Extremophiles (2017) 21:109–120
DOI 10.1007/s00792-016-0888-9

SPECIAL FEATURE: ORIGINAL PAPER

11th International Congress on Extremophiles

Genetic background of enhanced radioresistance in an anhydrobiotic insect: transcriptional response to ionizing radiations and desiccation

Alina Ryabova[1] · Kyosuke Mukae[2,3] · Alexander Cherkasov[1] · Richard Cornette[3] · Elena Shagimardanova[1] · Tetsuya Sakashita[4] · Takashi Okuda[3] · Takahiro Kikawada[3,5] · Oleg Gusev[1,3,6,7]

Received: 12 August 2016 / Accepted: 20 October 2016 / Published online: 2 November 2016

Abstract It is assumed that resistance to ionizing radiation, as well as cross-resistance to other abiotic stresses, is a side effect of the evolutionary-based adaptation of anhydrobiotic animals to dehydration stress. Larvae of *Polypedilum vanderplanki* can withstand prolonged desiccation as well as high doses of ionizing radiation exposure. For a further understanding of the mechanisms of cross-tolerance to both types of stress exposure, we profiled genome-wide mRNA expression patterns using microarray techniques on the chironomid larvae collected at different stages of desiccation and after exposure to two types of ionizing radiation—70 Gy of high-linear energy transfer (LET) ions (^{4}He) and the same dose of low-LET radiation (gamma rays). In expression profiles, a wide transcriptional response to desiccation stress that much exceeded the amount of up-regulated transcripts to irradiation exposure was observed. An extensive group of coincidently up-regulated overlapped transcripts in response to desiccation and ionizing radiation was found. Among this, overlapped set of transcripts was indicated anhydrobiosis-related genes: antioxidants, late embryogenesis abundant (LEA) proteins, and heat-shock proteins. The most overexpressed group was that of protein-L-isoaspartate/D-aspartate *O*-methyltransferase (PIMT), while probes, corresponding to LEA proteins, were the most represented. Performed functional analysis showed strongly enriched gene ontology terms associated with protein methylation. In addition, active processes of DNA repair were detected. We assume that the cross-tolerance of the sleeping chironomid to both desiccation and irradiation exposure comes from a complex mechanism of adaptation to anhydrobiosis.

Keywords Bioinformatics and evolutionary relationship of enzymes · Molecular biology · Radiation tolerance · Anhydrobiosis

Introduction

Among abiotic factors in extreme environments, desiccation is one of the most severe stresses for cells, with a

Communicated by H. Atomi.

This article is part of a special feature based on the 11th International Congress on Extremophiles held in Kyoto, Japan, September 12–16, 2016.

Electronic supplementary material The online version of this article (doi:10.1007/s00792-016-0888-9) contains supplementary material, which is available to authorized users.

✉ Takahiro Kikawada
kikawada@affrc.go.jp

✉ Oleg Gusev
oleg.gusev@riken.jp

[1] Institute of Fundamental Biology and Medicine, Kazan Federal University, Kazan, Russia

[2] Graduate School of Science and Engineering, Saitama University, Saitama, Japan

[3] Anhydrobiosis Research Group, Institute of Agrobiological Sciences, NARO, Tsukuba, Japan

[4] Takasaki Advanced Radiation Research Institute, National Institutes for Quantum and Radiological Science and Technology, Takasaki, Japan

[5] Department of Integrated Biosciences, Graduate School of Frontier Sciences, The University of Tokyo, Kashiwa, Chiba, Japan

[6] Center for Life Science Technologies, RIKEN, Yokohama, Japan

[7] RIKEN Innovation Center, RIKEN, Yokohama, Japan

potentially lethal outcome. Anhydrobiosis is an ametabolic state of an organism, which occurs as a response to extreme water loss (Leprince and Buitink 2015). Desiccation tolerance is common for the majority of plant seeds (Koster and Leopold 1988; Hoekstra et al. 2001a, b), many vegetative tissues of higher plants (resurrection plants) (Hoekstra et al. 2001a; Watanabe 2006; Gechev et al. 2012; Dinakar and Bartels 2013; Griffiths et al. 2014; Challabathula et al. 2015), and microorganisms, for example *Saccharomyces cerevisiae* (Potts et al. 2005; Calahan et al. 2011). It is also observed among several invertebrates, such as tardigrades (Halberg et al. 2013), bdelloid rotifers (Lapinski and Tunnacliffe 2003), nematodes (Shapiro-Ilan et al. 2014), embryos of some crustaceans (Artemia, Triops) (Ito et al. 2013; Hand and Menze 2015), and the largest anhydrobiotic animal known today—larvae of *Polypedilum vanderplanki* (Diptera) or the sleeping chironomid (Hinton 1960; Watanabe 2006). In a dehydrated state, many of these species are able to survive for a quite extended period of time-up to thousands of years for some plant seeds (Shen-Miller et al. 1995; Watanabe 2006). In addition, many anhydrobionts have shown an outstanding endurance to various abiotic stresses. Such cross-reaction manifests between desiccation and other negative external influences—extreme fluctuation of temperatures, hypoxia, hydrostatic pressures, chemicals, vacuum, and different types of ionizing radiation exposure (Crowe and Crowe 1992; Somme 1996; Watanabe 2006; Rebecchi et al. 2007; Gusev et al. 2010, 2014; McGill et al. 2015).

The larvae of *P. vanderplanki* enter into an anhydrobiotic state through an appropriate slow regime of desiccation, which lasts for 2 days or longer, and recover to their normal physiological state within an hour of rehydration (Kikawada et al. 2005; Cornette et al. 2010). The previous studies of high-LET and low-LET irradiation of both wet and dehydrated larvae, apart from obvious enhanced radioresistance compared with other midge species, showed a higher tolerance of completely dehydrated larvae to both types of radiation exposure. Nevertheless, radiotolerance could be enhanced even in wet larvae (Watanabe et al. 2007). It has been suggested that the radioresistance mechanism is associated with an ability to withstand water loss and takes place during both the induction and the recovery phase of anhydrobiosis (Watanabe et al. 2007; Gusev et al. 2010). A similar effect of simultaneous desiccation and radiotolerance is observed among other anhydrobionts (Billi et al. 2000; Cox and Battista 2005; Watanabe 2006; Gladyshev and Meselson 2008; Gusev et al. 2010).

There are three, not mutually exclusive, pathways for explaining the protective mechanisms underlying the successful withstanding of anhydrobiosis. First, the formation of a physiological barrier and creating a glass-like framework by replacing water via the accumulation of non-reducing sugar trehalose directly preserves cellular structure and biomolecules from dehydration damage. Late embryogenesis abundant (LEA) proteins and heat-shock proteins (HSP) act together to prevent protein aggregation and to refold the denatured proteins. Integral membrane proteins—aquaporins—contribute to water loss and accelerate a protective vitrification of cells (Hengherr et al. 2009; Cornette and Kikawada 2011). It is worth noting that not all anhydrobiotic animals use a strategy of accumulating non-reducing disaccharide to avoid water loss damage. In tardigrades and bdelloid rotifers, no increase in the concentration of trehalose during desiccation was detected (Lapinski and Tunnacliffe 2003; Hengherr et al. 2008). Second, drying stress increases the formation of reactive oxygen species (ROS) resulting in lipid peroxidation, denaturation of proteins, and nucleic acid damage with severe consequences on overall metabolism (França et al. 2007). Thus, oxidative stress seems to be the most damage-generating producer for biostructures, so an effective antioxidant system is quite necessary for desiccation tolerance. In extensive studies of antioxidant activity in anhydrobionts, a wide range of ROS scavenging tools was shown, for example enzyme systems, such as peroxidases, superoxide dismutases, catalases, and antioxidants like glutathione, etc. (França et al. 2007; Cornette and Kikawada 2011; Gusev et al. 2014). Third, high performance of nuclear DNA lesions repair might also be responsible for successfully undergoing to an anhydrobiotic state (Gusev et al. 2010).

Like the desiccation process, ionizing radiation affects cells in two ways: by directly injuring cellular components (mostly high-LET irradiation), or indirectly by generating of ROS (mostly low-LET irradiation) (Han and Yu 2010; Gusev et al. 2010). Hence, both hazards cause a similar trend in molecular damage, eventually leading to severe DNA lesions (Mattimore and Battista 1996; Musilova et al. 2015). Therefore, it can be assumed that some part of the protective mechanisms towards anhydrobiosis confers an incidental resistance to radiation (Musilova et al. 2015). This question is much better investigated among prokaryotes, especially in the famous polyextremophile bacterium *Deinococcus radiodurans*, remarkable for its capacity to resist doses of ionizing radiation, which are lethal for most other living creatures, as well as other types of stress exposure (Mattimore and Battista 1996; Tanaka et al. 2004). However, with the increasing complexity of the organism, the cellular machinery conformably becomes more complicated. In metazoa, a more sophisticated genome and repair system is supplemented with cell- and tissue-specific organization (Cromie et al. 2001).

Thus, understanding the mechanism of cross-resistance to anhydrobiosis and irradiation in animals seems to be a more difficult task on the one hand, but more promising on the other hand. The first steps in the clarification of the effects of

anhydrobiosis on radiation tolerance were produced with the sleeping chironomid *P. vanderplanki* by evaluating a short-term larval survival, metamorphosis, and reproduction after high- and low-LET irradiation between dry and hydrated larvae (Watanabe et al. 2006a, b). Later, the structural changes in the nuclear DNA of desiccated and irradiated larvae were analyzed (Gusev et al. 2010). In this study, we focused on alterations in expression patterns of the most renowned stress-resistant groups of enzymes, such as LEA proteins, antioxidants, HSPs, and DNA repair proteins.

Materials and methods

Insect rearing

P. vanderplanki were reared on a 1 % agar diet containing 2 % commercial milk under controlled light (13 h light: 11 h dark) and temperature (27–28 °C) conditions according to the previous report (Watanabe et al. 2002). Final instar larvae of approximately 1 mg wet body weight were used for all experiments. The procedure of desiccation to induce anhydrobiosis is as previously described (Watanabe et al. 2003). For rehydration, dry larvae were placed in dishes with 27–28 °C distilled water. Larvae for the RNA expression analysis were sampled according to the time (in hours) passed from the irradiation, beginning of desiccation and of rehydration, correspondingly.

Irradiation procedures

For gamma rays irradiation, approximately 100 hydrated larvae were placed in a plastic vial (Sumilon MS-4503, Sumitomo Bakelite Co., Tokyo, Japan) with 1 ml water. The samples were irradiated with 70 Gy of gamma rays from a ^{60}Co source at 60 Gy/min; 70 Gy is the half-inhibition gamma-ray dose for adult emergence in hydrated larvae. For helium-ions irradiation, hydrated larvae were placed on the bottom of a plastic Petri dish (diameter 50 mm, height 10 mm). The dish was covered with polyimide film and sealed with Parafilm (Alcan Packaging, Chicago, IL) to avoid drying. The samples were exposed to 70 Gy of a 50 MeV ^{4}He (LET∞ = 16.2 keV/μm) ion beam delivered from the azimuthally varying-field (AVF) cyclotrons at the Takasaki Ion accelerators for Advanced Radiation Application (TIARA) facility of the Japan Atomic Energy Agency (JAEA) (Watanabe et al. 2006a, b). Control samples were fake irradiated and manipulated in parallel with the test samples. Both irradiated and non-irradiated larvae were then reared in distilled water.

The irradiation dose in both cases was equal—70 Gy. At this dose, both types of radiation do not have any significant inhibitory effects on the further development of larvae (Watanabe et al. 2006b). In addition, the DNA fragmentation level in non-irradiated dried larvae after rehydration is analogous to that of wet larvae irradiated with 70 Gy of helium-ions (Gusev et al. 2010). We used helium ions as the source of high-LET radiation and gamma rays for low-LET radiation, because these types of ionizing radiation have a similar relative biological effectiveness (RBE) for *P. vanderplanki* larvae (Watanabe et al. 2006a, b).

Target cDNA for quantification

All clones of target genes used were selected by analysis of the Pv-EST database (Cornette et al. 2010; Kikawada et al. 2006). The full-length cDNAs were subcloned into pCR-4Blunt-TOPO vector (Invitrogen, Carlsbad, CA) and the resulting plasmids were used as templates for the calibration controls of real-time PCR reactions. DNA sequences were analyzed with the Vector NTI 10.3 software (Invitrogen) and CLC Main Workbench 6 (Qiagen Bioinformatics, Aarhus, Denmark).

Quantitative real-time PCR

The qRT-PCR analysis was conducted using RNA samples independent of those samples used in microarray experiments. Total RNA from hydrated, dehydrating, rehydrated, and irradiated larvae was extracted using Trizol (Invitrogen) and the RNeasy Mini Kit (Qiagen, Hilden, Germany), and reverse transcribed using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Basel, Switzerland). Real-time PCR was performed using a CFX96™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA) with SYBR Premix Ex Taq (Tli RNaseH Plus) (TaKaRa, Ohtsu, Japan). Amplifications were performed using 1 × SYBR Premix Ex Taq and 10 pmol of each primer. *P. vanderplanki* EF1-alpha cDNA served as an internal standard for data normalization and quantification. The expression of each gene was tested in triplicate in each of three biologically independent experiments. The cycling conditions were: 30 s activation at 95 °C, 40 cycles of 5 s at 95 °C, and 30 s at 60 °C. Melting curves from 65 to 95 °C, rising by 0.5 °C at each step, and pausing 5 s after each step, and accompanying software, were used for qRT-PCR data normalization and quantification. Used primer pairs are shown in Supplementary material (Table S1).

Microarray analysis

Tissues were homogenized using disposable pestle homogenizers. Total RNA was isolated from each homogenized tissue using Trizol (Invitrogen) and the RNeasy Mini Kit (Qiagen). Two replicate samples for each treatment were applied. The RNA quantity and purity were determined

by NanoDrop ND-1000 spectrophotometer at 260/280 nm (Thermo Fisher Scientific). The integrity of total RNA was assessed with an Agilent Bioanalyzer 2100 and RNA 6000 Nano LabChip Kit (Agilent Technologies).

cRNA preparation

A 400 ng of aliquot of total RNA extracted from desiccating and irradiated larvae 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). Labeled cRNA was purified with RNeasy Mini columns (Qiagen). The quality of each cRNA sample was verified by total yield and specificity calculated based on NanoDrop ND-1000 spectrophotometer measurement (Thermo Fisher Scientific).

Microarray hybridization

Custom microarrays for *P. vanderplanki* (4 × 44 k format) were prepared by Agilent Technologies Japan. Probe design for the microarrays was performed with a Web-based application, eArray (Agilent) using 16652 genes selected from Pv-EST database (Cornette et al. 2010). Further experiments were carried out according to manufacturer's manual (Agilent): labeled cRNAs were used for hybridization via the in situ hybridization kit plus (Agilent Technologies). 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.

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 imported to the Subio Platform (v.1.19) and normalized using turning into log ratio and global normalization. Then, the ratio-to-wet (D-cont.) and non-irradiated samples (G-cont.; He-cont.) as a control were produced to obtain fold change values. Identification of differentially expressed genes was performed using fold change threshold ≥3 and *P* value less than 0.05, obtained by Student *t* test.

Probe set annotation and functional analysis

Corresponding EST-sequences obtained from Pv-EST database were added to BLASTx search against three protein databases (TrEMBL, *Drosophila melanogaster,* and *Caenorhabditis elegans*) with *e* value cutoff 10e-5. Gene ontology (GO) mapping, merging, and summarizing annotations were performed with the Blast2go software (https://www.blast2go.com/). For the GO enrichment analysis, we applied BinGO plugin for Cytoscape platform utilized hypergeometric distribution test with Benjamini and Hochberg correction.

Results

Primary desiccation- and irradiation-responsive gene expression profiling

To perform a comprehensive determination of *P. vanderplanki* stress-driven gene expression, we profiled genome-wide expression patterns in the chironomid larvae collected at different stages of desiccation (D) 24, 48 h, rehydration (R) 24 h, and after 0.5, 3, and 12 h of two types of ionizing radiation exposure—gamma rays (G) and helium-ion irradiation (He).

According to the analysis of differential gene expression, 2222 (13.34 % of the total) probes were significantly up-regulated to at least one kind of stress, and 2617 (15.72 % of the total) probes were significantly down-regulated overall. Desiccation caused the largest changes in the transcription profile of the larvae—1705 (10.24 %) probes were found to be up-regulated, and 2211 (13.28 %) probes were down-regulated, respectively (Fig. 1). In contrast, ionizing radiation showed a lower transcription response. Thus, in response to gamma-ray exposure, 774 (4.65 %) probes were up-regulated and 452 (2.71 %) down-regulated; in helium-ion-irradiated larvae, and 286 probes (1.72 %) were up-regulated and 147 (0.88 %) down-regulated. The

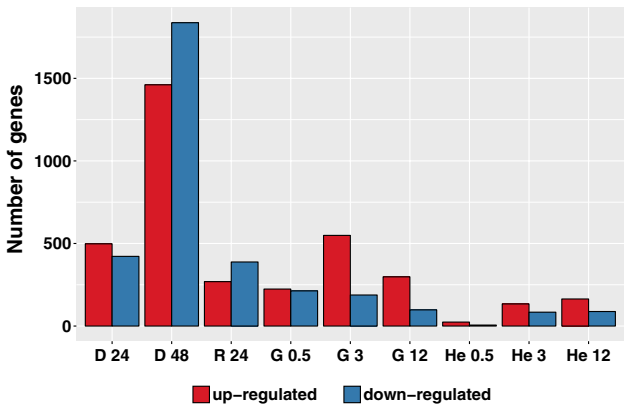

Fig. 1 Overview of differentially (fold change ≥3 compared with control samples) expressed probes among exposure of desiccation–rehydration cycle (*D* and *R*, respectively), gamma (*G*) and helium-ion (*He*) irradiation. *Red bars* present down-regulated transcripts, *blue bars* up-regulated transcripts

prevalence of down-regulated probes was specified only during desiccation stress response in checkpoints D48 and R24.

Remarkably, the overall number of up-regulated probes in D48 is many times more important than in other kinds of stress and time checkpoints. Gene response to gamma-ray irradiation reached the maximum at 3 h and declined, while response to helium-ion irradiation exposure showed a slow ongoing increase. To achieve a summary list of up-regulated expressed sequence tags (EST) of each stress exposure, we combined significant up-regulated probes from time checkpoints to a unified stress set—desiccation (D), gamma rays (G), and helium-ion irradiation (He). The comparison of the probes' expression of stress sets revealed 104 overlapped transcripts induced in response to desiccation and both types of irradiation.

To extend the set of the transcripts with similar expression patterns in different stress conditions and to investigate general trends in the activation template, we have subjected the obtained ESTs database (16,652 probes) to principal component analysis (PCA) (Fig. 2). The analysis showed that the general orientation of the axes was different between tested groups. The sum of the two first components explained the main part of data variability—69.2 %. The transcripts most positively correlated (with PCA scores more than 0.015) with principal component 1 (PC1), which explained 57.1 % of variability and was chosen for further analysis. Among this set of transcripts, the major part (93.21 %) showed a significant up-regulation on at least one-time checkpoint during the desiccation time-course. A proportion of these transcripts, which increase expression level more than three times upon radiation exposure, was less (G 45.9 %; He 19.67 %, respectively), but the general trend in their expression—activating in response to each type of stress, is the same. In contrast, probes positively correlated with the second principal component (explained 12.1 %) showed another expression pattern—decreasing expression in water loss conditions.

To understand the cross-tolerance mechanism between desiccation and radioresistance, the list of overlapped up-regulated transcripts and transcripts obtained from PCA had been sorted according to the estimated function in cell stress response. Apart from unknown genes and transcripts corresponding to different metabolic processes, five groups of the most important anhydrobiosis-related participants, well characterized in the previous papers (Cornette et al. 2010; Gusev et al. 2014), were found: late embryogenesis abundant proteins (LEA), protein-L-isoaspartate/D-aspartate *O*-methyltransferase (PIMT), thioredoxins (TRX), glutathione peroxidases (GPx), and heat-shock proteins (HSP). The most part of these desiccation responsive genes essentially showed an increase of their expression after each type of ionizing radiation exposure. The abundance of these stress-resistance groups is represented in Table 1.

All these groups of transcripts had a sufficient representation in both lists of significant stress-responsive probes, especially in the case of genes encoding LEA proteins, PIMT, and TRX (68.49, 68.18, and 41.46 %, respectively), which could demonstrate an involvement of these groups in the irradiation response processes.

We compared the changes in expression profiles of five stress-responsive groups. Notably, the expression of probes demonstrated mainly similar patterns in all kinds of stress exposure (Fig. 3). The most significant similarity is

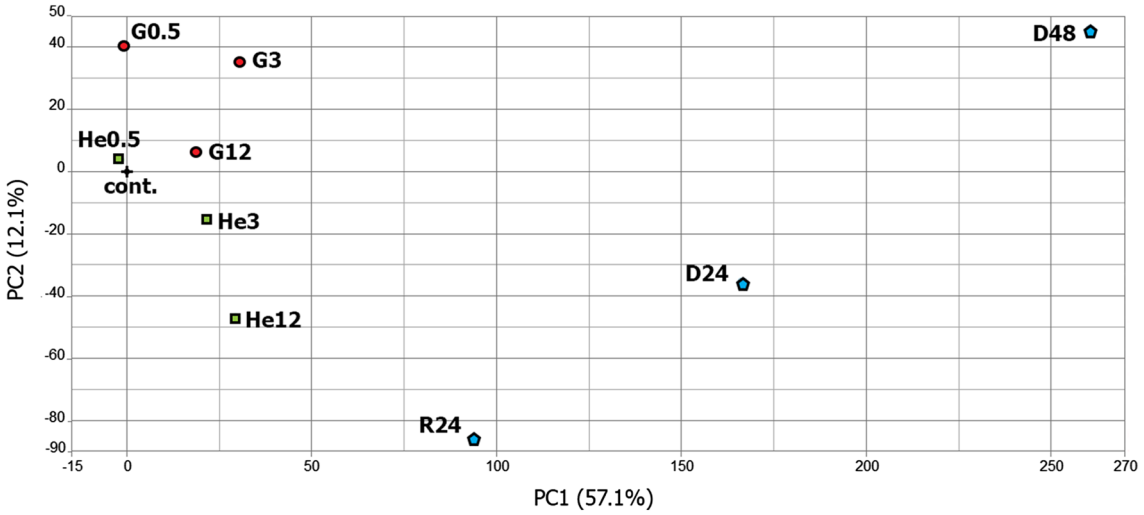

Fig. 2 Principal component analysis (PCA) based on normalized expression values. *Plot* shows two first principal components which cover 57.1 and 12.1 % of total variance, respectively. *Blue asterisks* represent desiccation time-course samples, and gamma- and helium-ion-irradiated samples are labeled as *red circles* and *green squares*, respectively

Table 1 Representation of transcripts, associated with well-known anhydrobiosis-responsive genes, in different selections

	Total number of probeset	Selected by PCA	Selected by overlapping in D-G-He up-regulated list
LEA	73	50	12
PIMT	22	15	15
Thioredoxin	41	17	3
GPx	25	4	1
HSP	61	9	1
Others	11,507	170	38
Unknown	4923	162	34
Total	16,652	427	104

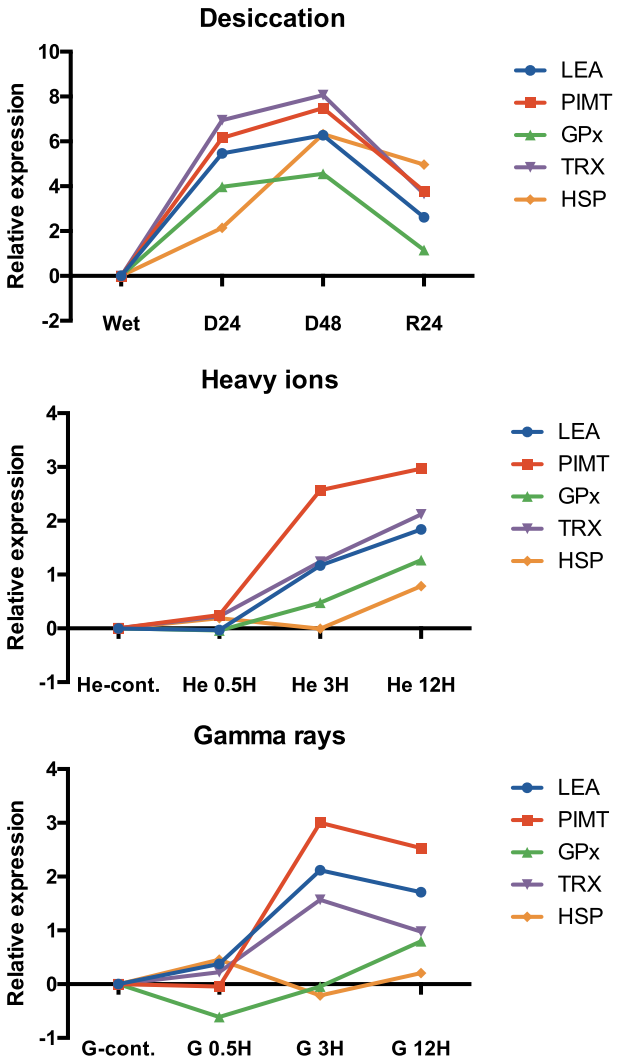

Fig. 3 Mean expression profiles of microarray probes for stress-responsive groups of transcripts

observed among the LEA proteins, PIMT, and TRX group. GPx and HSP groups show a lesser degree of similarity, especially in response to gamma rays.

Functional analysis of the response to different stresses

Resistance to complete desiccation, as well as remarkable tolerance to different types of irradiation, is a unique feature of *P. vanderplanki*, separating it from other insect species. Since the process of desiccation and rehydration induces DNA damage (Tanaka et al. 2004; Gusev et al. 2010), we assumed that some of the proteins needed to repair ionizing radiation-induced damage, including oxidation of biomolecules and double-strand breaks, would be identical to proteins used to mend the damaging effect of desiccation. The overlap in the larvae response to each stress should specify gene products that directly participate in the compensation of cellular damage, potentially identifying novel proteins critical to this process.

To apply the functional analysis, GO annotation of all microarray transcripts was performed. Transcript sequences obtained from Pv-EST database were subjected to a similarity search using BLASTx against TrEMBL, *Drosophila melanogaster* (Flybase) and *Caenorhabditis elegans* (Wormbase), and InterProScan. All BLASTx results were mapped to the Uniprot database separately to assign GO terms, and then merged into one resulting annotation. To avoid non-specific GO terms for the Diptera family, we used filtering by taxa. Approximately 6209 sequences from a total number of ESTs showed no significant similarity across all databases, 1439 sequences had BLASTx hits, but GO terms were not assigned. As a result, the final annotation contained 9005 ESTs with GO terms and was used as a background in the enrichment analysis.

To identify biological functions that occur in response to desiccation and two types of ionizing radiation exposure, and transform wide-scale expression changes into functional context, GO term enrichment analysis was accomplished using a set of genes acting similarly in response to three stress factors, obtained by PCA. The list of significantly enriched (P value <0.05) GO terms was added to the REVIGO software to reduce redundant terms. The filtered list was plotted in two-dimensional space related to GO term semantic similarity (Fig. 4a, b). The distribution

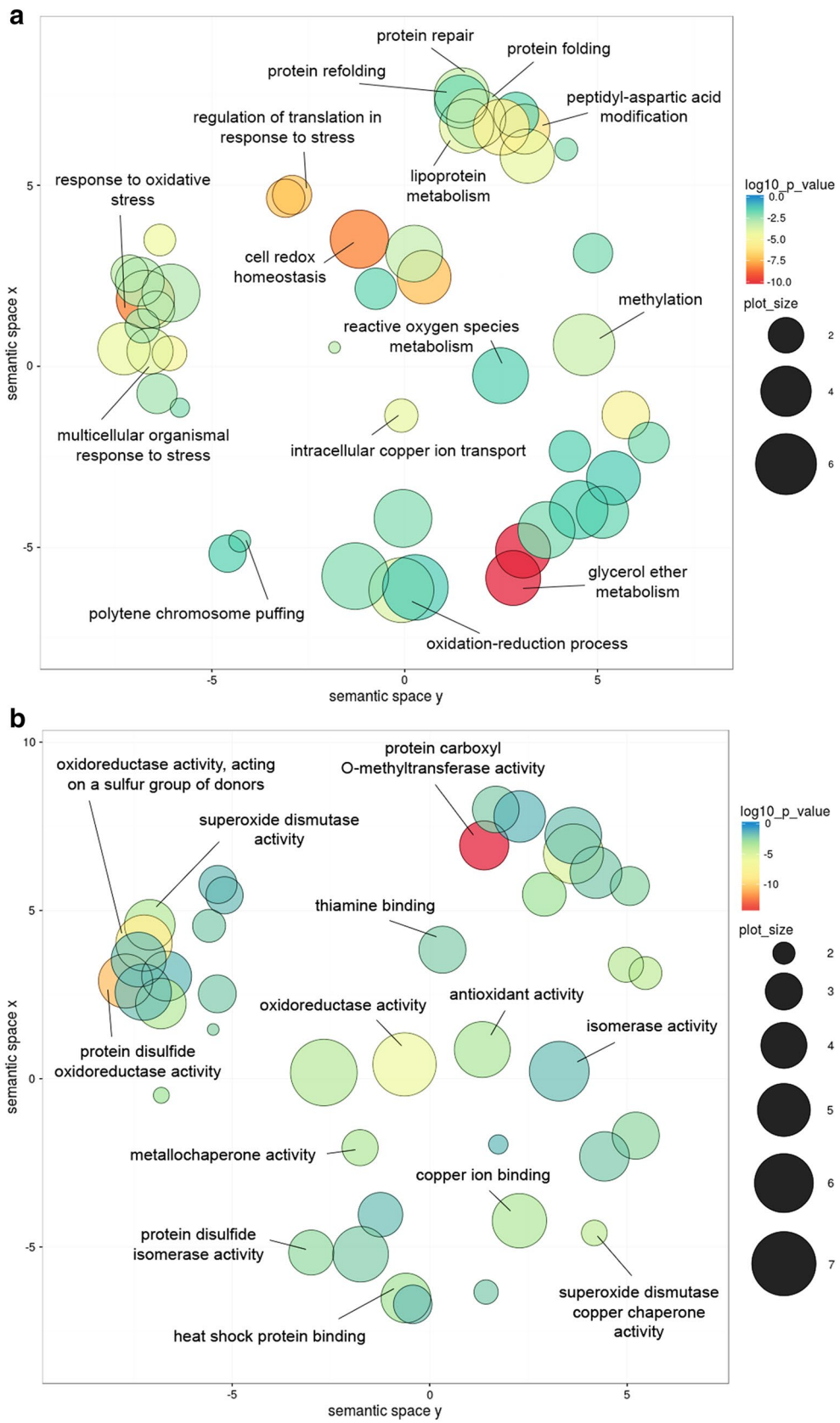

Fig. 4 Scatterplot visualization of abundance of enriched GO terms from the PCA analysis. GO terms were condensed by remaining redundant terms and *plotted* in two-dimensional space according to semantic similarity. *Color* of *bubbles* indicates log *P* value; distance between bubbles displays relatedness between GO terms. **a** biological processes and **b** molecular functions

of GO groups in desiccating larvae was in good agreement with our previous data, based on the estimation of total number of EST clones and clusters (Cornette et al. 2010).

The analysis showed the presence of several closely related clusters of enriched biological process GO terms (Fig. 4a). The first cluster is associated with biological processes in response to different abiotic and biotic factors. Most of the enriched terms in this cluster are "response to oxidative stress", "multicellular response to stress", "removal superoxide radicals", and others (Table S2). Another cluster contains terms of biological processes connected to protein modification: "peptidyl-aspartic acid modification", "protein repair", and "protein refolding".

Such important stress-related biological processes as "oxidation–reduction process", "reactive oxygen species metabolism" and "cell redox homeostasis" are also significantly enriched. Overall, most of the enriched biological processes act for neutralization of damage caused by reactive oxygen forms.

The main direction of significantly enriched molecular functions is involved in different types of antioxidant activity at various levels of GO terms: from general functions, such as "antioxidant activity", to more granular, such as "protein disulfide oxidoreductase activity" encompassed by TRX, "superoxide-dismutase activity" and related "copper chaperone activity", and "copper binding", which corresponds to Cu–Zn superoxide-dismutase (Fig. 4b). Furthermore, a cluster of similar GO terms was found, reporting to different transferase molecular functions, and the most enriched GO term in this cluster is "protein carboxyl O-methyltransferase activity".

Next, we performed GO enrichment using up-regulated transcripts of each time checkpoint of three stress exposure sets (D, G, and He) to assess the abundance of different GO terms during the stress time-course. The whole number of enriched terms in biological processes and molecular functions was 284 and 251, respectively. Obtained GO terms were filtered according to their presence in at least two stress exposure sets (Table S3). Using Venn diagrams, we found some identical processes and functions occurring in response to all kinds of stress (Fig. 5).

The distribution of enriched GO terms showed several notable findings, which is similar to results obtained with the enrichment of PCA gene set. Such expected biological processes as "protein repair", "peptidyl-aspartic acid modification", "protein methylation", "multicellular organismal response to stress", "protein alkylation", "glycerol ether metabolic process", "cell redox homeostasis", and "ether metabolic process", inducted by stress exposure, were represented in most checkpoints. Also significantly enriched GO terms, associated with stress response—"response to stress", "defense response", "methylation", "macromolecule methylation", and "glyoxylate catabolic process"—were either marked in at least one-time checkpoint in each of the stress factor sets. Interestingly, the biological processes associated with repair of DNA lesions—"non-recombinational repair" and "double-strand break repair via non-homologous end joining"—were enriched only in gamma and helium-ion irradiation gene lists.

Several enriched GO terms belonged to molecular function. The most significantly enriched GO terms in molecular function, which were marked in all stress sets with lowest *P* value, were associated with methyltransferase activity. Another group of GO terms that were enriched in all types of stress exposure was relevant to disulfide oxidoreductase activity. In addition, the GO term related to trehalose synthesis was enriched in D48 (expected) and G0.5-G3 (unexpected) time checkpoints. Some important stress-responsive GO terms showed valued enrichment in two stress exposure sets, for example: "heat-shock protein binding" (D-He), "Hsp90 protein binding" (D-He), "glutathione peroxidase activity" (D-G), and others.

qRT-PCR validation of microarray results

To confirm the changes in the transcriptional activity detected for transcripts associated with LEA4, LEA7, PIMT3, PIMT4, TRX8, and GPx (GPTx) in the microarray experiments, qRT-PCR was used to indicate relative levels of mRNA accumulation. Results of qRT-PCR experiments showed strong coherence with the microarray data (Fig. 6).

Discussion

The vast majority of living organisms are extremely sensitive to changes in hydration regime. Even small fluctuations in water content can cause large disturbances in a cell metabolism, osmotic shock, and finally lead to death. In addition, cells are exposed to extensive oxidative stress that increases the frequency of severe DNA lesions, especially the occurrence of potentially lethal double-strand breaks. Ionizing radiation as well as desiccation affects cells in a very aggressive manner, provoking damage to cell structures and biomolecules directly or indirectly according to the type of radiation (Watanabe et al. 2006a, b). It was shown that some radiation resistant organisms are also resistant to desiccation (Mattimore and Battista 1996; Billi et al. 2000), and vice versa. *P. vanderplanki* larvae have an extraordinary ability to withstand complete water loss and high-dose radiation exposure (Gusev et al. 2010). Therewith, the relatively large size of the sleeping chironomid, which is not typical of the majority of extremophiles, implies the presence of more complex mechanism of extended resistance. Based on the previous theoretical and experimental data (Kikawada et al. 2005; Watanabe et al. 2006a, b; Gusev et al. 2010), in this

work, we focused on the origin of this cross-tolerance phenomenon, trying to provide a broader view of comparable details of gene response to desiccation and two types of ionizing radiation.

We showed that transcriptional response in the expression profile of the desiccation–rehydration stages much exceeded the amount of up-regulated transcripts in response to both types of irradiation. It is quite natural, because desiccation implies not only damage and repair processes, but also general changes of the physiological status. However, a wide group of overlapped transcripts, coincidently up-regulated in each stress exposure, was allocated. Among this, overlapped set of transcripts was found the major anhydrobiosis-related groups of biomolecules: antioxidants, late embryogenesis abundant (LEA) proteins and heat-shock proteins. Their expression patterns were quite similar within the same stress, which may indicate that anhydrobiosis-related genes were activated and operate together as connected stress-responsive machinery.

The most overexpressed group was that of the protein-L-isoaspartate/D-aspartate *O*-methyltransferase (PIMT). Activity of these proteins is directly linked with stress resistance and lifespan (Furuchi et al. 2010; Khare et al. 2011; Desrosiers and Fanelus 2011). The structure and the number of methyltransferase-coding genes are a unique feature of *P. vanderplanki*: 14 PIMT-coding genes (instead of the usual 1–2), each of which is tissue-specific and has its own functional specialization. Currently, there is no identified homology among other insects (Gusev et al. 2014). In addition, performed functional analysis showed strongly enrichment of GO terms, associated with protein methylation, in response to all stress exposures. PIMT are probably involved in the repair of damaged proteins, which are probably generated by both types of irradiation, as during the desiccation process.

The most widely represented group among anhydrobiosis-related overlapped transcripts was LEA proteins (Table 1). During desiccation, highly hydrophilic LEA proteins act together with HSP for the protection of molecular structures against aggregation and denaturation, and also participate as one of the general components to the formation of a molecular shield as a physical guard against negative influence. The expected role of LEA proteins after exposure to irradiation is more ambiguous. A role of chaperone for damaged proteins could be expected. In addition, LEA was proposed to act as ion scavengers and such a function could participate to cope with ROS generated after gamma irradiation.

Remarkably, genes involved the metabolism of another anhydrobiosis-related physical protectant—trehalose—significantly reacted in response to desiccation and gamma rays, but not during helium-ion irradiation. While entering anhydrobiosis, larvae of *P. vanderplanki* generate a sufficient amount of trehalose for stabilizing biomolecules by replacing water with a glass-like sugar structure. Production of trehalose under gamma rays exposure is quite possible due to the proposed biological effect of low-LET irradiation. As a general model for the indirect action of radiation implies, the main cause of serious radiation-induced damage to cell is intracellular water that frequently produces ROS (Han and Yu 2010; Mattimore and Battista 1996). It is more typical for biological effects of low-LET radiation (gamma rays), but not so much for high-LET radiation (helium-ions) (Hall and Giaccia 2006). The abundance of trehalose

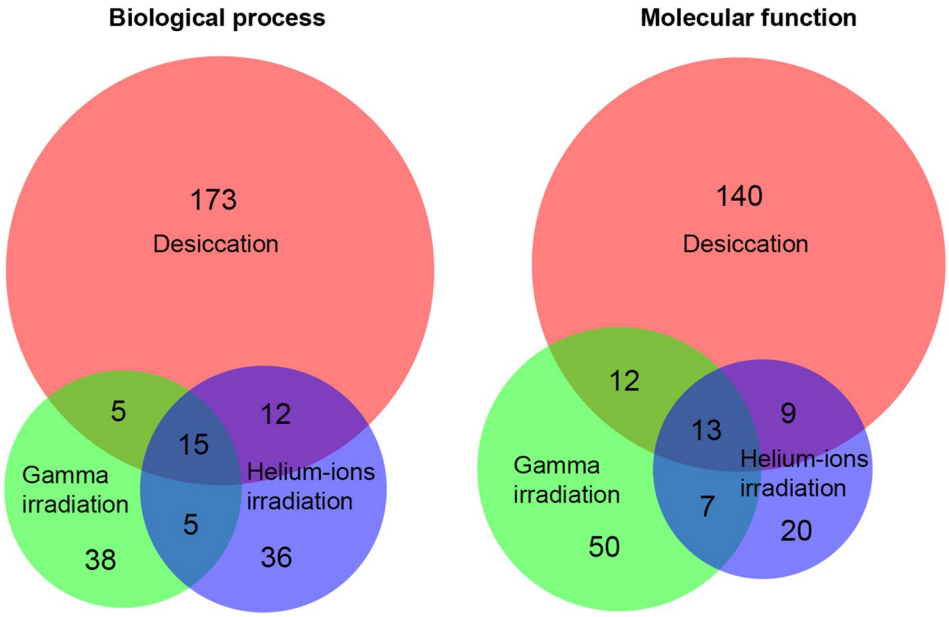

Fig. 5 Venn diagrams of biological processes and molecular functions of GO terms, enriched in at least one-time checkpoint

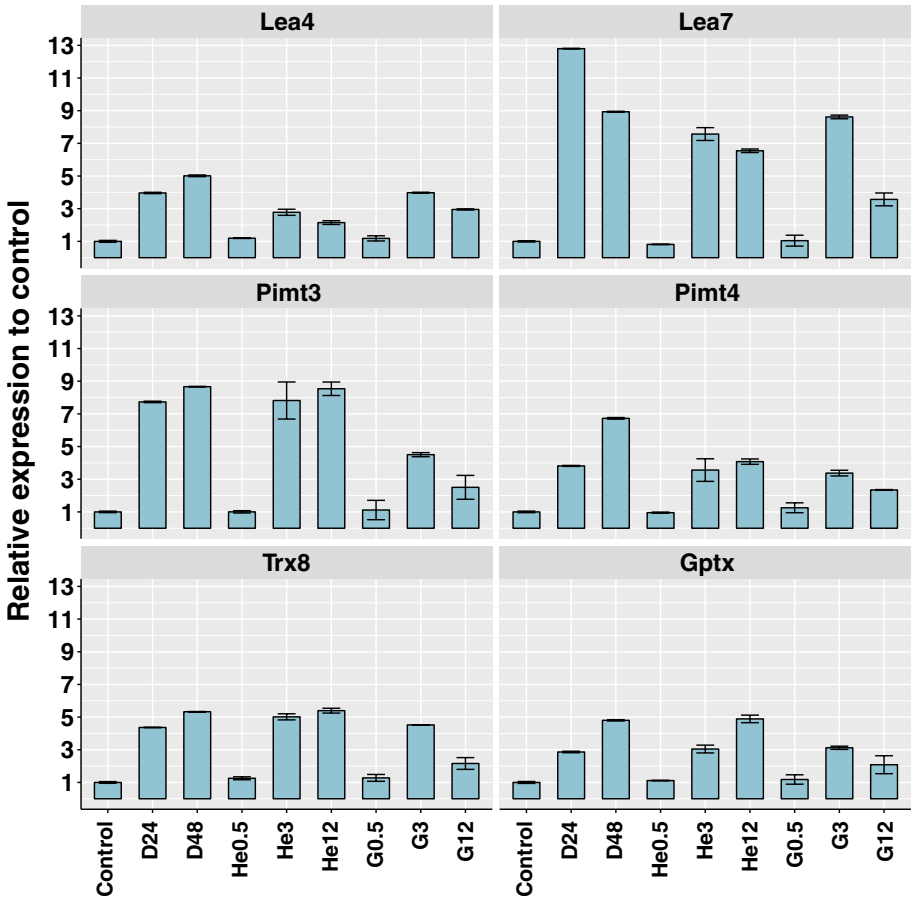

Fig. 6 Results of quantitative real-time PCR for transcripts associated with anhydrobiosis-responsive genes. Data represent the mean ± SEM of 3 samples

seems to possess a radioprotective action by reducing ROS formation in an absence of intracellular water. Such shielding function of trehalose against gamma rays exposure had been suggested by Yoshinaga et al. (1997). However, this assumption requires further studies in vitro.

Generation of ROS accompanies almost all types of external stress influence due to dysfunction of enzymatic reactions, eventually provoking DNA damage (França et al. 2007). Therefore, scavenging of rapidly increasing ROS becomes the main goal for acceptable survival. The previous studies (Cornette et al. 2010; Cornette and Kikawada 2011; Gusev et al. 2014) showed enhancing antioxidant activity with a dramatic increase in the expression level of oxidative stress-responsive genes during stages of anhydrobiosis. Our data demonstrated the same trend: the major active member of *P. vanderplanki* larvae's antioxidant system, which is critical for redox regulation of protein function—the thioredoxin metabolism group, including glutathione peroxidase—was detected in abundance throughout the desiccation process, reaching its maximum in a completely dehydrated state. Their representation during high–low-LET irradiation exposure was also significant.

Despite of mostly adverse effect of ROS for cells, they play an important role as intracellular signaling molecules with regulatory functions and act as a trigger for activation of gene expression and antioxidant systems (Schmitt et al. 2014). In the case of *P. vanderplanki* larvae, the initial stage of desiccation should provoke rapid accumulation of ROS, which might induce a wide transcriptional response of anhydrobiosis-related protectants. Possibly, drastic increasing of ROS by ionizing radiation exposure can generate a transcriptional response similar to water loss. This could explain the presence of highly up-regulated transcripts for anhydrobiosis-related genes in larvae exposed to gamma rays.

In addition, active DNA repair processes were detected in response to each kind of stress exposure. While the predominant repair pathway in anhydrobiosis is still unclear, an estimated core pathway for double-strand break repair in both high–low-LET irradiations was performed via non-homologous end joining (Moskalev et al. 2015).

As a conclusion, it is safe to assume that the cross-tolerance of the sleeping chironomid to ionizing radiation exposure comes from the evolutionary-based anhydrobiosis-associated mechanism of stress-resistance. This well-organized mechanism acts partially to eliminate negative effects obtained from other types of external stress impact, which is not typical to larvae's living environment. According to

the previous studies (Kikawada et al. 2005; Watanabe et al. 2006a, b; Gusev et al. 2010), we suppose that the adaptation of *P. vanderplanki* larvae to anhydrobiosis makes them capable to desiccate at any time or to induce multiple anhydrobiosis-related gene associations as soon as the first signs of stress appear. The latter seems to be more possible. Both desiccation process and ionizing radiation exposure are accompanied by oxidative stress. Rapid generating of ROS could be such a first signal, which activates the stress-resistance machinery, due to its universality and production speed. Thus, tolerance to ionizing radiation is a side effect of adaptation to anhydrobiosis.

Acknowledgments This work was supported in part by the Grants-in-Aid from MEXT/JSPS KAKENHI (Grant Number 16K07308, 25252060, 16K15073, and 15H05622) and a part of this study is also the result of the project "Characterization of the Mechanisms Underlying the Radiation Resistance Associated with Cryptobiosis" carried out under the Grant "Strategic Promotion Program for Basic Nuclear Research" by MEXT. Bioinformatics analysis was supported by Russian Science Foundation Grant for international groups (No. 14-44-00022).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Billi D, Friedmann E, Hofer KG, Caiola MG, Ocampo-Friedmann R (2000) Ionizing-radiation resistance in the desiccation-tolerant cyanobacterium *Chroococcidiopsis*. Appl Environ Microbiol 66(4):1489–1492. doi:10.1128/AEM.66.4.1489-1492.2000

Calahan D, Dunham M, DeSevo C, Koshland DE (2011) Genetic analysis of desiccation tolerance in *Sachharomyces cerevisiae*. Genetics 189(2):507–519. doi:10.1534/genetics.111.130369

Challabathula D, Puthur JT, Bartels D (2015) Surviving metabolic arrest: photosynthesis during desiccation and rehydration in resurrection plants. Ann N Y Acad Sci 1365(1):89–99. doi:10.1111/nyas.12884

Cornette R, Kikawada T (2011) The induction of anhydrobiosis in the sleeping chironomid: current status of our knowledge. IUBMB Life 63:419–429. doi:10.1002/iub.463

Cornette R, Kanamori Y, Watanabe M, Nakahara Y, Gusev O, Mitsumasu K, Kadono-Okuda K, Shimomura M, Mita K, Kikawada T, Okuda T (2010) Identification of anhydrobiosis-related genes from an expressed sequence tag database in the cryptobiotic midge *Polypedilum vanderplanki* (Diptera; Chironomidae). J Biol Chem 285(46):35889–35899. doi:10.1074/jbc.M110.150623

Cox MM, Battista JR (2005) *Deinococcus radiodurans*–the consummate survivor. Nat Rev Microbiol 3(11):882–892. doi:10.1038/nrmicro1264

Cromie GA, Connelly JC, Leach DRF (2001) Recombination at double-strand breaks and DNA ends: conserved mechanisms from phage to humans. Mol Cell 8(6):1163–1174

Crowe LM, Crowe JH (1992) Anhydrobiosis: a strategy for survival. Adv Space Res 12(4):239–247

Desrosiers RR, Fanelus I (2011) Damaged proteins bearing L-isoaspartyl residues and aging: a dynamic equilibrium between generation of isomerized forms and repair by PIMT. Curr Aging Sci 4(1):8–18

Dinakar C, Bartels D (2013) Desiccation tolerance in resurrection plants: new insights from transcriptome, proteome and metabolome analysis. Front Plant Sci 4:482. doi:10.3389/fpls.2013.00482

França MB, Panek AD, Eleutherio EC (2007) Oxidative stress and its effects during dehydration. Comp Biochem Physiol A Mol Integr Physiol 146(4):621–631

Furuchi T, Sakurako K, Katane M, Sekine M, Homma H (2010) The role of protein L-isoaspartyl/D-aspartyl *O*-methyltransferase (PIMT) in intracellular signal transduction. Chem Biodivers 7(6):1337–1348. doi:10.1002/cbdv.200900273

Gechev TS, Dinakar C, Benina M, Toneva V, Bartels D (2012) Molecular mechanisms of desiccation tolerance in resurrection plants. Cell Mol Life Sci 69(19):3175–3186. doi:10.1007/s00018-012-1088-0

Gladyshev E, Meselson M (2008) Extreme resistance of bdelloid rotifers to ionizing radiation. PNAS 105(13):5139–5144. doi:10.1073/pnas.0800966105

Griffiths CA, Gaff DF, Neale AD (2014) Drying without senescence in resurrection plants. Front Plant Sci 5:36. doi:10.3389/fpls.2014.00036

Gusev O, Nakahara Y, Vanyagina V, Malutina L, Cornette R, Sakashita T, Hamada N, Kikawada T, Kobayashi Y, Okuda T (2010) Anhydrobiosis-associated nuclear DNA damage and repair in the sleeping chironomid: linkage with radioresistance. PLoS One 5(11):e14008. doi:10.1371/journal.pone.0014008

Gusev O, Suetsugu Y, Cornette R, Kawashima T, Logacheva MD, Kondrashov AS, Penin AA, Hatanaka R, Kikuta S, Shimura S, Kanamori H, Katayose Y, Matsumoto T, Shagimardanova E, Alexeev D, Govorun V, Wisecaver J, Mikheyev A, Koyanagi R, Fujie M, Nishiyama T, Shigenobu S, Shibata TF, Golygina V, Hasebe M, Okuda T, Satoh N, Kikawada T (2014) Comparative genome sequencing reveals genomic signature of extreme desiccation tolerance in the anhydrobiotic midge. Nat Commun 5:4784. doi:10.1038/ncomms5784

Halberg KA, Jørgensen A, Møbjerg N (2013) Desiccation tolerance in the tardigrade *Richtersius coronifer* relies on muscle mediated structural reorganization. PLoS One 8(12):e85091. doi:10.1371/journal.pone.0085091

Hall EJ, Giaccia AJ (2006) Radiobiology for the radiologist, 6th ed. Philadelphia: Lippincott Williams&Wilkins p 546

Han W, Yu PKN (2010) Ionizing radiation, DNA double strand break and mutation. Chapter 7. In: Advances in genetics research, Vol. 4. Nova Science Publishers, Inc., New York

Hand SC, Menze MA (2015) Molecular approaches for improving desiccation tolerance: insights from the brine shrimp *Artemia franciscana*. Planta 242(2):379–388. doi:10.1007/s00425-015-2281-9

Hengherr S, Heyer AG, Köhler HR, Schill RO (2008) Trehalose and anhydrobiosis in tardigrades–evidence for divergence in responses to dehydration. FEBS J 275(2):281–288

Hengherr S, Worland MR, Reuner A, Brummer F, Schill RO (2009) High-temperature tolerance in anhydrobiotic tardigrades is limited by glass transition. Physiol Biochem Zool 82:749–755. doi:10.1086/605954

Hinton HE (1960) A fly larva that tolerates dehydration and temperatures of −270 °C to +102 °C. Nature 188(4747):336–337. doi:10.1038/188336a0

Hoekstra FA, Golovina EA, Buitink J (2001a) Mechanisms of plant desiccation tolerance. Trends Plant Sci 6(9):431–438. doi:10.1016/S1360-1385(01)02052-0

Hoekstra FA, Golovina EA, Tetteroo FA, Wolkers WF (2001b) Induction of desiccation tolerance in plant somatic embryos: how exclusive is the protective role of sugars? Cryobiology 43(2):140–150

Ito C, Goto SG, Numata H (2013) Desiccation and heat tolerance of eggs of the Asian tadpole shrimp. Triops granarius. Zoolog Sci 30(9):760–766. doi:10.2108/zsj.30.760

Khare S, Linster CL, Clarke SG (2011) The interplay between protein L-isoaspartyl methyltransferase activity and insulin-like signaling to extend lifespan in *Caenorhabditis elegans*. PLoS One 6(6):e20850. doi:10.1371/journal.pone.0020850

Kikawada T, Minakawa N, Watanabe M, Okuda T (2005) Factors inducing successful anhydrobiosis in the African Chironomid *Polypedilum vanderplanki*: significance of the larval tubular nest. Integr Comp Biol 45(5):710–714

Kikawada T, Nakahara Y, Kanamori Y, Iwata K-I, Watanabe M, McGee B, Tunnacliffe A, Okuda T (2006) Dehydration-induced expression of LEA proteins in an anhydrobiotic chironomid. Biochem Biophys Res Commun 348(1):56–61. doi:10.1016/j.bbrc.2006.07.003

Koster KL, Leopold AC (1988) Sugars and desiccation tolerance in seeds. Plant Physiol 88(3):829–832

Lapinski J, Tunnacliffe A (2003) Anhydrobiosis without trehalose in bdelloid rotifers. FEBS Lett 553(3):387–390. doi:10.1016/S0014-5793(03)01062-7

Leprince O, Buitink J (2015) Introduction to desiccation biology: from old borders to new frontiers. Planta 242(2):369–378. doi:10.1007/s00425-015-2357-6

Mattimore V, Battista J (1996) Radioresistance of *Deinococcus radiodurans*: functions necessary to survive ionizing radiation are also necessary to survive prolonged desiccation. J Bacteriol 178:633–637

McGill LM, Shannon AJ, Pisani D, Félix MA, Ramløv H, Dix I, Wharton DA, Burnell AM (2015) Anhydrobiosis and freezing-tolerance: adaptations that facilitate the establishment of *Panagrolaimus* nematodes in polar habitats. PLoS One 10(3):e0116084. doi:10.1371/journal.pone.0116084

Moskalev A, Zhikrivetskaya S, Krasnov G, Shaposhnikov M, Proshkina E, Borisoglebsky D, Danilov A, Peregudova D, Sharapova I, Dobrovolskaya E, Solovev I, Zemskaya N, Shilova L, Snezhkina A, Kudryavtseva A (2015) A comparison of the transcriptome of *Drosophila melanogaster* in response to entomopathogenic fungus, ionizing radiation, starvation and cold shock. BMC Genom 16(Suppl 13):S8. doi:10.1186/1471-2164-16-S13-S8

Musilova M, Wright G, Ward JM, Dartnell LR (2015) Isolation of radiation-resistant bacteria from Mars analog Antarctic dry valleys by preselection, and the correlation between radiation and desiccation resistance. Astrobiology 15(12):1076–1090. doi:10.1089/ast.2014.1278

Potts M, Slaughter SM, Hunneke FU, Garst JF, Helm RF (2005) Desiccation tolerance of prokaryotes: application of principles to human cells. Integr Comp Biol 45(5):800–809. doi:10.1093/icb/45.5.800

Rebecchi L, Altiero T, Guidetti R (2007) Anhydrobiosis: the extreme limit of desiccation tolerance. ISJ 4:65–81

Schmitt FJ, Renger G, Friedrich T, Kreslavski VD, Zharmukhamedov SK, Los DA, Kuznetsov VV, Allakhverdiev SI (2014) Reactive oxygen species: re-evaluation of generation, monitoring and role in stress-signaling in phototrophic organisms. Biochim Biophys Acta 1837(6):835–848. doi:10.1016/j.bbabio.2014.02.005

Shapiro-Ilan DI, Brown I, Lewis EE (2014) Freezing and desiccation tolerance in entomopathogenic nematodes: diversity and correlation of traits. J Nematol 46(1):27–34

Shen-Miller J, Mudgett MB, Schopf JW, Clarke S, Berger R (1995) Exceptional seed longevity and robust growth: ancient sacred lotus from China. Am J Bot 82(11):1367–1380. doi:10.2307/2445863

Somme L (1996) Anhydrobiosis and cold tolerance in tardigrades. Eur J Entomol 93(3):349–357

Tanaka M, Earl AM, Howell HA, Park MJ, Eisen JA, Peterson SN, Battista JR (2004) Analysis of Deinococcus radiodurans's transcriptional response to ionizing radiation and desiccation reveals novel proteins that contribute to extreme radioresistance. Genetics 168:21–33. doi:10.1534/genetics.104.029249

Watanabe M (2006) Anhydrobiosis in invertebrates. Appl Entomol Zool 41(1):15–31. doi:10.1303/aez.2006.15

Watanabe M, Kikawada T, Minagawa N, Yukuhiro F, Okuda T (2002) Mechanism allowing an insect to survive complete dehydration and extreme temperatures. J Exp Biol 205:2799–2802

Watanabe M, Kikawada T, Okuda T (2003) Increase of internal ion concentration triggers trehalose synthesis associated with cryptobiosis in larvae of *Polypedilum vanderplanki*. J Exp Biol 206:2281–2286

Watanabe M, Sakashita T, Fujita A, Kikawada T, Horikawa DD et al (2006a) Biological effects of anhydrobiosis in an African chironomid, *Polypedilum vanderplanki* on radiation tolerance. Int J Radiat Biol 82:587–592

Watanabe M, Sakashita T, Fujita A, Kikawada T, Nakahara Y et al (2006b) Estimation of radiation tolerance to high LET heavy ions in an anhydrobiotic insect, *Polypedilum vanderplanki*. Int J Radiat Biol 82:835–842

Watanabe M, Nakahara Y, Sakashita T, Kikawada T, Fujita A, Hamada N, Horikawa DD, Wada S, Kobayashi Y, Okuda T (2007) Physiological changes leading to anhydrobiosis improve radiation tolerance in *Polypedilum vanderplanki* larvae. J Insect Physiol 53(6):573–579

Yoshinaga K, Yoshioka H, Kurosaki H, Hirasawa K, Uritani M, Hasegawa M (1997) Protection by trehalose of DNA from radiation damage. Biosci Biotechnol Biochem 61(1):160–161