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Research paper

Identification and analysis of up-regulated proteins in *Lissorhoptrus oryzophilus* adults for rapid cold hardening

Shuang Yang, Xinxin Zhang, Jinxin Wang, Shang Wang, Yu Pan, Juhong Zhang, Jinghui Xi*

College of Plant Science, Jilin University, ChangChun 130062, PR China

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ABSTRACT

Rapid cold hardening (RCH) is a central physiological adaptation in which brief chilling significantly enhances the cold tolerance ability of insects. However, the mechanism of rapid cold stress response remains unknown for rice water weevils. To elucidate the mechanism, a comparative proteomic analysis was applied with two-dimensional gel electrophoresis (2-DE) between RCH and control treatment samples. A total of 21 protein spots were found to be up-regulated in rice water weevil adults for the RCH condition. As an initial work, 16 of these proteins were identified using MALDI TOF/TOF MS. In addition, the expression patterns of 9 proteins (including 2 HSPs that were not identified in the 2-DE experiment) were confirmed by qPCR analysis. Using the geneMANIA app in cytoscape, we constructed a protein-protein interaction network analysis of these up-regulated proteins. Gene ontology (GO) function annotation shows that a "response to heat process," including 6 HSPs, was related to the identified proteins. These up-regulated, RCH responsive proteins could possibly serve as potential biomarkers to study the molecular mechanisms of the *L. oryzophilus* response to cold stress.

1. Introduction

Many species of insects die due to low temperature exposures, such as exposure to freezing surroundings or cold shock. Rapid cold hardening (RCH), an adaptation that responds to low temperatures (0–5 °C) within minutes to hours, could decrease insect mortality induced by cold shock (Teets and Denlinger, 2016). RCH is possessed by many insect species, such as *Locusta migratoria*, *Neoseiulus californicus*, *Drosophila melanogaster* and *Plutella xylostella* (Cui et al., 2014; Ghazy and Amano, 2014; Overgaard et al., 2014; Park and Kim, 2014). Insects possess RCH to enhance their cold tolerance within a thermally variable environment throughout their lifespan, which is thought to act as a supplementary mechanism for overwintering and diapause (Michaud and Denlinger, 2006; Powell and Bale, 2004). To counter the injuries caused by cold shock, protective physiological and/or biochemical effects must be gained during the RCH period. Thus, the adjustments associated with RCH are important, but the basic mechanisms remain elusive.

To fully uncover and describe the effects that RCH elicits in insects, different approaches are used in cold related studies. Most recently, systematic and comprehensive technologies, such as proteomic and transcriptomics, have been utilized by researchers to obtain candidate proteins and genes in mRNA levels associated with RCH. Changes induced by the RCH of brain protein profiles in *Sarcophaga crassipalpis* were achieved by taking advantage of 2D gels and LC-MS/MS combination methods, and 14 high abundance proteins related to RCH were identified (Li and Denlinger, 2008). In *Drosophila melanogaster*, two dimension difference gel electrophoresis, (2D-DIGE) proteomics combined with GC/MS were performed to investigate the critical proteins in RCH (Overgaard et al., 2014). RNA-Seq display general mRNA profile changes matching various investigations of large-scale data of ecological and physiological changes that do not require genetic models. A recent research obtained the differential expression profiles of mRNAs between cold-treated and non-treated specimens in a New Zealand alpine stick (Dunning et al., 2013).

Temperature is a critical factor that constrains the geographic distribution and seasonal activity of insects and influences the spread of the invasive pests (Bale and Hayward, 2010). The rice water weevil

Abbreviations: L. oryzophilus, *Lissorhoptrus oryzophilus*; Dmel, *Drosophila melanogaster*; RCH, rapid cold hardening; 2-DE, two-dimensional gel electrophoresis; GO, Gene ontology; 2D-DIGE, two dimension difference gel electrophoresis; RWW, rice water weevil; MALDI-TOF/MS, Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry; CHAPS, 3-[(3-Cholamidopropyl)dimethylammonio]propanesulfonate; DTT, DL-Dithiothreitol; Hsp, Heat shock protein; qPCR, quantitative Real-Time PCR; KEGG, Kyoto Encyclopedia of Genes and Genomes; RP, ribosomal protein; ACD, medium-chain specific acyl-CoA dehydrogenase; ITP, Inositol 1,4,5-trisphosphate receptor; MACF1, Microtubule-actin cross-linking factor 1; ADF, Actin-depolymerizing factor; FACR, fatty acyl-CoA reductase; AK, arginine kinase; MLC2, Myosin regulatory light chain 2; ATPase, ATPase family AAA domain-containing protein 3; dUTPase, deoxyuridine 5′-triphosphate nucleotidohydrolase

* Corresponding author.
E-mail addresses: yangshuang14@mails.jlu.edu.cn (S. Yang), xinxinz16@mails.jlu.edu.cn (X. Zhang), wangshang13@mails.jlu.edu.cn (S. Wang), jhxi1965@jlu.edu.cn (J. Xi).

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(RWW), *Lissorhoptrus oryzophilus* Kuschel (Coleoptera: Curculionidae), is a destructive rice pest that is native to the United States. It has invaded temperate rice growing regions of north eastern Asia and in recent decades its distribution has increased. Its invasive populations are exclusively composed of parthenogenetic female weevils, including those in mainland China (Aghaee and Godfrey, 2014). Biology studies suggest that temperature has an effect on oogenesis (Shi et al., 2007). These traits make the weevil an attractive case study species to researchers studying how temperature impacts the reproduction in RWW between origin and invasive habitats.

This study aims to explore the changes induced by RCH in protein levels across cold-treated and control samples. In this study, 2-DE combined with MALDI-TOF/MS were used to uncover the critical proteins (> 1.5-fold change) that differentially expressed themselves in response to RCH. This study revealed proteins up-regulated in RCH by female adults.

2. Materials and methods

2.1. Insects and rapid cold hardening

L. oryzophilus adult samples were captured from paddy fields located in Changchun city, Jilin province, northeast China (43°88′N, 125°35′E).

2.1.1. Effects of the rapid cold hardening on cold shock

The normal treatment maintained at 25 °C and the RCH treatment expose to 0 °C for 2 h. Each treatment contained 20 adults, placed in one petri dish (5 replicates per treatment) and treated with a cold shock (− 8 °C, 2 h). After a 2 h recovery period at 25 °C, weevils able to move were judged to have survived. All temperature treatments were proceeded in a temperature controllable refrigerator BCD-242S at 40% relative humidity (RH) (Haier, Qingdao, China) in the dark. The experimental protocol described above was shown in the Fig. 1(A).

2.1.2. 2-DE experiment

Normal samples were maintained at 25 °C, while RCH samples were exposed to 0 °C for 2 h. Then all samples were stored at − 80 °C. Each treatment contained 100 samples and was repeated three times for further experiments.

2.2. Two-dimensional gel electrophoresis and image analysis

Protein extraction and 2-DE were performed using the protocol of phenol extraction and methanol/ammonium acetate precipitation described by our lab (Bi et al., 2016). The extraction sample pellet was solubilized with lysis buffer (5 M urea, 2 M thiourea, 2% CHAPS, 20 mM DTT, 3.3 mM Tris-HCl (pH 7.4), 2% IPG buffer, 0.002% bromophenol blue). The protein concentrations were determined prior to electrophoresis analysis using the Bradford method (Bradford, 1976). Dry IPG strips (180 × 3 × 0.5 mm, pH 4–7 linear, GE) were rehydrated for 16 h in a 340-μL lysis buffer containing 300 μg proteins. IEF was performed on the 2-DE system (GE Healthcare, Piscataway, NJ, USA) at 20 °C with a current limit of 50 μA. The second dimension was performed in 1-mm-thick 12.5% (*w/v*) polyacrylamide slab gels. At least three biological replicates were performed for each sample. After 2-DE separation, the proteins on the gel were visualized by silver staining.

Gels were normalized using the total spot density of each gel. The abundance of individual protein spots was determined as percent volume and calculated using the Image Master 2D Platinum software (GE Healthcare, Piscataway, NJ, USA). Spots with fold change > 1.5-fold change of the percent volume values between two samples were chosen. After that, One-way ANOVA analysis results with $p < 0.05$ were considered statistically significant differences. To identify the protein spots, Coomassie Brilliant Blue R-350 staining was applied to the preparing gels.

2.3. Protein identification by mass spectrometry

Protein spots were excised from the preparative gels and destained with 100 mM NH_4HCO_3 in 30% ACN. After removing the destaining buffer, the gel pieces were lyophilized and rehydrated in 30 μL of 50 mM NH_4HCO_3 containing 50 ng trypsin. After overnight digestion at 37 °C, the peptides were extracted three times with 0.1% TFA in 60% ACN. Extracts were pooled and lyophilized. MS spectra were obtained using the 4800 Plus MALDI TOF/TOF™Analyzer (AB SCIEX) operating in a result-dependent acquisition mode. Peptide mass maps were acquired in positive ion reflector mode (2 kV accelerating voltage) with 1000 laser shots per spectrum. Monoisotopic peak masses were automatically determined within the mass range 800–4000 Da with a signal-to-noise ratio minimum set to 10 and a local noise window width of *m/z* 250. In MS/MS-positive ion mode, the spectra were averaged, the collision energy was 2 kV, and the default calibration was set. Monoisotopic peak masses were automatically determined with a signal-to-noise ratio minimum set to 5 and a local noise window width of *m/z* 250. The MS and MS/MS spectra were analyzed using MASCOT (http://www.matrixscience.com) searching the NCBI database (http://www.ncbi.nlm.nih.gov/) with the following parameter as previously reported by our lab (Bi et al., 2016).

2.4. Network construction

The protein-protein interaction networks associated with differentially expressed proteins between RCH and the control treatment were predicted using GeneMANIA with default parameters and algorithms

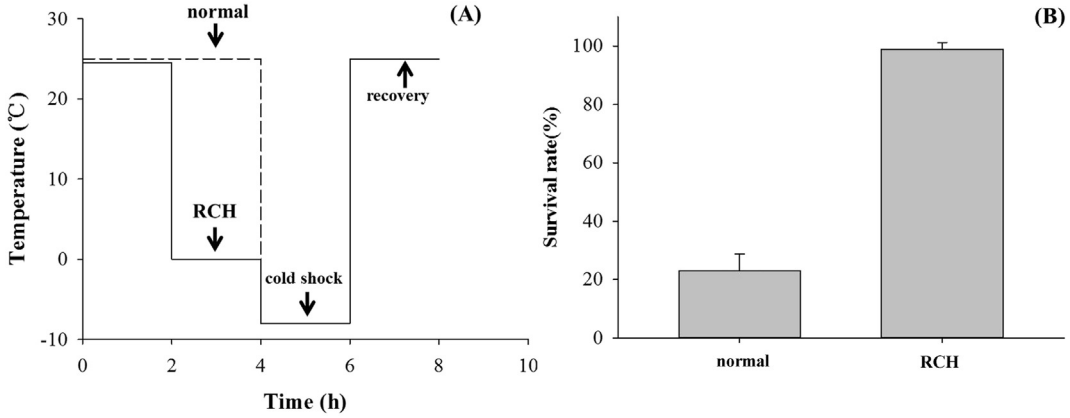

Fig. 1. (A) Experimental protocol of RWW treated with RCH (0 °C, 2 h) or normal samples (25 °C, 2 h) to cold shock (2 h at − 8 °C). (B) Survival rate (mean ± SEM) of RCH and normal samples exposing to cold shock (− 8 °C, 2 h).