Original article

Tocotrienol-Rich Fraction Modulates Genes Expression in Oxidative Stress-induced *Caenorhabditis e legans*

Goon Jo Aan[1], Noralisa Abdul Karim[2], Mohd Shahril Aszrin Zainudin[3], Suzana Makpol[4]

Abstract:

Objective: The study was done to determine the effect of Tocotrienol rich fraction (TRF) on the expression of RNAs in *C. elegans* under oxidative stress. ***Methods:*** The nematodes were divided into 4 groups and treated accordingly: control; TRF; hydrogen peroxide (H_20_2); TRF treatment before and after H_20_2-induction (TRF+H_20_2+TRF). Expressions of RNAs were analyzed with Affymetrix Genechip *C. elegans* Genome Array and Genespring GX11 software where differentially expressed genes were further analyzed using gene ontology (GO). Selected genes (unc-15, cit-1.2, ftn-1, rsks-1, unc-4 and daf-12) were analyzed with RT-qPCR to validate the results. ***Results:*** TRF modulated the expression of 314 genes involved in determination of adult lifespan, regulation of growth and lipid modification. A total of 440 genes involved in RNA metabolic processes, transcription, growth and differentiation of muscle and nerve cells were differently expressed following H_20_2 induction. TRF treatment before and after H_20_2-induction resulted in 438 differentially expressed genes involved in RNA metabolic processes, transcription, response to xenobiotic stimulus and protein amino acid phosphorylation. ***Conclusion***: TRF modulates the expression of genes involved in the regulation of lifespan in *C. elegans*.

Keywords: tocotrienol; C.elegans; antioxidant; oxidative stress

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Introduction

One unique feature in organism life cycle is the aging process. It occurs when the physiological system experiences a progressive functional decline which leads to poor maintenance of homeostasis and consequently death[1]. Many studies have been conducted to investigate the process and a number of theories have emerged to explain the underlying mechanism. Of the many theories, the free radical theory of aging is widely accepted because many experimental studies conducted since its introduction supported the hypothesis that free radicals cause senescence. Hydrogen peroxide (H_2O_2) is one of the most abundant ROS in living cells [2]. It is produced as a by-product of aerobic metabolism and it has been demonstrated to be involved in apoptosis pathway, induction of intracellular oxidative stress and acceleration of aging [3,4].

It has been shown in many organisms ranging from invertebrates to humans that the increase in oxidative damage to lipid, protein and DNA correlated with increasing age[5,6]. Among major model organism, nematode *Caenorhabditis elegans* (*C. elegans*) is well-suited for the aging research. *C. elegans* offers great advantage as a well-established model due to its short life cycle, large progeny production in a

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short time, ease of maintenance in laboratory as well as morphological simplicity which facilitates the aging study[7].Moreover, the completely sequenced genome of this nematode makes it preferable for the molecular genetic analysis to be carried out[8].There is also a strong conservation between *C. elegans* and mammals in cellular and molecular principles where 60-80% of human genes have been identified in *C. elegans*[9].

Many genes have been identified to control aging in *C. elegans*[10]. For example, mutants in *age-1* gene which encodes class-I phosphatidylinositol 3-kinase (PI3K), have been found to have long lifespan, increased resistance to stress and impaired reproduction[11]. Elsewhere, nematodes with mutation in the insulin/IGF*/daf-2* signaling pathway had prolonged lifespan as compared to the wild type[12]. Under normal conditions, *daf-2* gene in *C. elegans* has also been identified to signal the AGE-1 PI3K, PDK-1 and AKT-1/2 kinases to negatively regulate *daf-16*, a forkhead transcription factor FOXO which is essential for longevity and stress resistance. The function of this pathway in mediating longevity and metabolism is also well-conserved in *Drosophila sp.* and mammals[13,14].

Over the years, there has been a growing effort among researchers to understand the genetic basis of aging which shows promises that lifespan and aging process can be genetically manipulated. The search for compounds that can delay aging and extend lifespan in model organism is widely conducted. Therefore, studies on modulation of endogenous antioxidant defences with antioxidants supplementation can be regarded as promising strategies to delay aging.

Vitamin E is a lipid-soluble antioxidant which can be found naturally in vegetable oil and lipid-rich plant product[15].Vitamin E occurs in nature in eight different isoforms: α-, β-, γ- and δ-tocopherols and α-, β-, γ- and δ-tocotrienols. Tocotrienols differ from tocopherols in that they have an isoprenoid rather than a saturated phytyl side chain[16]. It is well-accepted that tocotrienols is a more potent antioxidant as compared to tocopherols[17,18]. Apart from its antioxidant properties, tocotrienols are beneficial to health due to its neuroprotective effect, anti-carcinogenic as well as cholesterol-lowering property[19-21]. It has also been demonstrated that tocotrienols play a role in regulating signal transduction of cell death pathway[22]. However, the molecular mechanism of how tocotrienols modulate the aging pathway is still scarce. Adashi and Ishii (2000) reported that tocotrienols reduced the accumulation of protein carbonyl and consequently extended the mean lifespan of *C. elegans*[23]. In our previous study, TRF at a concentration of 50 µg/ml was found to restore the mean lifespan and reduce the accumulation of lipofuscin in oxidative stress-induced *C. elegans*[24]. Based on these results, it was hypothesized that TRF treatment stimulated cellular regeneration that lead to restoration of lifespan in *C. elegans* by modulating the expression of genes in various metabolic pathways. To elucidate the molecular mechanism of lifespan extension by this antioxidant, the microarray technology is further used to analyze the changes in gene expression with TRF treatment in the present study.

Materials and methods

Nematode Strain and Culture Conditions

The wild type *C. elegans* strain (N2) were grown at 20°C on nematode growth medium (NGM), with *E. coli* OP50 as food source according to the method by Brenner (1974)[26]. All of the maintenance and handling procedures for the nematodes were conducted as described previously[26].

H_2O_2 -induction of oxidative stress and TRF treatment

The TRF Tri-E 70 was supplied by Sime Darby Bioganic, previously known as Golden Hope Bioganic (Selangor, Malaysia), which contains 70% of total vitamin E (15% α-tocopherol, 23% α-tocotrienol, 2% β-tocotrienol, 20% γ-tocotrienol and 11% d-tocotrienol). The optimum dose of H_2O_2(0.3 mM) for induction of oxidative stress, and the optimum concentration of TRF (50 µg/ml) to be treated in *C. elegans* were predetermined in our previous study[24]. The nematodes were divided into four groups and treated accordingly: control, H_2O_2 induction (H_2O_2), TRF treatment (TRF), and TRF treatment pre- and post- H_2O_2 induction (TRF+H_2O_2+TRF). The TRF treatments were given from hatching until day 3 of adulthood to determine the ability of *C. elegans* to recover from H_2O_2-induced oxidative stress during the developmental phase.

RNA Extraction and Quantification

At day 3 of adulthood, worms were isolated from NGM agar plates and washed several times with M9 buffer (3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, 1 ml 1 M $MgSO_4$, dH_2O to 1 L) to remove any contaminating *E. coli*. Total RNA from each group was extracted using TRIzol reagent following manufacturer's instruction (Invitrogen, USA), purified using RNeasy Mini Kit (Qiagen, USA) and assessed for RNA quality and quantity by using NanoDrop 1000 Spectrophotometer and Agilent 2100 Bioanalyzer. Through Agilent 2100

Bioanalyzer, the RNA integrity was assessed by RNA Integrity Number (RIN) that indicates the degree of degradation of isolated RNA (RIN 1 – 10; with 1 being the most degraded and 10 being the most intact)[27]. Samples with RIN of >7 were chosen for further experiments to ensure experimental accuracy.

C. elegans Whole Genome DNA Microarray

Purified total RNA was amplified and labelled with biotinylated nucleotide analog using Affymetrix GeneChip 3' IVT Express Kit according to the manufacturer's protocol. The biotin-labeled amplified RNA (aRNA) was then fragmented before being hybridized onto the genome array. The hybridization controls was used to monitor the hybridization quality, which composed of a mixture of biotinylated and fragmented cRNA of *bioB*, *bioC*, *bioD* and *cre* prepared in staggered concentrations (1.5, 5, 25, and 100pM respectively).Using Affymetrix Genechip *C. elegans* Genome Array, 22625 transcripts were screened for changes in expression patterns and levels. After 17 hours of hybridization, washing and scanning of the arrays was carried out using Gene Chip Hybridization, Wash and Stain Kit (Affymetrix). The performance of every chip was evaluated for indication of successful hybridization, washing and staining procedures using Affymetrix Expression Console software.

Analysis of Microarray Data

The probe intensity values generated by the Affymetrix scanner were stored in .CEL files format, and Robust Multichip Average (RMA) algorithm was applied to normalize all the data before further downstream data analyses being carried out in Genespring GX11 software. Briefly, the raw signals were log transformed and normalized using the Percentile shift normalization method, the value was set at 70th percentile. Probes with intensity values below 20th percentile were filtered out using the "Filter Probesets by Expression" option in Genespring GX11. The unpaired T-test was applied to find the candidates for differential expression, and genes with significant signal level between two different conditions (p-value cut off at 0.05) were collected. Expression ratios between pairs of compared conditions were log2 transformed. The differentially expressed genes were further subjected to hierarchical clustering analysis (Distance metric: Pearson centered; Linkage rule: Centroid) to group the samples according to the degree of similarity of their expression profile. Principle Component Analysis (PCA) was also performed to check whether samples from similar experimental condition were clustered together or not. Genes with log2 (fold change) of ≥1.2 (up-regulated) or ≤-1.2 (down-regulated) were selected as candidate genes for further functional analysis using Database for Annotation, Visualization and Integrated Discovery (DAVID) version 2.1. DAVID 2.1 tool has been widely used to elucidate the biological meanings of gene lists derived from microarray study through identification of enriched GO terms[28]. The differentially expressed genes were mapped to the Search Tool for the Retrieval of Interacting Genes (STRING) database to investigate the functional interaction among genes and proteins, thus identify the target genes for validation according to their confidence, evidence, actions or type of interaction in the protein networks.

RT-qPCR Analysis

Validation of selected differentially expressed genes (Table 4) primarily derived from microarray analysis were performed through RT-qPCR analysis (iCycler iQ5™ RT-PCR detection system, Bio-Rad, USA). Gene-specific primers were designed using the online tools, Primer3 (http://frodo.wi.mit.edu.primer3/) based on the complete mRNA sequences obtained from NCBI (http://www.ncbi.nlm.nih.gov/). RT-PCR analysis was carried out by using iQ™ SYBR Green Supermix kit (Bio-Rad, USA) and cDNA sample for each selected genes were synthesized to be used as a target for amplification step. The optimal annealing temperature for each target genes was assessed via

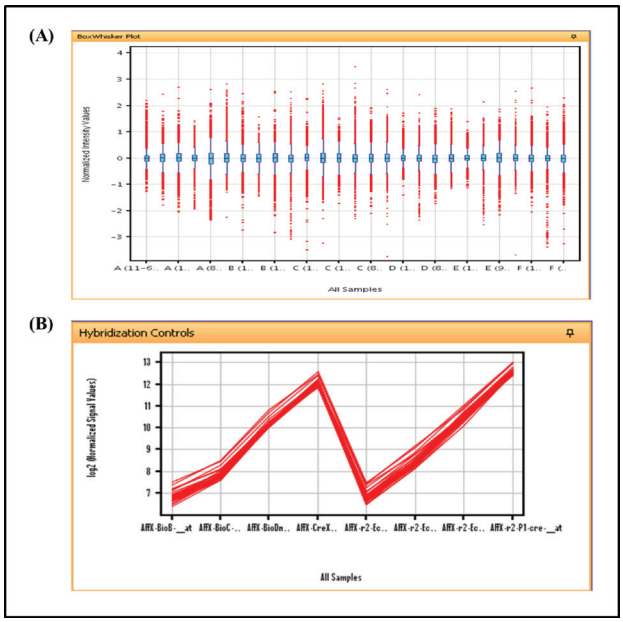

Fig. 1 (A) Box-Whisker plot shows the distribution of normalized intensity values of the probe sets within all samples. (B) The hybridization control plot represents the signal intensities of the control probes (bioB, bioC, bioD and cre) in staggered concentration in each sample.

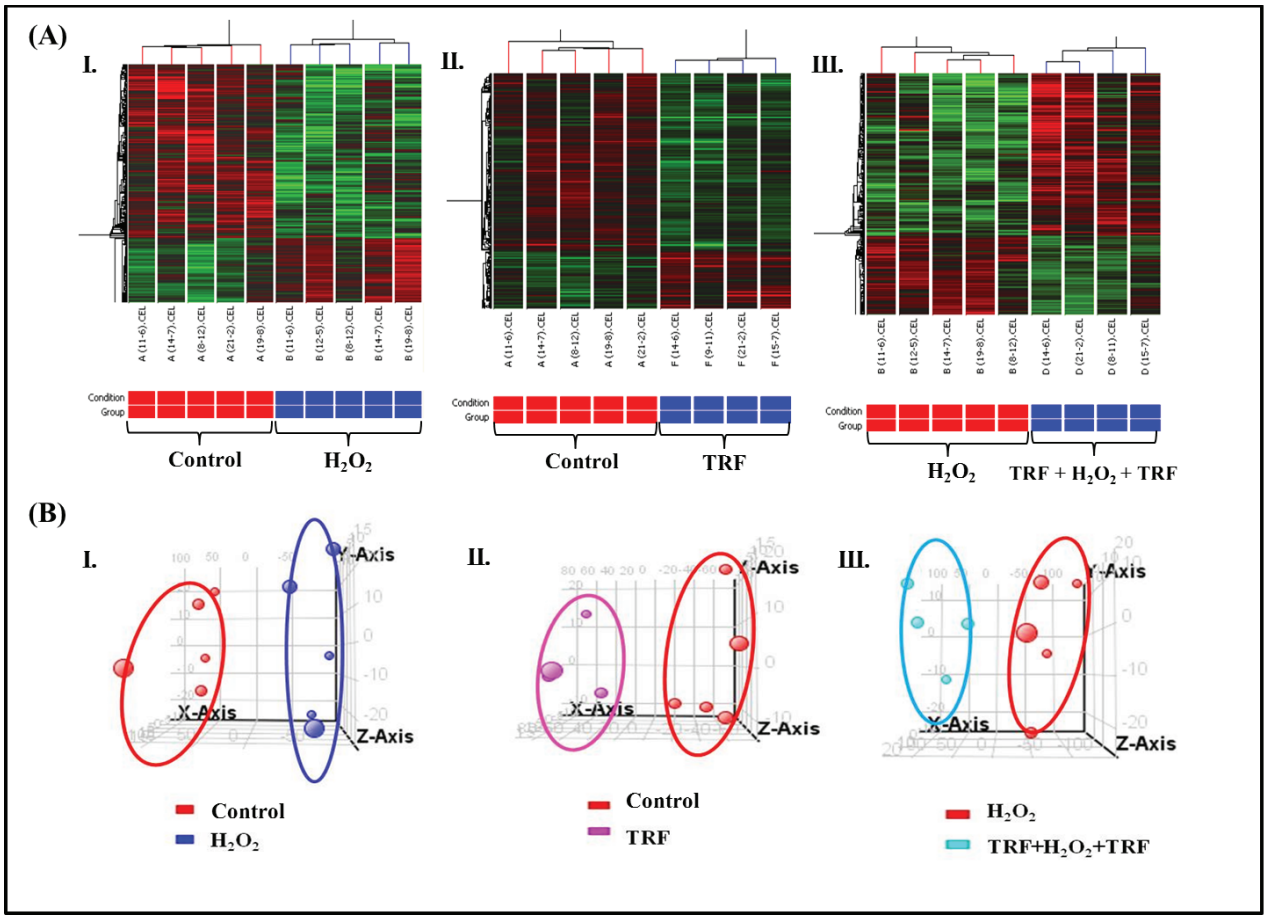

Fig. 2 (A) Heat maps illustrating the up-regulated genes (red entities) and down-regulated genes (green entities) generated from the hierarchical clustering between two experimental conditions. (B) PCA plots showing the grouping of samples with distinct experimental condition. Each dot represents a sample (array) and colored according to experiment grouping assigned in Genespring GX11. The projections of the samples on the first three principal components are shown. (C) Eigen values plot during PCA clustering between two experimental conditions.

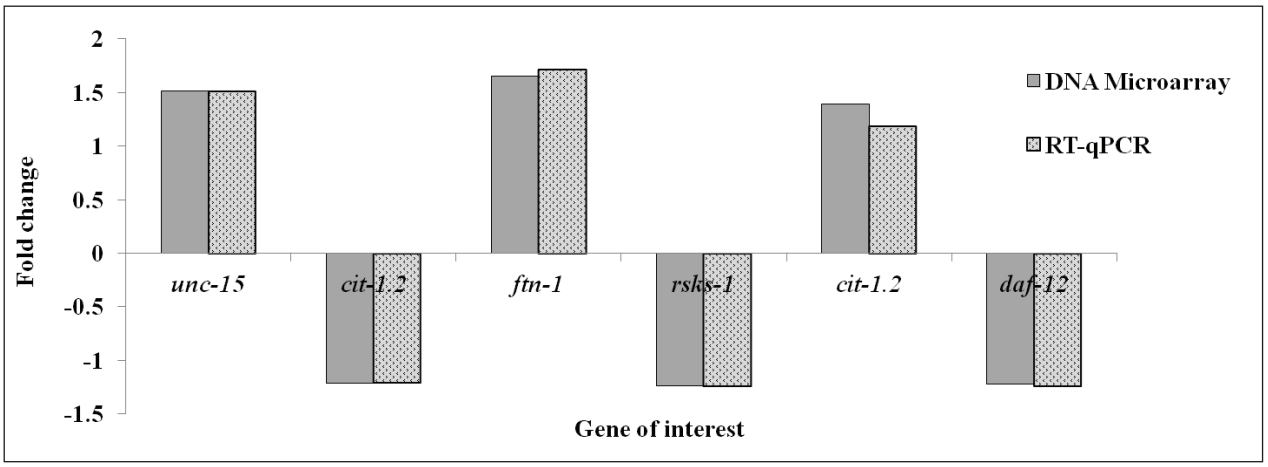

Fig. 3 Validation of microarray data using RT-qPCR assay on selected significant genes.

temperature gradient feature in the iQ5™ system. The relative expression values (REV) for all target genes were calculated based on the C_T value obtained from the amplification curve.

Ethical clearance: This research was approved by the ethics committee of UKM Molecular Biology Institute, Kuala Lumpur, Malaysia.

Results

Gene Expression Changes in C. elegans

The gene expression data was collected from four to five independent experiments (4-5 biological replicates), and box-whisker plot was used to

visualize the data after it was normalized and summarized through RMA algorithm in Genespring GX 11 Software (Fig. 1). Samples quality was assessed through hybridization controls which in turn depicts the hybridization quality. All samples were in good quality and the hybridization process was done properly as shown by the consistency of profile intensity plots generated and there was no deviation of signal from the expected intensity profile. Comparisons of genes expression level were done between the following three sets of interpretations: (i) H_2O_2 group versus control; (ii) TRF group versus control; and (iii) TRF+ H_2O_2+TRF group versus H_2O_2 group. Probes with intensity values below 20th percentile were filtered out, resulting in 17 937 genes for [H_2O_2 vs Control] interpretation, 18 022 genes for [TRF vs Control] interpretation, and 18 053 genes for [TRF+ H_2O_2+TRF vs H_2O_2] interpretation, respectively.

Based on statistical analysis (unpaired T-test; $p<0.05$; log2 fold change cut-off at 1.2), we found a total of 440 genes to be differentially expressed upon induction with H_20_2 in which 248 genes were up-regulated by 1.2 fold and 192 genes were down-regulated by 1.2. A comparison between control and TRF group showed 314 genes to be differentially expressed where 115 genes were found to be up-regulated and 199 genes to be down-regulated. In addition, TRF treatment before and after H_2O_2-induction (TRF+H_20_2+TRF group) resulted in 438 differentially expressed genes where 177 genes were up-regulated and 261 genes were down-regulated. Clustering analyses on differentially expressed genes showed that within group samples were clustered according to the degree of similarity of their expression profiles as shown in the dendrograms and that samples representing the same experimental condition were grouped closer together in the PCA plots (Fig. 2).

Gene Ontology Analysis on Differentially Expressed Genes through DAVID online tools

Lists of up- and down-regulated genes were uploaded to DAVID 2.1 tool. GO analysis was performed using Affymetrix probe set identifiers to calculate statistically enriched GO biological process annotations for the differentially expressed genes. The output of functional annotation analysis using DAVID 2.1 online tools appeared as a list of GO terms (mainly biological processes) that were significantly enriched (P-value <0.05). Besides that, the enrichment scores shown in the generated output represent the overall importance (enrichment) of gene groups.

Functional annotation analysis of 440 differentially expressed genes between control and H_2O_2 group showed that 24 GO terms were significantly enriched ($p<0.05$) (Table 1). Of these, the regulation of RNA metabolic process and regulation of RNA transcription involving a large number of genes (30-34 genes) were significantly affected. Interestingly, DAVID functional analysis on 314 significant genes that were changed upon TRF treatment showed that a large fraction of genes were involved in the regulation of growth and growth rate. On top of that, biological processes associated with lifespan determination, lipid modification and glycosylation, and also aging were enriched with TRF treatment (Table 2). TRF treatment before and after H_2O_2 induction also remarkably affected the biological processes in *C. elegans*. Functional analysis of 438 differentially expressed genes between H_2O_2 and TRF+H_2O_2+TRF groups revealed 11 statistically significant biological processes in which most of the genes were involved in the regulation of RNA metabolic process and transcription (Table 3).

Differentially expressed genes in selected biological terms were mapped to network of interactions using online tool, STRING (string-db.org) to explore the pattern of regulation of biological process based on the functional interactions between genes. Gene of interest (GOI) was selected from the interaction networks (see Supplementary data) for further validation by RT-qPCR assay (Table 4). The finding from RT-qPCR assay indicated that all GOIs showed similar expression pattern to that of those derived from microarray experiment and thus validated the microarray data (Fig. 3).

Discussions

Nematodes react to oxidative stress condition by launching the response mechanism through the induction of gene expression. In our previous study, exposure to exogenous H_2O_2 shortened the lifespan of *C. elegans* and increased the accumulation of oxidative biomarkers such as lipofuscin and 8-hydroxydeoxyguanosine [24]. Although little is known about H_2O_2-responsive genes in *C. elegans*, we believe that exposure to exogenous H_2O_2 might regulate the changes in *C. elegans* genes expression based on several lines of evidences that explained its important roles in the activation of signaling pathways to stimulate cell proliferation[29,30] and differentiation in multicellular organisms[31,32].

Differential genes expression analysis using unpaired t-test in Genespring revealed that H_2O_2 affected the transcription process of genes such as *cit-1.2*

which has been reported to affect the lifespan of *C. elegans* through RNA interference studies[33]. *cit-1.2* gene encodes the protein cyclin-T2 that belongs to the cyclin family[34]. It stimulates RNA and protein binding as well as regulates the enzyme activity and transcription. Cyclin T/CDK9 complex forms the transcription factor, P-TEFb that stimulates transcriptional elongation [35]. According to Kohoutek et al. (2009), a decreased in cyclin-T2 expression down-regulates the expression of genes involved in transcription and metabolism[36]. Reduced expression of cyclin-T2 was found to down-regulate a group of genes that are involved in response to stimuli and stress, muscle development, negative regulation of transcription and metabolism and signal transduction[37]. This gene has been found to have a pro-longevity effect on *C. elegans*[33].

Hundreds of genes have been identified to be involved in the longevity of *C. elegans*[38]. Though it was uncertain if tocotrienol could modulate the expression of these genes, previous studies on the molecular aspect of tocotrienol reported that it modulated genes expression in some disease models[39-41]. GO analysis in this study indicated that TRF affected the expression of genes involved in important age-related biological processes such as determination of adult lifespan and regulation of growth. Interestingly, many of these genes such as *daf-2*, *dod-20, dod-21*, *lbp-7*, *rsks-1* and *ftn-1* were found to be associated with aging and/or longevity in *C. elegans* according to the AnAge database which denotes the influence of genes to longevity based on longevity records and life history traits of organism and species[42].

TRF treatment significantly increased *ftn-1* expression and decreased *rsks-1* expression ($p<0.05$) as compared to control group.*ftn-1* encodes *FTN-1* or feritin which is essential for normal lifespan under iron stress conditions and has been reported to be essential for embryogenesis[34]. Previously,*ftn-1* gene expression was found to be directly proportional to the lifespan of *C. elegans*. Mutations in *ftn-1* gene was found to shorten the lifespan of *C. elegans*[43], while increased expression of *ftn-1* extended the lifespan of yeast[44,45]. *rsks-1* encodes a putative ribosomal protein S6 kinase (S6K) that is orthologous to human p70S6K protein. *rsks-1* is an age-related gene that has anti-longevity effect in which increased expression leads to shortened lifespan of *C. elegans*[38]. In this study, *ftn-1* and *rsks-1* genes were thought to affect *C. elegans* lifespan via several signals pathways that is triggered by the reduction of DAF-2 signaling. Expression of *ftn-1* gene is coordinately regulated by insulin/IGF-1 and HIF signaling pathways, previously known to interact in the regulation of stress resistance and lifespan[46]. On the other hand, *rsks-1* expression is regulated by TOR signaling which acts as a downstream target of insulin/IGF signaling that is known to be important in modulating aging and other age-related diseases[47]. The fact that TRF modulated the expression of these aging related genes known to be involved in different biological processes indicates a promising role of TRF in lifespan extention.

Pre and post treatment of TRF modulated the expression of 438 genes in H_2O_2 induced nematodes. Some of these genes have been reported to be associated with aging process in *C. elegans*. For instance, RNA interference of *cit-1.2* gene[33] and mutation in *daf-12*, *unc-4* and *unc-26* genes were found to affect the lifespan of *C. elegans*[48-50]. Based on network interaction analysis through STRING online tool, *cit-1.2* and *daf-12* genes were found to be involved in the regulation of aging process. The increased expression of *cit-1.2* gene with TRF treatment in H_2O_2-induced nematodes supports our previous finding that TRF restores the lifespan of oxidative stress-induced *C. elegans*[24].

In contrast with *cit-1.2* gene, *daf-12* gene was down-regulated in TRF+ H_2O_2+TRF group as compared to H_2O_2 group. *daf-12* encodes a member of the steroid hormone receptor superfamily that affects dauer formation downstream of the TGF- and insulin signaling pathways[34] *daf-12* is essential for normal development of nematodes, as well as responsible to ensure survival under harsh conditions and most importantly it is involved in modulating aging process in nematodes[51]. This gene showed anti-longevity effect in which its activation leads to a shorter lifespan of hermaphrodites[52]. Mutations of this gene can extend the lifespan of *C. elegans* by almost 4 times more than normal lifespan[48].

Conclusion

Besides having antioxidant properties that can protect against oxidative damage, TRF also modulated the expression of genes in the insulin/IGF-1 signaling pathway and its downstream pathways involved in the regulation of lifespan in *C. elegans*.

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Conflict of Interest : None

Authors's contribution:

Data gathering and idea owner of this study: Goon JA, Abdul Karim N, Zainudin MSA

Study design: Goon JA, Abdul Karim N, Zainudin MSA, Makpol S

Data gathering: Goon JA, Abdul Karim N, Zainudin MSA, Makpol S

Writing and submitting manuscript: Goon JA

Editing and approval of final draft: Goon JA, Abdul Karim N, Zainudin MSA, Makpol S

Biological process (GO term)	*P*-value	Enrichment score	Differentially expressed genes
Regulation of RNA metabolic process	1.71E-04	1.56	*ceh-43, end-1, nhr-213, puf-9, nhr-233, nhr-181, nhr-88, dmd-3, K04C1.3, nhr-117, ref-2, C34D1.1, nhr-161, tab-1, nhr-184, nhr-90, C07E3.6, nhr-78, C34E11.2, sel-7, T22H9.4, asd-2, C33D3.3, ceh-10, Y92H12BL.1, nhr-201, F49E12.6, elt-6, sdc-1, dmd-7, nhr-11, nhr-2, mls-2*
Cellular component assembly in morphogenesis	3.47E-04	2.65	*unc-15, mup-2, unc-60, unc-89, unc-27*
Myofibril assembly	3.47E-04	2.65	*unc-15, mup-2, unc-60, unc-89, unc-27*
Actomyosin structure organization	3.47E-04	2.65	*unc-15, mup-2, unc-60, unc-89, unc-27*
Regulation of transcription, DNA-dependent	3.67E-04	1.56	*dmd-7, T22H9.4, ceh-43, nhr-2, K04C1.3, C33D3.3, nhr-181, F49E12.6, C43H8.1, nhr-11, nhr-213, mls-2, Y46E12A.4, nhr-184, nhr-117, nhr-90, ceh-10, tab-1, end-1, C34E11.2, nhr-161, nhr-201, nhr-88, nhr-233, sdc-1, C34D1.1, C07E3.6, elt-6, dmd-3, nhr-78*
Regulation of transcription	4.49E-04	1.56	*dmd-7, T22H9.4, ceh-43, nhr-2, K04C1.3, C33D3.3, ngn-1, nhr-181, F49E12.6, C43H8.1, nhr-11, nhr-213, mls-2, Y46E12A.4, nhr-184, nhr-117, R155.4, nhr-90, ceh-10, tab-1, end-1, C34E11.2, mdt-29, nhr-161, nhr-201, nhr-88, nhr-233, sdc-1, C34D1.1, C07E3.6, elt-6, dmd-3, nhr-78, cit-1.2*
Striated muscle cell differentiation	5.63E-04	2.65	*unc-15, mup-2, unc-60, unc-89, unc-27*
Striated muscle cell development	5.63E-04	2.65	*unc-15, mup-2, unc-60, unc-89, unc-27*
Muscle cell development	7.01E-04	2.65	*unc-15, mup-2, unc-60, unc-89, unc-27*
Muscle cell differentiation	0.001	2.65	*unc-15, mup-2, unc-60, unc-89, unc-27*
Cellular component morphogenesis	0.002	2.65	*lad-2, unc-15, rac-2, mls-2, unc-60, unc-27, dgn-1, mup-2, unc-129, unc-89*
Cellular macromolecular complex subunit organization	0.004	1.11	*unc-26, unc-15, unc-60, hil-2, unc-89, hil-3, tbb-4, his-24*
Neuron development	0.011	1.60	*lad-2, mup-2, rac-2, mec-8, unc-129, dgn-1*
Cell morphogenesis involved in neuron differentiation	0.013	1.60	*lad-2, mup-2, rac-2, unc-129, dgn-1*
Axonogenesis	0.013	1.60	*lad-2, mup-2, rac-2, unc-129, dgn-1*
Skeletal myofibril assembly	0.013	1.60	*unc-15, unc-60, unc-89*
Cell morphogenesis involved in differentiation	0.015	1.60	*lad-2, mup-2, rac-2, unc-129, dgn-1*
Actin cytoskeleton organization	0.015	1.60	*unc-15, mup-2, unc-60, unc-89, unc-27*
Neuron differentiation	0.017	1.60	*lad-2, mup-2, rac-2, mec-8, unc-129, dgn-1*
Neuron projection morphogenesis	0.017	1.60	*lad-2, mup-2, rac-2, unc-129, dgn-1*

Macromolecular complex subunit organization	0.018	1.11	*unc-26, unc-15, unc-60, hil-2, unc-89, hil-3, tbb-4, his-24*
Neuron projection development	0.021	1.60	*lad-2, mup-2, rac-2, unc-129, dgn-1*
Axon guidance	0.033	1.60	*lad-2, rac-2, unc-129, dgn-1*
Cellular macromolecular complex assembly	0.034	1.11	*unc-15, hil-2, unc-89, hil-3, tbb-4, his-24*

The count indicates the number of observations from the input of the 440 genes. Terms are listed in decreasing order of significance (P-value).

Biological process (GO term)	***P*-value**	**Enrichment score**	**Differentially expressed genes**
Aging	0.004	2.42	*C32H11.9, dod-21, lbp-7, C36E8.1, ugt-1, ftn-1, dod-20, tir-1, thn-1, DAF-2, ges-1, C43H8.1, rsks-1*
Multicellular organismal aging	0.004	2.42	*C32H11.9, dod-21, lbp-7, C36E8.1, ugt-1, ftn-1, dod-20, tir-1, thn-1, DAF-2, ges-1, C43H8.1, rsks-1*
Determination of adult lifespan	0.004	*2.42*	*C32H11.9, dod-21, lbp-7, C36E8.1, ugt-1, ftn-1, dod-20, tir-1, thn-1, DAF-2, ges-1, C43H8.1, rsks-1*
Fatty acid metabolic process	0.009	*1.42*	*acs-2, F58F9.7, acs-2, mlcd-1, fat-3*
Lipid modification	0.021	*1.42*	*ugt-1, F58F9.7, ugt-9, ugt-25, ugt-33*
Positive regulation of growth	0.028	*1.49*	*W08A12.4, T21D12.9, T07A9.9, pup-2, egl-15, D2030.3, T16G1.6, ugt-1, tir-1, Y71H2AM.17, F26A1.14, ZK858.1, DAF-2, Msi3p, ulp-4, ngp-1, C45G9.5, C36E8.1, unc-13, Y56A3A.2, Y105E8A.25, C27H5.4, nhr-8, T23B12.2, hid-1, egl-18, plc-1, ptr-20, mrt-2, fat-3, spc-1, sma-1, F19C7.1, C43H8.1, enol-1, elo-3, skr-15, ddr-2, F56C9.7, F49H6.5, C35E7.8, vha-2, vha-3, fkh-5, clec-210*
Regulation of growth	0.031	*1.49*	*W08A12.4, T21D12.9, T07A9.9, pup-2, egl-15, D2030.3, T16G1.6, ugt-1, tir-1, Y71H2AM.17, F26A1.14, ZK858.1, DAF-2, Msi3p, ulp-4, ngp-1, C45G9.5, C36E8.1, unc-13, Y56A3A.2, Y105E8A.25, C27H5.4, nhr-8, T23B12.2, hid-1, egl-18, plc-1, ptr-20, mrt-2, fat-3, spc-1, sma-1, F19C7.1, C43H8.1, enol-1, elo-3, skr-15, ddr-2, F56C9.7, F49H6.5, C35E7.8, vha-2, vha-3, fkh-5, clec-210, unc-76*
Positive regulation of growth rate	0.035	*1.49*	*W08A12.4, T07A9.9, pup-2, egl-15, D2030.3, T16G1.6, ugt-1, tir-1, Y71H2AM.17, F26A1.14, ZK858.1, DAF-2, Msi3p, ulp-4, ngp-1, C45G9.5, C36E8.1, unc-13, Y56A3A.2, Y105E8A.25, C27H5.4, nhr-8, T23B12.2, hid-1, egl-18, plc-1, mrt-2, fat-3, sma-1, F19C7.1, C43H8.1, enol-1, elo-3, skr-15, ddr-2, F56C9.7, F49H6.5, C35E7.8, vha-2, vha-3, fkh-5, clec-210*
Regulation of growth rate	0.035	*1.49*	*W08A12.4, T07A9.9, pup-2, egl-15, D2030.3, T16G1.6, ugt-1, tir-1, Y71H2AM.17, F26A1.14, ZK858.1, DAF-2, Msi3p, ulp-4, ngp-1, C45G9.5, C36E8.1, unc-13, Y56A3A.2, Y105E8A.25, C27H5.4, nhr-8, T23B12.2, hid-1, egl-18, plc-1, mrt-2, fat-3, sma-1, F19C7.1, C43H8.1, enol-1, elo-3, skr-15, ddr-2, F56C9.7, F49H6.5, C35E7.8, vha-2, vha-3, fkh-5, clec-210*
Lipid glycosylation	0.043	*1.42*	*ugt-1, ugt-9, ugt-25, ugt-33*

The count indicates the number of observations from the input of the 314 genes. Terms are listed in decreasing order of significance (P-value).

Biological process (GO term)	*P*-value	Enrichment score	Differentially expressed genes
Regulation of transcription, DNA-dependent	1.46E-05	3.22	*nhr-212, C33D3.3, fos-1, F49E12.6, nhr-143, nhr-45, tbx-39, nhr-182, nhr-206, cog-1, nhr-117, nhr-109, nhr-10, lin-42, nhr-194, nhr-124, nhr-97, C43H8.1, nhr-230, unc-4, daf-12, fax-1, nhr-201, nhr-88, sknr-1, nhr-102, nhr-233, C34D1.1, C29F9.5, lin-28, nhr-241, C50A2.4, dmd-3, nhr-173*
Regulation of RNA metabolic process	1.60E-05	3.22	*nhr-212, C33D3.3, fos-1, F49E12.6, nhr-143, nhr-45, tbx-39, nhr-182, nhr-206, cog-1, nhr-117, nhr-109, nhr-10, lin-42, nhr-194, nhr-124, nhr-97, C43H8.1, nhr-230, unc-4, daf-12, fax-1, nhr-201, nhr-88, sknr-1, nhr-102, nhr-233, C34D1.1, C29F9.5, lin-28, nhr-241, C50A2.4, dmd-3, nhr-173*
Regulation of transcription	1.31E-04	3.22	*nhr-212, C33D3.3, fos-1, F49E12.6, nhr-143, nhr-45, tbx-39, nhr-182, hlh-16, nhr-206, cog-1, nhr-117, nhr-109, nhr-10, lin-42, nhr-194, nhr-124, nhr-97, C43H8.1, nhr-230, unc-4, daf-12, fax-1, nhr-201, nhr-88, sknr-1, nhr-102, nhr-233, C34D1.1, C29F9.5, lin-28, nhr-241, C50A2.4, dmd-3, cit-1.2, nhr-173*
Transcription	0.002	3.22	*nhr-212, F49E12.6, nhr-143, nhr-45, nhr-182, nhr-206, nhr-117, nhr-109, nhr-10, nhr-194, nhr-124, nhr-97, nhr-230, unc-4, daf-12, fax-1, nhr-201, nhr-88, nhr-102, nhr-233, nhr-241, cit-1.2, nhr-173*
Vesicle organization	0.005	1.01	*unc-26, K09B11.9, unc-11*
Response to xenobiotic stimulus	0.007	2.12	*cyp-35A3, cyp-35A4, cyp-35A5*
Protein amino acid phosphorylation	0.02	1.39	*cdk-4, Y38H8A.4, ZK666.8, pak-1, cst-2, C18H7.4, nekl-1, R09D1.12, ZK632.3, C45G9.1, R90.1, ZC373.4, , F28C10.3, , ZC123.4, kin-3, C09B9.4, ZK596.2, akt-2*
Regulation of development, heterochronic	0.02	1.01	*daf-12, lin-14, lin-28*
Di-, tri-valent inorganic cation transport	0.029	1.01	*W02B12.9, unc-68, ncx-2*
Protein localization	0.035	1.39	*K09B11.9, sec-15, vps-26, cdc-42, Y71G12B.11, atg-4.2, F38E11.5, Y71G12B.11, pac-1, F08G12.1, Y116A8C.10, unc-68, elks-1, 4R79.2, apt-9*

Gene symbol	NCBI Accession No.	Forward sequence (5'→3')	Reverse sequence(5'→3')	Amplicon size (bp)
*pmp-3**	NM_001269679	GTTCCCGTGTTCATCACTCAT	ACACCGTCGAGAAGCTGTAGA	115
unc-15	NM_001136291	AGGACTTGAACAAGCACGTC	TCGAGTTGAACCTTCTGGTC	106
ftn-1	NM_072543	AGAAAGACGAGTGGGGAACT	TCGAATGTACCTGCTCTTCC	168
rsks-1	NM_067046	GAAATCGTCGTCTCTCTGGA	TCCCTCGATTTCCTCCTTAC	138
cit-1.2	NM_181926	AGTCGAGCAAGAGTTGATGG	GAATGGTGGTGAAGAGGATG	108
daf-12	NM_001047774	GATCCAGTCATCCACAGTCC	CTGACGTCGTCGACTCTCTT	153

**C. elegans* reference gene

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