatbody at 25$^{o}$C. To obtain differentially expressed cDNAs, two rounds of hybridizations were performed, following the manufacturer’s instructions of the cDNA Subtraction kit. The subtracted cDNAs obtained from the second PCR amplification were cloned into pDrive vector (Qiagen GmbH, Hilden, Germany). The subtracted PCR products generated by SSH were cloned into pGEMT

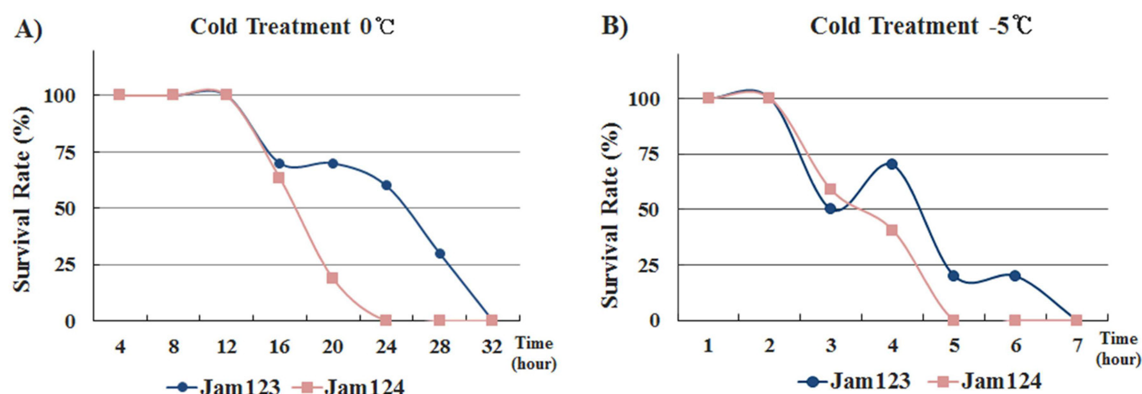



**Fig 1.** In the silkworm breed (5th instar fifth-day) after cold (low temperature) stress controlling the survival rate which follows at time. A) Jam123 and Jam124, 0°C, B) Jam123 and Jam124, -5°C

vector (Promega, Charbonnies, France). A total of 960 clones were selected from the LB plates and grown in liquid LB medium. Bacterial cultures were blotted onto Hybond blotting nylon membrane (Amersham Biosciences, Piscataway, NJ, USA). The membrane was hybridized with double-stranded cDNA pools of equal specific activity derived from the subtracted or un-subtracted tester mRNA in radiation hybridization buffer for 15–8 h at 72°C. DNA hybridizations were hybridized at 65°C during 48 h with radiolabelled probe(Prime-It II Random Primer Labeling Kit (stratagene, USA)). Membranes were washed and then exposed to X-ray films. The signals of corresponding clones from two hybridizations were compared and the positive clones were selected. All the positive clones were sequenced by ABI (ABI, USA).

### Cell viability by MTS assay

The *Bombyx mori* cell line BmE21 was maintained at 26°C in GRACE insect medium supplemented with 10% (v/v) fetal bovine serum (Gibco-BRL). For the MTS assay, the CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (Promega) was used following the manufacturer's instruction. Briefly, at 3 h before each of the desired time points, 10 μL of the MTS reagent was added into each well and cells were incubated at 26°C. The absorbance was detected at 490 nm with a Microplate Reader (TECAN, Switzerland). All the experiment was repeated three times. Blank equivalents with no ascorbic acid were performed in parallel. BmE21 cells were seeded into 96-well plates at a density of 1×104 per well (100 μL) and treated with the following. Wells with serum free medium were used as negative control. The cells were cold(4°C) treated for 1d, 3d, 7d, or 15d.

## Results and Discussion

### Temperature and time for the induction of cold (low temperature) stress

To investigate by cold(low temperature) stress condition for receiving, silkworm breeds JAM123 JAM124 of day 5 of the fifth instar, and female to male 20 0 °C, -5 °C processing. JAM 124 strain 0°C for 3 h, JAM123 strain 0 °C for four h, low temperature stress survival were examined (Fig. 1a). JAM124 for 12 h until a 100% survival, and thereafter at 24 h were all lethal. JAM123 for 12 h showed 100% survival, and 32 h thereafter at all lethal. Two strains -5°C cold stress survival results, after all of 2 h was 100% survival. JAM 124 strain 5 h, 7 h JAM123 strain all mortalities were (Fig. 1b)).

### Silkworm hemolymph protein expression analysis due to cold(low temperature) stress induced

JAM123 in order to investigate the changes of hemolymph protein by the Cold(low temperature) stress treatment and then, the time according to SDS-PAGE analysis (Fig. 2). A result, 8 h after cold-stress handling approximately 45kDa about the size of a specific protein (Fig. 2. arrows) were generated.

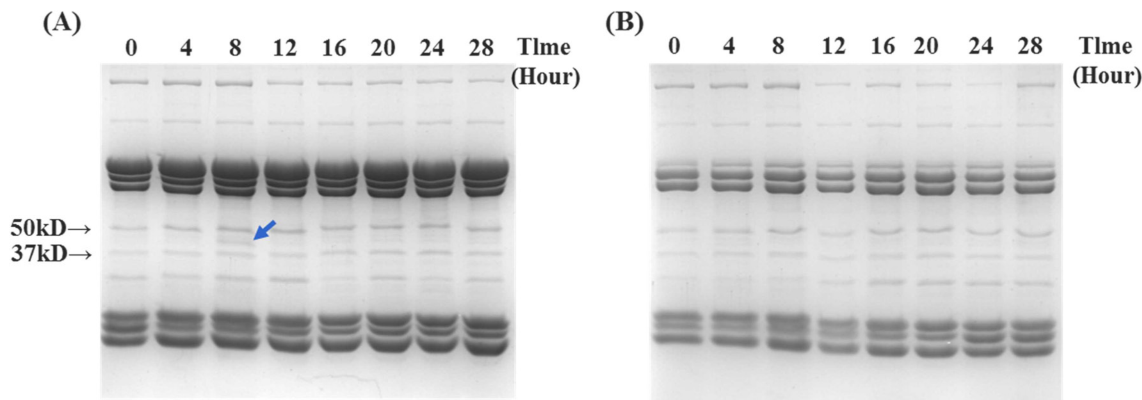



**Fig 2.** Representation analysis of the hemolymph protein which follows at low temperature control time. (Jam123, 5th instar fifth-day). A) female, B) male

## Identification of up-regulated genes cold(low temperature) stress by SSH

SSH was conducted to identify the specific genes that are induced by low temperature. Cold stress treated silkworm fat body (Tester) and untreated silkworm fat body (Driver) from the subtraction cDNA gene bank was constructed(Fig. 3a,b) .

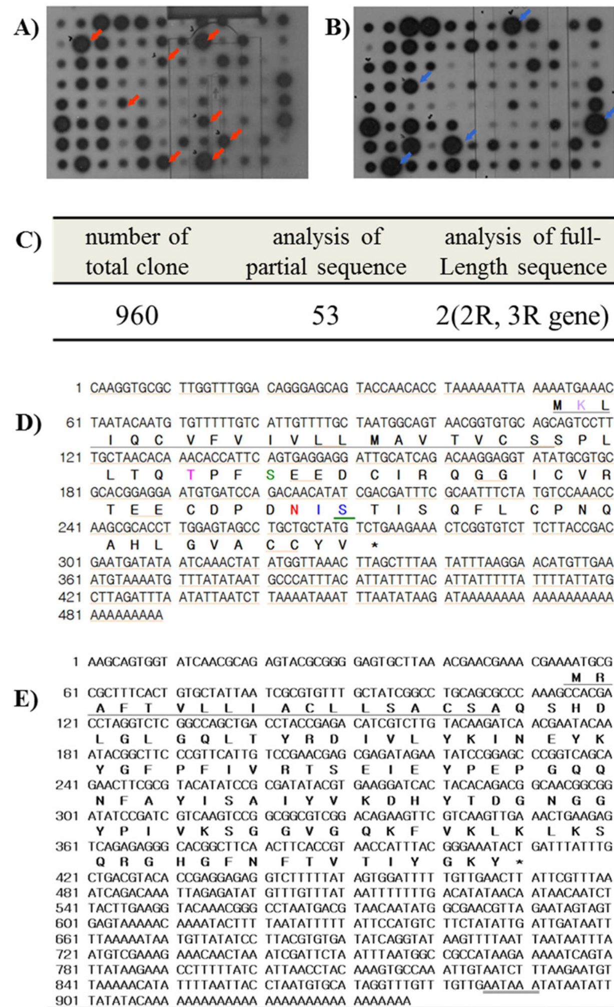
Made Subtraction cDNA genebank from clone 960 were randomly selected. Differentiate screening results, up-regulated 15clone, down-regulated 38clones were selected (Fig. 3c). Cold stress-related selection of these clones partial nucleotide sequence analysis of the BLAST alignments were performed(Fig. 37d,e).

## Identification of the genes which related cold stress, cell viability of tranfected BmE21

Two novel gene cold-vector tranfected genes for insect cells, in order to investigate the related stress with was constructed. Build a vector pIZT+2R+HisTaq and pIZT+3R+HisTaq were tranfected in the Silkworm cultured cells BmE21.

As demonstrated previously, cold-stress treatment significantly improved cell viability in vector pIZT_2R and pIZT_3R gene BmE21 (Fig. 4a). The mean values for cell viability increased by 2-fold in the pIZT_2R, pIZT_3R ,respectively, cold 7d(Fig. 4b).

Stress of various environmental changes cause a variety reaction in vivo, in insects. And, these environmental stresses involve ultraviolet, pH, salinity and temperature. Among them, the genetic research for Heat Shock Protein (HSP) which expressed by high temperature in cell, have been




| number of total clone | analysis of partial sequence | analysis of full-Length sequence |
|---|---|---|
| 960 | 53 | 2(2R, 3R gene) |

**Fig 3.** Suppression subtractive hybridization. Red and black arrows indicate up-regulated clones by the forward and reverse probes, respectively. A) subtraction. Normal silkworm Up-regulated, B) Cold treated silkworms Up-regulated, C) Identification of up-regulated genes cold-stress by SSH, D) 2R-gene full sequence (ACCESSION No. GQ149511), E) 3R-gene full sequence ACCESSION(No. GQ338156)

carried away vividly. This gene has important role in folding involved chaperone gene of protein and Apoptosis in cell (A.Li and D. L.Denlinger, 2008; Yi SX, *et al*., 2007, Qi Xu *et al*., 2011).

Otherwise, low temperature cause some genetic errors in cell that it called Cold Shock Protein (CSP). This CSP has studied in plant, bacteria, fish and partly insects(fruit fly: Drosophila melanogaster, flesh fly: Sarcophaga nodosa), there are deficient report in lepidopteran insects. (B.J. Sinclair *et al*., 2007 ; Goto SG. 2001; Shy-Xia Yi *et al*., 2007), In bacteria, CSP genes are role in secondary structure of RNA and DNA, and it makes stable transfer and translation, efficient DNA replication, reduction of membrane fluidity. Furthermore, it has function of RNA chaperones as stably transcription, translation at low ambient temperature.

Therefore, discovering by the silkworm in the cold(low temperature) stress provides important clue of the homeostatic mechanism of an organism in the utilization and low temperature environment of new genetic resources.

In the present study, we focus on discovering the genes that are expressed by the cold(low temperature) stress in the silkworm. In cold(low temperature) stress test, we found 100% survive from cold stress at $0^{o}C$ up to 12h and $-5^{o}C$ up to 2h, and then, survive rate was rapidly decrease in silkworm(Fig. 1)

JAM123 strain is more strongler than JAM124 strain during cold(low temperature) stress test, there are no differences between male or female(data not shown). In the future, there is a need to cold-sensitive research in a variety of silkworm strains. To investigate difference of cold(low temperature) stress level. we perfoemed SDS-PAGE for specific protein and we deteted a specific protein at $0^{o}C$ up to 8h(Fig. 2).

Thereafter two whole genes have selected by SSH analysis which expressed specific genes constructed cold(low temperature) stress(Fig. 3). To inverstigate the association between two genes and cold(low temperature) stress, we constructed pIZT expression vector that called pIZT+2R/3R+HisTaq for insect cell(Fig. 4).

Transgenic silkworm cell was produced transforming this vector in BmE21 cell. we performed MTS assay for cell viability after cold(low temperature) stress treatment on 7days(Fig. 4). This means both genes are concern with cold(low temperature) stress. In Further study, there are necessary to prove that molecular and physiological characteristics study and to investigate biological function

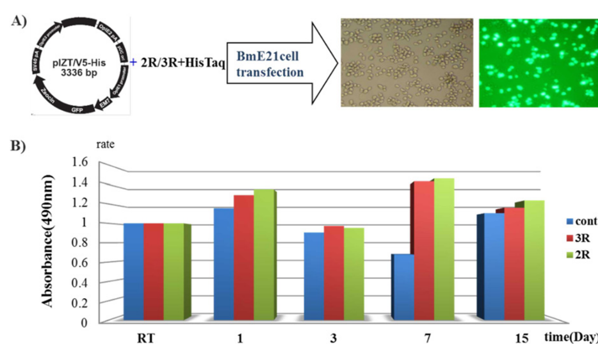



**Fig 4.** Constructed pIZT expression vector that called pIZT+2R/3R+HisTaq for insect cell. Performed MTS assay for cell viability after cold(low temperature) stress treatment at time-course measurements. A) Transfected cell : pIZT+2R/3R+HisTaq, B) MTS assay

research in selected genes. And it would be possible to analyze the basic physiological functions which related with low temperature mechanism in insects.

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