Elucidation of mechanisms underlying desiccation tolerance with exhaustive gene analysis

Kyosuke Mukae1,2, Richard Cornette3, Oleg Gusev2, Rie Hatanaka2, Takashi Okuda2, and Takahiro Kikawada2

1Department of Regulatory Biology, Saitama University, 255 Shimo-Okubo, Sakura-ku, Saitama City, Saitama 338-8570, Japan
2Anhydrobiosis Research Group, Insect Mimetics Research Unit, National Institute of Agrobiological Sciences, 1-2 Ohwashi, Tsukuba, Ibaraki 305-8634, Japan

Upon dehydration, the sleeping chironomid (Polypedilum vanderplanki) larvae decrease water content and simultaneously increase trehalose content, to eventually enter into a state of a metabolically suspended animation, i.e., anhydrobiosis, after 48 h of the treatment. Intriguingly, the desiccated larvae can resume the activity once rehydrating. In addition to trehalose content elevation, expression of genes encoding stress-related proteins, such as HSP and LEA proteins, are thought to be deeply involved in desiccation tolerance. However, fine molecular mechanisms underlying anhydrobiosis have remained to be elucidated. Exhaustive gene expression analysis using microarray technique showed that a large number of genes annotated as related to protein degradation mechanisms, including ubiquitin-proteasome system, ER-associated protein degradation and lysosomal hydrolases was upregulated in the larvae upon dehydration. Based on the result, we expected that protein degradation might play an important role to invoke desiccation tolerance.

(Received Aug. 22, 2011; Accepted Sep. 30, 2011)

INTRODUCTION

The sleeping chironomid (Polypedilum vanderplanki) larvae are the largest multicellular animals known to tolerate almost complete desiccation1,2. The larvae inhabit temporary rock pools in the semi-arid regions in African continent. When the rock pools dry up, the larvae undergo complete desiccation but can resume the activity within a few hours once water become available. In the larvae, desiccation induces expression of desiccation tolerance-related genes encoding, for instance, heat shock protein (HSP), late embryogenesis abundant (LEA) proteins, and trehalose metabolic enzymes3-5. Meanwhile, desiccation also causes fragmentation of DNA6 through overproduction of reactive oxygen species (ROS), which may lead to denaturation of proteins. On rehydrated process, damaged DNA is likely to be recovered by several DNA repair enzymes6; however, the fate of unnecessary desiccation tolerance-related and/or denatured proteins still remains obscure. In order to reveal the remaining question of the mechanisms underlying anhydrobiosis of P. vanderplanki, we conducted exhaustive gene expression analysis using microarrays. Consequently, we discuss roles for protein degradation in desiccation tolerance.
MATERIALS AND METHODS

1. Insect rearing

*P. vanderplanki* larvae were collected from rock pools in Nigeria. Larvae were reared for successive generations under controlled light (13 h light: 11 h dark) and temperature (27°C).

2. Dehydration and rehydration of the larvae

Final instar larvae of approximately 1 mg wet body weight were used for all experiments. Larvae were placed on a filter paper with 0.44 ml of distilled water in a glass Petri dish (diameter 65 mm, height 20 mm) and set in a desiccator (20 x 20 x 20 cm) with 1 kg of silica gel. Desiccated larvae were immersed in distilled water. Treatment with dehydration (D) or rehydration (R) was applied for 48 h or 24 h, respectively.

3. RNA isolation

Total RNA from hydrated (D0), dehydrated (D24, D48), and rehydrated (R24) larvae was extracted using RNAsiso (Takara Bio) and the RNeasy Mini Kit (Qiagen). Two replicate samples for each treatment were conducted. The RNA quantity and purity were determined by NanoDrop ND-1000 spectrophotometer at 260/280 nm. The integrity of total RNA was assessed with an Agilent Bioanalyzer 2100 and RNA 6000 Nano LabChip Kit.

4. cRNA preparation

A 400 ng of aliquot of total RNA was reverse transcribed into cDNA using the Agilent’s Quick Amp Labeling Kit. The synthesized cDNA was transcribed into complementary RNA (cRNA) and labeled with Cyanine 3-CTP (10 mM). Labelled cRNA was purified with RNeasy Mini columns. The quality of each cRNA sample was verified by total yield and specificity calculated based on measurement with NanoDrop ND-1000 spectrophotometer.

5. Microarray hybridization

Custom microarrays for *P. vanderplanki* (4 x 44k format) were prepared by Agilent Technologies Japan. Probe design for the microarrays was performed with a web-based application, eArray (Agilent) using 16,652 genes selected from Pv-EST database. Further experiments were carried out according to manufacturer’s manual (Agilent). Briefly, labeled cRNAs were used for hybridization using the in situ hybridization kit plus. Arrays were incubated at 65°C for 17 h in Agilent’s microarray hybridization chamber. After hybridization, arrays were washed according to the Agilent protocol.

6. Image processing and data analysis

Arrays were scanned at 5-μm resolution using DNA Microarray Scanner with the Agilent Scan Control software. The fluorescent intensities of each feature were extracted using the Feature Extraction Software with default parameter. The raw intensity data were normalized using D0 as control sample through the Subio Platform software. Information for the gene classification was retrieved from the KEGG (Kyoto Encyclopedia of Genes and Genomes; http://www.genome.jp/kegg/).

RESULTS AND DISCUSSION

The microarray used here represents a total 16,652 genes. The genes expressed more than 2-fold or less than 0.5-fold were selected as the upregulated or downregulated genes, respectively. Among 16,652 genes, expressions of 9,803 genes in D24, D48 and R24 samples were altered over 2-fold compared with expression levels in D0 sample, indicating that dehydration and rehydration led to dramatic alternation in gene expressions (Fig. 1).

As shown in Venn diagrams (Fig. 2), upregulated or downregulated genes were classified into 14 groups on
the basis of their expression patterns during D24/ D48/ R24 stages of anhydrobiosis. In total 5,143 upregulated and 5,110 downregulated genes were identified by microarray analysis. The D48 sample contained the largest number of genes with altered expression. This result showed that the degree of the alternation for gene expression was rather large on the completely desiccated state.

![Classification of upregulated (A) and downregulated (B) genes identified with the microarray analysis.](image)

Fig. 2. Classification of upregulated (A) and downregulated (B) genes identified with the microarray analysis.

Upregulated genes in D48 sample were classified into ten functional categories. Categories of genes coding stress-related proteins and a number of metabolic systems were upregulated (Fig. 3). It raises the possibility that the metabolic systems such as nucleotide metabolism and transport system related to acquisition of desiccation tolerance. In the larvae, genomic DNA is known to be damaged during desiccation and then repaired upon rehydration\(^5\). Transport system is also likely to play an important role in the desiccation tolerance. For example, trehalose transporter TRET1 governs proper distribution of trehalose as a compatible solute and an anhydro-protectant upon dehydration\(^6\). Upregulation of genes coding nucleotide metabolism and transport system shown on the microarray analysis would provide evidence for a hypothesis that DNA repair upon rehydration and the transport of trehalose is taking place in the larvae.

In this study, we found that the groups of genes related to the protein synthesis/ phosphorylation and protein degradation represent a major part of transcripts with altered expression (Fig. 3). Desiccation tolerance-related proteins such as LEA protein are synthesized de-novo on dehydration process\(^4\), and DNA repair enzymes such as Rad 51 are synthesized de-novo on rehydration\(^5\). The processes of de-novo syntheses are reflected in our observation that the genes coding protein synthesis/ phosphorylation was mostly upregulated (Fig.3).

![Functional classification of genes upregulated in D48.](image)

Fig. 3. Functional classification of genes upregulated in D48.

So far, there have been no reports that protein degradation related to acquisition of desiccation tolerance. We further divided genes related to protein degradation into three groups. These three groups are: I. Endoplasmic Reticulum Associated Protein Degradation (ERAD), II. Ubiquitin-Proteasome system, and III. Lysosome system. Members of group I and II are contributed to selective degradation of proteins\(^9\).

In this study, members of group I and II, including ubiquitin enzyme (E1, E2, E3), 26S proteasome coding genes were upregulated in D48 sample. Members of group III contributed to non-selective degradation of proteins\(^10\) were significantly upregulated in D48 sample. For instance, serine and cysteine protease, and aminopeptidase were comprised in the group III. Taken together, various genes coding protein degradation were induced by dehydration (Fig. 4).
Fig. 4. The number of upregulated genes coding protein degradation.

Summarizing our previous and present results, we suggest that the chironomid larvae need to de-novo synthesize a large variety of proteins in consequence of acquire desiccation tolerance. Losing exogenously nutrient supply on dehydration and rehydration processes, the chironomid larvae would have to degrade existing proteins and then might receive amino acid source from the existing proteins. As another possibility, denatured proteins should accumulate on the both of processes and then be degraded or eliminated. Therefore, protein degradation might have two physiological functions in both dehydration and rehydration processes of the larvae.

Further studies will be required to understand the precise mechanism of how the protein degradation associates with desiccation tolerance.

REFERENCES