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Screening of Calorie Restriction-induced Genes in the Rotifer *Brachionus Plicatilis**

Aung Kyaw Swar Oo

Abstract

Rotifers play an important role in an aquatic ecosystem and are used as model organisms for various study areas. Although many studies have been conducted to identify environmental factors that influence rotifer populations, the molecular mechanisms involved still remain to be elucidated. The availability of food resource is also a one of the most important environmental factors that fluctuate rotifer populations over time. In this study, gene(s) differentially expressed by calorie restriction in the monogonont rotifers, *Brachionus plicatilis*, was analyzed, where a calorie-restricted group was fed 3 h/day and a well-fed group fed *ad libitum*. A subtracted cDNA library from the calorie-restricted rotifers was constructed using suppression subtractive hybridization (SSH). One hundred sixty three expressed sequence tags (ESTs) were identified, which included 109 putative genes with a high identity to known genes in the publicly available database as well as 54 unknown ESTs. A total of 38 different genes were obtained among 109 ESTs. Gene ontology study showed the differentially expressed genes related to cellular structure, transport, and division; DNA synthesis; metabolism; transcription; RNA biosynthesis; and other functions were 24, 11, 36, 5, 3, 18% respectively, whereas genes with functionally unclassified were 3%.

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Introduction

In an ecosystem all the organisms living together in a specific habitat rely on each other. The amount of energy within the ecosystem is always maintained at a constant level and is hardly created nor declined. The energy flow through a food web and a food chain is carried out by various trophic levels in a particular system. Therefore, individuals in every trophic level are important for the sustainable ecosystem in which they live and for their own survival. Biotic and/or abiotic factors also play key roles for the survival of animal species. It is postulated that a number of animals are influenced by both population density-dependent and -independent factors, and their impacts are

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relative and depend on the physiological status of the population itself (Mori, 1988). The population of any animal species fluctuates depending on the changes in their surrounding environments and within their own population. These fluctuations strongly influence humans in various ways such as food resources, public health, economy and environment.

Nowadays population dynamics studies have become popular and animals from diverse taxa with different economic values of importance are used as model organisms in these studies. The rotifer *Brachionus plicatilis* is a small zooplankton approximately 0.3 mm in length that inhabits brackish waters. It is an economically important species as a live food organism in aquaculture (Hagiwara *et al.*, 2001) because it has a rich nutritional profile and a suitable size for larval fish and shrimp. It reproduces either sexually or asexually with one of the highest population growth rate among metazons. Cyclically parthenogenetic organisms provide a valuable model for investigating the relationships between reproductive mode and population structure, and the maintenance of genetic variation in natural populations (Bell, 1982; Hebert, 1987). There are several reports on obligate parthenogenetic strains (reproduce only asexually) in *Brachionus* sp. (Bennett and Borass, 1989; Fussmann *et al.*, 2003) and Ishikawa strain of *B. plicatilis* used in the present study has been known to reproduce asexually: mictic females or males are not observed (Yoshinaga *et al.*, 2000). The parthenogenetic rotifer is now widely used as a model organism in population dynamics studies and in the laboratory it shows a typical sigmoid growth curve as observed with other laboratory-reared model organisms (Yoshinaga *et al.*, 2001a).

Environmental factors, either climatic or biological, cause an alternation in the individual life history parameters of the rotifer, such as reproductive pattern and life span, leading to the fluctuation of population (Yoshinaga *et al.*, 2003). The rotifer subjected to calorie restriction (CR) in a feeding schedule of 3 h/day showed its life span two times longer than that subjected to well fed, and offspring production concomitantly decreased about ten times under CR (Yoshinaga *et al.*, 2003). Thus, CR is one of the biological factors that shift a mode of reproduction and concomitantly a mode of life span in the rotifer. Such trade-off between lifetime fecundity and life span is proposed as an alternative life history strategy of the rotifer under starved conditions to maintain its population size stable (Yoshinaga *et al.*, 2000). It is also proposed that the effects of CR on the starvation tolerance are transmitted from parents to their offspring in the rotifer (Yoshinaga *et al.*, 2001b).

Molecular approaches to the effects of CR on life span have been conducted using rotifer as a model organism. It is widely accepted that life span is regulated by the interaction between oxidative stress and an enzymatic antioxidation. The major antioxidant enzyme, superoxide dismutase (SOD), catalyzes decomposition of reactive oxygen species (ROS), which provokes massive damages to DNA, proteins, and lipids (Finkel and Holbrook, 2000). The accumulated mRNA levels of manganese-SOD (Mn-SOD), which functions in mitochondria, were found to increase in calorie-restricted, long-lived rotifer (Kaneko *et al.*, 2005). It has been also claimed that the dietary restriction (another term of CR) retarded the rate of nuclear division in the gastric glands and vitellarium (yolk-secreting gland) of the rotifer *Asplanchna brightwelli* (Verdone-Smith and Enesco, 1982). Although it is generally accepted that an energy saving through the suppression of reproduction during the period of food shortage is a prerequisite to a longer life span and maintaining the population size, little is known at present about the genes expressed under CR.

Suppression subtractive hybridization (SSH) is a powerful, reliable technique to identify differentially expressed genes that are involved in physiological processes of both aquatic invertebrates (Brown *et al.*, 2006; Soetaert *et al.*, 2006) and vertebrates (Reynders *et al.*, 2006; Wang and Wu, 2007) responding to various environmental conditions. In this study, SSH was used to identify differentially expressed genes in calorie-restricted rotifer *B. plicatilis*.

Materials and Methods

Culture, feeding regimen, and sample collection

The parthenogenetic rotifer *B. plicatilis* (Ishikawa strain) was used in the present study. Rotifers were cultured using Brujewicz artificial seawater (BAS). The BSA, consisting of 454 mM NaCl, 10 mM KCl, 10 mM $CaCl_2$, 27 mM $MgSO_4$, 26 mM $MgCl_2$, 2.4 mM $NaHCO_3$ and 0.8 mM NaBr, was sterilized and filtered by 0.45-μm filter. The half-diluted BAS culture media were used in the present study. The rotifers were precultured under a batch culture system at 25°C using a cool-incubator (HCRCS2V150W-A1202, Ikuta Industries, Tokyo, Japan) and subjected to a continuous feeding with commercially available concentrated algae *Nannochloropsis oculata* (Nikkai Center, Tokyo, Japan). Eggs deposited on the bottom of the precultures were

collected and hatched out. Neonates were cultured under the same conditions as mentioned above in total darkness except during observation and subsequently divided into two groups: one for well-feeding (WF) as the control and the other for CR. CR was imposed by periodical food limiting at a 3 h/day feeding regimen, while WF was instituted by feeding at *ad libitum*. Culture media were changed daily at the beginning of the CR period. The rotifers in the CR group were transferred using a plankton net (50 μM mesh size) into a fresh medium without algae, whereas those in the WF group were into a fresh medium previously suspended with food algae. Samples were collected on day 2, when the reproductive performance between the WF and CR groups was clearly distinct, using the plankton net and washed two times with fresh BAS.

Total RNA extraction

Total RNAs were extracted using Isogen (Nippon Gene, Toyama, Japan) according to the manufacturer's protocols with a little modification (Fig. 1). Harvested rotifers (about 40,000 ind/200ml) were homogenized with 1 ml of Isogen in 1.5 ml tubes. The tubes were then swirled using a vortex and stored at room temperature for 5 minutes. An aliquot of 0.2 ml of 99.5% chloroform was added into the tubes, which were subsequently shaken vigorously for 15 seconds and stored at room temperature for 2 to 3 minutes. After storage, the tubes were centrifuged at 16,000xg for 15 minutes at 4℃. The uppermost aqueous layers containing the extracted RNAs were transferred into new tubes, added with 0.5 ml of 99.5% isopropanol, and stored at −20℃ overnight to precipitate RNAs. The precipitated RNAs were collected by centrifugation at 16,000xg for 30 minutes at 4℃. All aqueous phase was discarded and the precipitated RNAs were washed with 1 ml of 70% ethanol. The tubes were again centrifuged at 4,600xg for 10 minutes at 4℃. Alcohol was discarded and the tubes were dried briefly till the alcohol residues were completely evaporated. Finally, the RNA pellets were re-suspended into sterile distilled water.

Poly $(A)^+$ RNA isolation

Poly $(A)^+$ RNAs were isolated from the total RNAs using Oligotex-dT30 (super) mRNA purification Kit (TaKaRa, Otsu, Japan) according to the manufacturer's instructions. RNA integrity was examined by using agarose gels containing 1% formaldehyde. The quantity and quality of RNA were determined by absorbance at A260 and at A260/280 using a DU® 530 Life Science UV/Vis spectrophotometer (Beckman Instruments, Inc., Fullerton, CA,

USA).

SSH library construction and plasmid isolation

cDNAs from the CR group were used as a tester, whereas cDNAs form the WF group were used as a driver, and the driver cDNAs were subtracted from the tester cDNAs (Fig. 2). SSH was carried out using PCR-Select^{TM} cDNA Subtraction Kit (TaKaRa) with minor modifications. PCR amplification was conducted using a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The optimized cycles for the primary and secondary PCRs were 27 and 15, respectively. The resulting subtracted cDNAs were ligated to the pGEM-T vectors using pGEM-T Vector Systems (Promega, Madison, WI, USA) and transformed into *Escherichia coli* strain JM109. White colonies were randomly picked up from the subtracted cDNA library and the presence of inserts was checked by agarose gel electrophoresis. The clones with inserts were grown overnight in Luria-Bertani (LB) broth media containing ampicillin (200 μg/ml) at 37 °C under shaking. Plasmid isolation was accomplished using GenElute Plasmid Miniprep Kit (Sigma, St. Louis, MO, USA).

Sequencing and search for homologous sequences

The purified plasmid DNAs with inserts were subjected to PCR labeling using BigDye^{TM} Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and sequencing was performed using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). Adaptor and vector sequences flanking either side of partial sequences were detached using SeqEd v1.0.3 software (Applied Biosystems). Finally, modified expressed sequence tags (ESTs) were submitted to the National Center for Biotechnology Information database (NCBI) using the blastx program in the Basic Local Alignment Search Tool (BLAST) to search any known gene counterparts with homologous sequences in the database.

Results

Reproductive performance under calorie restriction

The WF group on day 2 showed observable reproduction and most individuals carried at least two eggs. However, reproduction in the CR group was indistinct and very few individuals carried one egg. Thus, the numbers of eggs and individuals carrying eggs were remarkably different between the CR

and WF groups, which were regarded as a visual parameter for distinguishing the effects of CR.

SSH and sequence homology

After SSH, different band patterns were seen between the subtracted and unsubtracted PCR products. The bands with the molecular weights of 612, 495, 345, and 210 bp were predominantly observed in the subtracted PCR products (Fig. 3). Subtracted PCR products were subcloned into the pGEM-T vectors and inserted clones were subjected to sequencing.

Randomly collected 163 clones containing inserts were sequenced and submitted to the NCBI database using the blastx program in BLAST. Among 163 ESTs submitted, 109 ESTs (66.9%) retrieved their homologous sequences, whereas other 54 ESTs (33.1%) showed no significant similarity to known genes in the database. Sequence alignment of the ESTs with the same gene products was conducted using the ClustalW multiple sequence alignment program, yielding 38 different genes among 109 ESTs (Table 1).

Gene ontology study showed one group of gene having unknown function (3%) and 6 functional groups of gene related to cellular structure, transport, and division (24%), DNA synthesis (11%), metabolism (36%), other functions (18%), transcription (5%), and RNA biosynthesis (3%) were observed among the differentially expressed genes (Fig. 4).

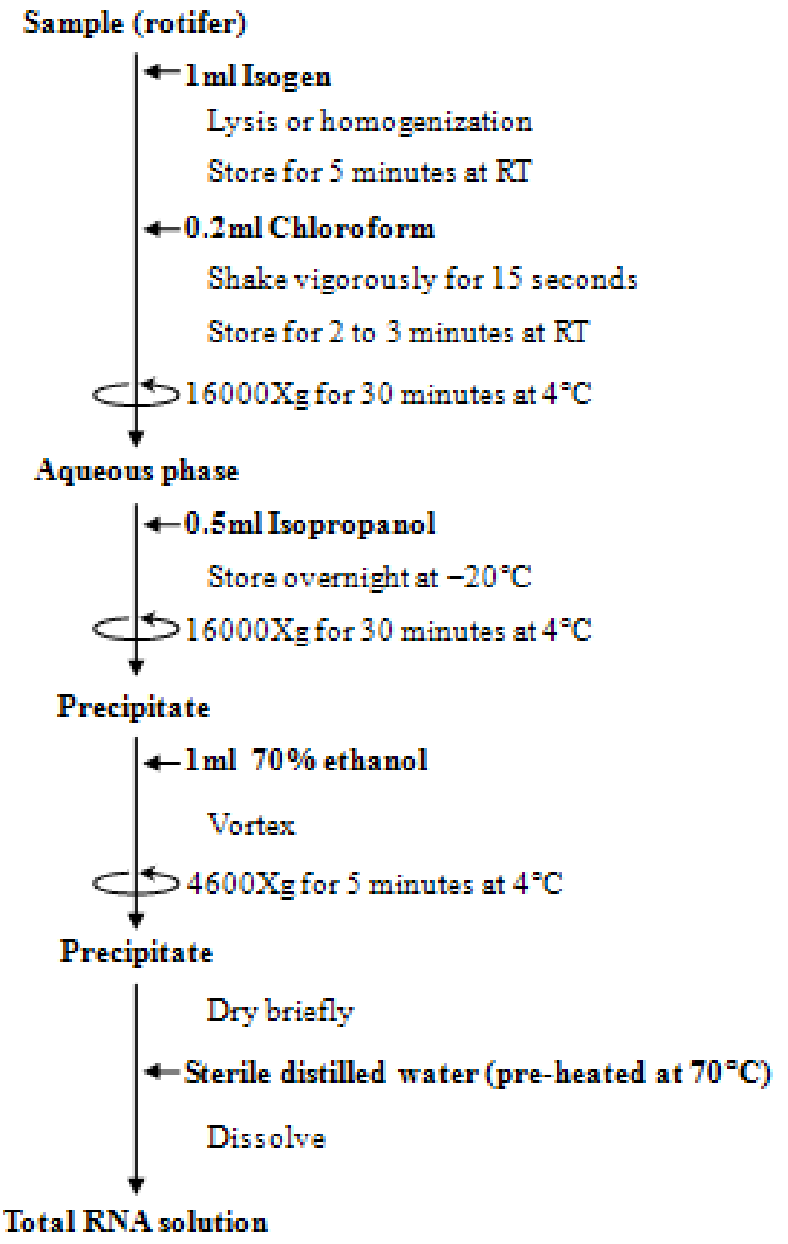

Fig. 1. General outline for total RNA extraction. RT, room temperature

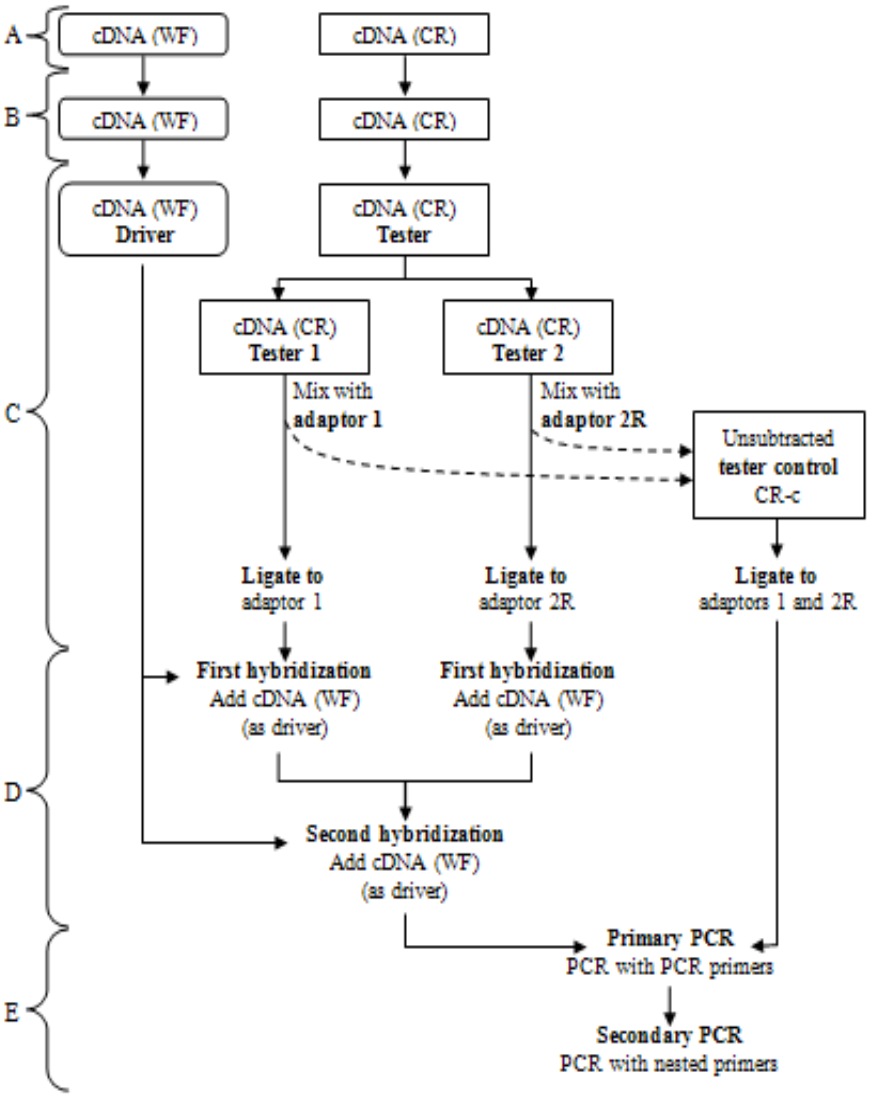

Fig. 2. Basic steps in SSH (A-E) and type of cDNA subtraction performed for the present study. SSH technique basically covers cDNA synthesis (A), enzyme digestion (B), adaptor ligation (C), hybridization (D), and PCR amplification (E). As the present study was focused on calorie restriction (CR) cDNA prepared from the CR samples were used as a tester, whereas those prepared from the control well-fed (WF) samples were used as a driver. The tester cDNAs were subtracted from the driver cDNAs. CR-c, CR unsubtracted tester control; PCR, polymeric chain reaction; cDNA, complementary DNA

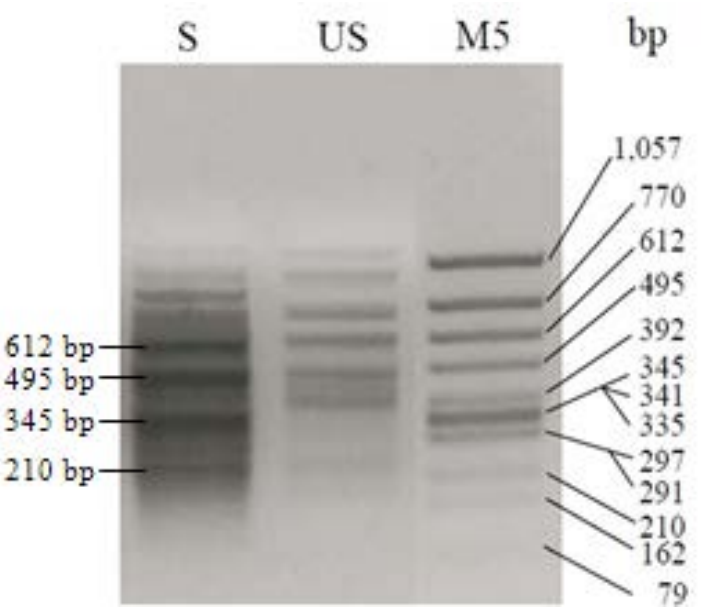

Fig. 3. PCR products resulted from SSH. PCR products were run on 2% agarose gel containing ethidium bromide. S and US stand respectively for the subtracted and unsubtracted PCR products; M5, molecular weight marker 5

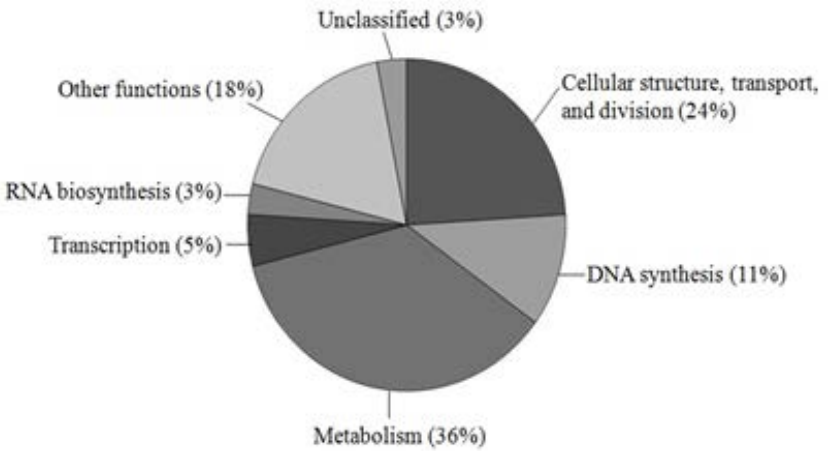

Fig. 4. Gene ontology analysis on 38 differentially expressed genes in calorie-restricted *Brachionus plicatilis* by functional classification for predicted proteins.

Table 1. Differentially expressed genes in calorie-restricted *Brachionus plicatilis* as revealed by SSH

Putative gene	Abbrevia-tion	Accession no.	E value[1]	Length of query sequence	Identity (%)
Genes related to cellular structure, transport, and division					
Lissencephaly-1	*Lis1*	XP_392399	4e-18	288	69
Dynein heavy chain domain 3	*Dnahc3*	XP_997725	1e-42	481	58
Beta 2 tubulin	*tub*	AAX09675	1e-37	301	97
Microtubule-associated protein EB 1	*MT-EB1*	BAC05521	4e-08	230	61
BRCA2 and CDKN1A-interact-ing protein, isoform BCCIPβ	*BCCIPβ*	XP_423888	3e-17	676	32
Calmodulin (synthetic construct)	*CaM64B*	AAD34268	1e-12	427	40
Genes related to DNA replication					
CDT1 protein	*CDT1*	AAH21126	0.031	303	31
Mismatch repair protein in mitosis and meiosis	*Msh6p*	NP_010382	8.6	424	38
DNA polymerase epsilon	*Ploe*	EAT45963	7.3	495	41
DNA polymerase sigma	*Pols*	NP_001012 968	1e-26	239	73
Genes related to metabolism					
Stom protein	*stom*	AAH91908	9e-46	550	65
Galactose-4-epimerase, UDP	*Gale*	EAT40915	3e-21	432	60
Glycogen phosphorylase	*Glase*	NP_001001 904	5e-12	158	67
β-galactosidase	*Glb*	XP_792349	2e-11	262	49

Putative gene	Abbrevia -tion	Accession no.	E value[1]	Length of query sequence	Identity (%)
2-isopropylmalate synthase	*IPS2*	YP_609867	0.76	343	78
Succinate dehydrogenase complex subunit D	*SDHD*	AAW70035	8.4	182	50
Iron regulatory protein	*Irp*	AAR15297	2e-09	428	51
Peptidylglycine-hydroxylating monooxygenase	*Phm*	NP_477225	9e-13	351	47
Genes related to other functions					
Tissue factor pathway inhibitor	*TFPI*	AAB26836	5e-14	384	38
Serine protease	*Ser*	EAT46744	3e-08	521	38
Multifunctional 14-3-3 family chaperone	*14-3-3*	ABF18291	1e-28	249	83
Serine/threonine phosphatase	*STPP*	AAD01260	2e-26	174	98
Serine/threonine protein kinase with TRP repeats	*PK-TRP*	YP_593054	8.6	200	38
Transposase	*Tsase*	NP_602772	1.3	382	54
Envelope glycoprotein	*gp*	ABA61554	5.0	459	45
Genes related to transcription					
Zinc finger protein	*Znf*	FAA00107	3e-05	307	48
EBF protein	*EBF*	XP_688771	5.4	304	42
Genes related to RNA biosynthesis					
NOL1/NOP2/Sun domain family 2 protein	*NSUN2*	XP_419023	2e-14	234	53
Predicted metal-dependent RNase	*COG1782*	ZP_00366321	5.6	494	42
Unknown					
Conserved hypothetical protein	*CHP*	EAT32996	0.26	369	27

[1]E value or expectation value — the number of the different alignments with scores equivalent to or better than raw score (S) that are expected to occur in the database search by chance.

Discussion

CR without essential nutrient deficiency is the only known experimental intervention that extends life span and retards age-related defects of various species across wide phylogenetic differences. In any organisms, adaptation to changes occurring in the environment is controlled by molecular-based mechanisms. Relevant expression or suppression of genes to time and conditions is a prerequisite to adjusting metabolism at the molecular level in all organisms. Proper gene expression confers energetically favorable mechanistic pathways in energy-demanding cells and hereby cells sustain their normal activities required for maintaining life.

SSH is a powerful technique to identify differentially expressed genes that involve in physiobiological processes of organisms under a particular condition. In the present study, SSH was used to identify genes induced by CR in the rotifer *B. plicatilis*. Annotated sequences were classified by Gene Ontology followed by manual adjustment, showing 6 functional groups among 38 differentially expressed genes (see Table 1).

Predicted metal-dependent RNase (*COG1782*) was most abundantly found (32 ESTs) among 109 ESTs encoding 38 different genes, but its putative function is largely unknown. Among genes related to cellular structure, transport and division, the number of EST encoding *Dnahc3* was 17, the second most abundant transcript. Dynein motor protein has several roles, in combination with other molecules, in cellular activities. In *Drosophila*, dynein is required during germline cell divisions and oocyte differentiation (McGrail and Hays, 1997). The dynein heavy chain gene is differentially expressed during development with the highest levels of transcripts in ovaries and embryos (Li *et al.*, 1994). Dynein localization along the oocyte cortex in wild-type *Drosophila* egg chambers is dependent on *Drosophila* Lis1, DLis1 (Swan *et al.*, 1999). It has been speculated that a membrane-associated protein, spectrin, is required for proper localization of DLis1 to the oocyte cortex in the *Drosophil*a ovary (Swan *et al.*, 1999). Lis1 interacts physically with β-spectrin *in vitro* (Wang *et al.*, 1995). In the present study, the gene encoding β-spectrin was also observed among 38 different genes. Based on these findings and the present results, expression of genes encoding Dnahc3, Lis1 and β-spectrin protein are probably attributable to reproductive suppression of the rotifer

under CR.

BCCIPβ is an isoform of BCCIP, a BRCA2 and CDKN1A (p21 or $p21^{Waf1/Cip1}$) interacting protein. BCCIPβ interacts with p21 *in vivo*, inhibits cell growth and delays progression of G1 to S phase (Meng *et al.*, 2004). It has been reported that CR decreases the rate of cell division as well as the total number of dividing cells in rat colonic mucosa (Albanes *et al.*, 1990). In the present study, *BCCIPβ* expression was observed by CR. Dietary restriction retards the rate of organ-specific nuclear division in the rotifer *A. brightwelli* (Verdone-Smith and Enesco, 1982). Cell division in the rotifer is known to occur only in their eggs and CR suppresses reproduction (Egami, 1972). Taken together, expression of BCCIPβ may regulate the metabolic shift of rotifers from reproduction to body maintenance under CR.

In this study, the genes encoding DNA polymerase epsilon (*Pole*), DNA polymerase sigma (*Pols*), and replication protein (*CDT1*) were expressed by CR. In *S. cerevisiae*, Pols is necessary to stimulate DNA polymerase activity of Pole holoenzyme (Edwards *et al.*, 2003) and this activity is also required for rapid and efficient chromosomal DNA replication in *Xenopus*, a higher eukaryote (Shikata *et al.*, 2006). Pole in *S. cerevisiae* localizes and functions at the replication forks (Hiraga *et al.*, 2005) and its expression peaks at G1/S (Sugino, 1995). CDT1 is also specifically recruited to chromatin during G1 and S phase (Bell and Dutta, 2002). Therefore, the expression of *Pole* and *CDT1* would affect cell cycle progression. Intracellular iron metabolism is important for cell-cycle progression and regulated by iron regulatory protein, Irp. *Irp* was expressed by CR in the present study. Expression of the genes that regulate cell-cycle progression (e.g., *GADD45* participating in growth arrest and DNA damage) are induced by iron chelators (Gao and Richardson, 2001). On the other hand, GADD45 arrests the cell cycle and is involved in DNA nucleotide excision repair (Kastan *et al.*, 1992; Levine, 1997). *GADD45B* expression is also inducible by tissue factor pathway inhibitor, TFPI (Shirotani-Ikejima *et al.*, 2002). *TFPI* was also expressed by CR in the present study and its expression might also related to cell cycle arrest at G1/S phase. NOL1/NOP2/Sun domain family 2 protein (NSUN2) is an RNA methyltransferase. NSUN2 has high sequence homology to mammalian Misu protein, which contains SUN domain. It has been reported that *Misu* expression is highest in S phase (Frye and Watt, 2006). Therefore, in the present study expression of *NSUN2* indicates that the cells in the rotifers were mostly in S phase of the cell cycle under CR.

In addition to its roles in cell cycle delays, BCCIP functions in genome stability through its direct interaction with homologous recombinational repair (HRR) protein, BRCA2 (Lu *et al.*, 2005), indicating BCCIP participates in DNA repair. Mismatches result from DNA replication errors and genetic recombination, and DNA damages are fixed in the genome if uncorrected (Crouse, 1996; Kolodner, 1996; Modrich and Lahue, 1996; Modrich, 1997). Mismatches are corrected by mismatch repair proteins, Msh2p and Msh6p (Alani, 1996; Iaccarino *et al.*, 1996). In this study *Msh6p* expression was observed under CR, suggesting that CR induces the expression of genes involved in DNA repairs and their expression is attributable to genome stability.

CR not only functions at cellular and molecular levels, but also has many effects on metabolism of various animals. Lowered plasma glucose content consequent to a variety of CR regimens has been demonstrated in mouse, rat, and non-human primates of different ages (Masoro *et al.*, 1992; Harris *et al.*, 1994; Kemnitz *et al.*, 1994; Cefalu *et al.*, 1995). The genes encoding glycogen phosphorylase (*Glase*) and β-galactosidase (*Glb*) were expressed by CR in this study. Glycogen is degraded for metabolic use by Glase, liberating glucose units from the liver cells into bloodstream. Glb (also called lactase) is commonly used to cleave lactose into galactose and glucose. Lactase activity is consistently higher in dietary restricted animals than their counterparts fed *ad libitum* (Maier *et al.*, 2007). The expression of these genes is likely to regulate the body glucose levels required for maintaining important metabolic processes under CR. Enzyme IPS2 in *S. cerevisiae* catalyzes leucine biosynthesis (Ryan *et al.*, 1973). Leucine is an essential amino acid and also a potent activator of serine/threonine kinase involved in many cellular processes, including protein synthesis, cell growth, and metabolism (Inoki *et al.*, 2005; Cota *et al.*, 2006). In this study, the gene encoding serine/threonine protein kinase with TRP repeats (*PK-TRP*) was also expressed together with *IPS2*.

The members of the 14-3-3 family mediate interactions between diverse components having different biological activities and 14-3-3 proteins have been implicated in the regulation of cell cycle (Stoica *et al.*, 2006). Because of its diverse biological functions, the expression of the gene would be important for various biological processes under CR.

The transposable element (TE) gene encodes transposase (Tsase), which confers translocation of TE in the genome. It has been observed that TEs are differentially expressed in black tiger shrimp *Penaeus monodon* exposed to

a range of environmental stressors (de la Vega *et al.*, 2007). In fish, the exprssion of *Tsase* is induced by external stimuli such as toxin, stress, and bacterial antigens (Krasnov *et al.*, 2005). In this study, *Tsase* and the gene encoding viral envelope glycoportein (*gp*) were also expressed by CR. Based on previous findings and the present results, *Tsase* expression seems necessary for responding to various environmental stressors.

Early B cell factor (EBF) is a transcription factor known to be responsible for the development of B lymphocytes. *Collier* (*col*, the *Dorsophila* ortholog of the vertebrate gene encoding EBF) has been implicated in developing lamellocytes, which function in cellular immune response to parasitization in *Drosophila* (Crozatier *et al.*, 2004). Therefore, the expression of *EBF* might protect the rotifer from various potential diseases under CR.

CR functions at physiological, cellular, and molecular levels. At cellular levels, CR mediates cell proliferation and inhibits the organ-specific cell proliferation (Lok *et al.*, 1990; Lu *et al.*, 1993). CR reduces the follicle size of reproductive females of tree lizard *Urosaurus ornatus* (French *et al.*, 2007). Verdone-Smith and Enesco (1982) reported that the rate of nuclear division in gastric glands and vitellarium (yolk-secreting gland) of the rotifer *A. brightwelli* was retarded by dietary restriction. A trade-off between life span and lifetime fecundity has been proposed as an alternative life-history strategy of *B. plicatilis* under starved conditions (Yoshinaga *et al.*, 2000).

Conclusion

The primary role of CR seems to postpone reproductive senescence upon the somatic maintenance, thereby animals gain an increased chance of survival with a reduced intrinsic rate of senescence. Based on previous studies and the present findings, we predict here that these aspects of CR may also be found in the calorie-restricted rotifer of *B. plicatilis* (Ishikawa strain). Most of the up-regulated genes in the present study may be important for the rotifer in maintaining their metabolic processes under CR. It is likely that there are other genes still remaining out of the present study due to differences in experimental procedures. However, the differentially expressed genes observed in the present study shed light into the molecular mechanisms that control the metabolic processes of a particular animal under CR.

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