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ORIGINAL CONTRIBUTION

Gene expression profiling to investigate tyrosol-induced lifespan extension in *Caenorhabditis elegans*

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Abstract
Purpose We have previously reported that tyrosol (TYR) promotes lifespan extension in the nematode *Caenorhabditis elegans*, also inducing a stronger resistance to thermal and oxidative stress in vivo. In this study, we performed a whole-genome DNA microarray in order to narrow down the search for candidate genes or signaling pathways potentially involved in TYR effects on *C. elegans* longevity.
Methods Nematodes were treated with 0 or 250 μM TYR, total RNA was isolated at the adult stage, and derived cDNA probes were hybridized to Affymetrix *C. elegans* expression arrays. Microarray data analysis was performed, and relative mRNA expression of selected genes was validated using qPCR.
Results Microarray analysis identified 208 differentially expressed genes (206 over-expressed and two under-expressed) when comparing TYR-treated nematodes with vehicle-treated controls. Many of these genes are linked to processes such as regulation of growth, transcription, reproduction, lipid metabolism and body morphogenesis. Moreover, we detected an interesting overlap between the expression pattern elicited by TYR and those induced by other dietary polyphenols known to extend lifespan in *C. elegans*, such as quercetin and tannic acid.
Conclusions Our results suggest that important cellular mechanisms directly related to longevity are influenced by TYR treatment in *C. elegans*, supporting our previous notion that this phenol might act on conserved genetic pathways to increase lifespan in a whole organism.

Keywords *Caenorhabditis elegans* · Extra-virgin olive oil · Longevity · Microarray · Tyrosol

Introduction

Extra-virgin olive oil (EVOO), the main source of fat in the Mediterranean diet, has been traditionally associated with a higher longevity in the human population [1–3]. Although health-protective effects of olive oil have been attributed to its high content of monounsaturated fatty acids, mainly oleic acid, it is now widely accepted that the particular abundance of different micronutrients, especially phenolic compounds, is also highly implicated in these effects. Tyrosol (TYR), a liposoluble, non-carboxyl mono-phenol, is one of the most representative phenols in olive leafs and in EVOO. This compound has been shown to exert antioxidant and anti-proliferative activities as well as the ability to inhibit pro-oxidation processes on human LDL particles in different studies [4–8]. Even though the protective role of EVOO phenols in different pathologies is currently the object of extensive research, there have been very few studies that explore in depth the molecular mechanisms by which olive oil may influence longevity [9], being often

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assumed that these effects are the result of the antioxidant potential of its phenolic compounds and other free-radical scavengers such as vitamin E [10]. In recent years, a variety of molecules of plant origin have been proven effective in slowing aging and extending lifespan in animal model organisms [11, 12], although the specific physiological and genetic mechanisms responsible for these effects are still not fully understood. Recently, our group has reported that TYR is capable of extending lifespan and increasing the oxidative and thermal stress resistance in the nematode *Caenorhabditis elegans* [13]. This model organism presents a series of advantages for biogerontological research, including its short life cycle [14–16] and a strong conservation in molecular and cellular pathways between worms and mammals. In this sense, comparison of the human and *C. elegans* genomes confirmed that the majority of human disease genes and disease pathways are present in *C. elegans* [17]. The signaling pathway mediated by *daf-2* insulin/IGF1 [18], the implication of reactive oxygen species [19], the perturbation of mitochondrial function [20] and dietary restriction [21] are among the known mechanisms involved in the regulation of lifespan in *C. elegans*. A preliminary genetic analysis in our previous study, by using various nematode mutant strains, identified *daf-2*, *daf-16* and *hsf-1* as candidate mediators of TYR-induced longevity and stress resistance, although our results did not exclude the participation of other genes in these effects [13]. Moreover, by using a proteomic approach, we further identified the main proteins differentially expressed in response to TYR [22]. Most of these proteins are involved in the regulation of cellular functions such as vitellogenesis and embryonic morphogenesis, protein synthesis, metabolism of fatty acids, alcohol and amino acids, the transport of proteins into nucleus, heat shock response, membrane transport, and detoxification.

In order to complete our understanding of the cellular mechanisms induced by TYR in *C. elegans*, in the present study, we used the Affymetrix expression microarrays to determine the *C. elegans* global mRNA expression pattern in response to TYR treatment. With this technology, the gene expression of 22,625 transcripts from the whole genome of this species has been measured, allowing the identification and analysis of the most significant changes induced by TYR. Microarray results were validated by performing real-time quantitative polymerase chain reaction (qPCR) of selected genes. With this analysis, we intend to identify important genes and cellular networks directly or indirectly related to TYR effects in vivo. Furthermore, a comparison with available microarray data from long-lived mutants and from previous studies using other polyphenolic treatments in *C. elegans* helped us to perform a more comprehensive analysis of the expression profile results obtained in this study.

Materials and methods

Chemicals

TYR was purchased from Extrasynthese (Lyon, France). Buffers and other solutions were made with reagents obtained from the best quality commercial sources.

Strains and culture conditions

The *C. elegans* strain *fer-15*(*b26*) used in this study was obtained from the *Caenorhabditis* Genetics Center (CGC, Minneapolis, MN, USA) which is supported by NIH funding. We used this strain, which is sterile at 25 °C, because it was the strain used as *wild type* in our previous lifespan experiments [13] and we wanted to reproduce the exact experimental conditions to the ones used in that study. Furthermore, the sterility phenotype facilitates the RNA isolation from synchronized adult nematodes avoiding progeny interference and minimizing internal hatching. Worms were propagated at 20 °C on solid nematode growth media (NGM) seeded with the *E. coli* strain OP50. To prepare TYR treatment, TYR (2-(4-hydroxyphenyl) ethylalcohol) was dissolved in ethanol and added to the liquid NGM medium previously autoclaved and cooled to 50 °C to a final concentration of 250 μM. The medium was immediately dispensed into Petri dishes that were kept protected from light and stored at 4 °C until use. A final ethanol concentration of 0.1 % (v/v) was maintained in both control- and TYR-containing plates. Nematodes were exposed to the treatment starting from the egg stage until their collection for the experiments.

C. elegans growth for microarray experiments and total RNA isolation

Caenorhabditis elegans were maintained on NGM and *E. coli* OP50 as previously described [23]. For microarray experiments, we reproduced the same experimental conditions that were conducted to demonstrate lifespan extension by TYR treatment [13]. Briefly, *fer15*(*b26*) nematodes were synchronized and raised at 25 °C on NGM plates containing either 250 μM TYR or vehicle (control). We collected nematodes on the fourth day of adulthood, since at this stage we detected significant effects of tyrosol on stress resistance in our previous study [13]. Nematodes were collected from the NGM plates in M9 buffer, washed three times and pelleted by centrifugation for RNA isolation. After centrifugation, worms were resuspended in 350 μl of TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), flash-frozen in liquid nitrogen and thawed at 37 °C three times for disruption, and total RNA was purified using the RNeasy Mini kit (Qiagen, Venlo, Netherlands) following the

manufacturer-recommended protocol. Final volume of isolated RNA was 50 µl per biological sample.

DNA synthesis and microarray hybridization

RNA quality was analyzed with the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) using the RNA 6000 nano kit. All RNA samples were of sufficient quality for gene array analysis with RNA integrity number (RIN) >7. A total amount of 50 ng of RNA was used as the template for cDNA synthesis and in vitro transcription to synthesized biotin-modified aRNA using the GeneChip® 3′ IVT Express Kit (Affymetrix, 901228). aRNA was purified from unincorporated nucleotides and other reaction components using the RNeasy Mini Kit (Qiagen). A total of 15 µg of biotin-labeled aRNA was fragmented following the instructions described in the Affymetrix manual (P/N 702646 Rev.8) and hybridized to *C. elegans* GeneChip® Genome Arrays (Affymetrix, 900383). They were processed and scanned using Affymetrix instrumentation and with hybridization, washing and scanning parameters provided by the manufacturer.

Microarray gene expression data analysis

Computational and statistical analyses were carried out using the R software (http://www.r-project.org/) and the appropriate Bioconductor packages (http://www.bioconductor.org/) run under R. In order to remove all the possible sources of variation of a non-biological origin between arrays, densitometry values between arrays were normalized using the robust multiarray (RMA) normalization function implemented in the Bioconductor affylmGUI package. Statistically significant differences between groups were identified using the rank product nonparametric test implemented in the Bioconductor Rank-Prod package. Those genes showing a corrected p value <0.05 were selected as significant.

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus database [24] and are accessible through GEO Series accession number GSE57664 (http://www.ncbi.nlm.nih.gov/geo). Functional annotations were carried out using DAVID (http://david.abcc.ncifcrf.gov). Protein–protein interaction network was obtained using STRING (http://string-db.org) with default parameters.

Comparison with literature datasets to screen for overlaps

We performed a comparison between our list of differentially expressed genes (DEGs) and a previously published list of DEGs in response to tannic acid and quercetin treatments in *C. elegans* [12]. We also compared our data with the published expression profiles obtained from the long-lived mutants *daf-12* (rh273) [25], TGF-beta [26] and *daf-2* [27, 28]. For each dataset, we grouped all the DEGs with a fold change (FC) higher than 1.5 and compared it with our list of TYR DEGs (also with a FC > 1.5). We used the tool Venny (http://bioinfogp.cnb.csic.es/tools/venny/) to look for overlapping genes between different groups.

Real-time qPCR validation

In order to validate the microarray hybridizations and analysis, we chose five of the ten over-expressed genes with the highest FC value (*msp-81*, *msp-77*, *msp-56*, *flp-15* and *ins-2*) plus one of the two under-expressed genes (B0024.4) and quantified their relative mRNA expression level using qPCR. Briefly, cDNA was synthesized from 1 µg of total RNA using the Maxima First Strand cDNA Synthesis Kit for real-time qPCR (Thermo Scientific, Waltham, MA, USA). qPCR was performed in a Mx3000 QPCR System (Stratagene, Agilent Technologies) using Kapa SYBR FAST qPCR kit (Kapa Biosystems, Wilmington, MA, USA) with 1 µl cDNA in a 10-µl reaction volume using the following gene-specific oligonucleotide primers: *msp-81* (NM_069358.3), CAACGACCGTATC ACTGTTGA (F) and TTCTTGCGACGAACCATACC (R); *msp-77* (NM_069380.2), AGACTTGGAGTTGATCCA CCA (F) and TTGGTATCCTCTTGTCCGAAG (R); *msp-56* (NM_069361.4), CTGTTTCCTGCGATGC TTTT (F) and TCACGACGGAATTGTTTGG (R); *flp-15* (NM_067419.2), ACAATTCGGTCGGAACAATC (F) and AGATGGTCCACGTCGTTTTC (R); *ins-2* (NM_062793), GAGTCAACTCCAACTCCAAACC (F) and TTGAACGTCACATTGTTTTATGC (R); B0024.4 (NM_073247.3), AGCAAATGTTCTTTCCTGTCGT (F) and TGCTGGAACACTTCCCACAA (R); *ama-1* (NM_068122.6), AAGCTATAGCCCTTCGTCGC (F) and CGAGGATGGAGTGTACGTCG (R). Cycle thresholds of amplification, expression levels of the target genes normalized to the housekeeping gene *ama-1* and relative FC for transcripts were calculated using the MxPro software (Stratagene). All samples were run in triplicate. p values were calculated using a two-tailed Student's t test.

Results

Identification of genes differentially expressed in response to TYR

A principal component analysis (PCA) was imposed on the complete normalized dataset to capture the cluster structure prior to further analysis, showing that the three independent replicates of each experimental trial are located closely together, underlining the presence of a sound technical

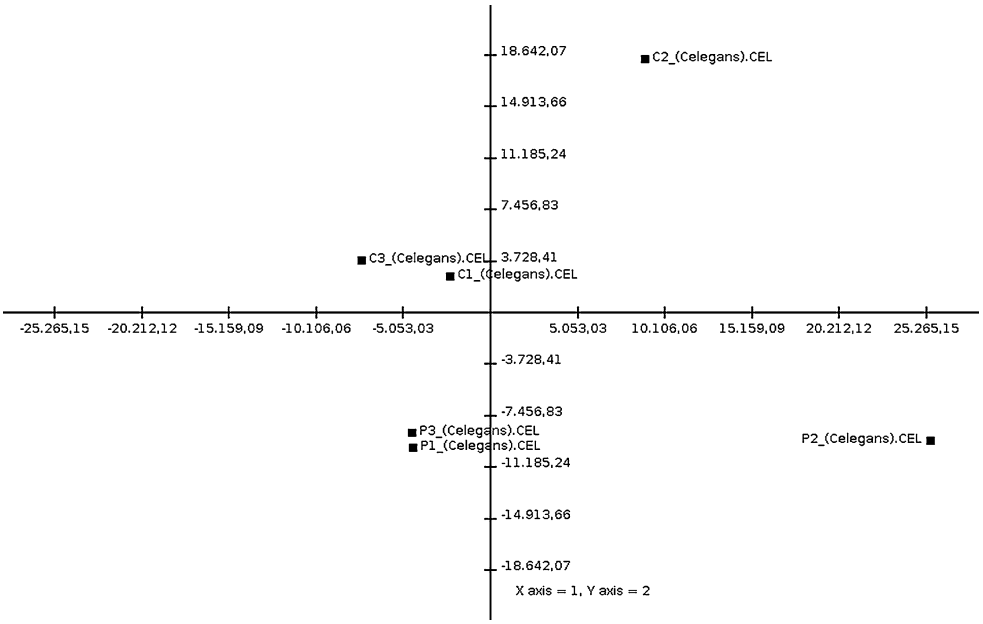

Fig. 1 A PCA plot derived from biological replicates of independent *C. elegans* samples treated with 250 μM TYR (P1, P2 and P3) and non-treated controls (C1, C2 and C3)

reproducibility and overall differences between groups in the gene expression profile (Fig. 1).

We detected 208 genes differentially expressed (206 up-regulated and two down-regulated) between control and TYR-treated worms (Supplementary Material 1). Most of the over-expressed genes are involved in the regulation of growth and body morphogenesis (*nspd-5*; *nspd-4*) and transcription (*ceh-5*; *ces-2*) among other biological processes related to reproduction (*msp-79*; *msp-81*; *msp-51*), lipid storage (*msp-56*; *igcm-3*; *acs-2*), metabolism (*hch-1*; *skr-21*) and signal transduction (*cfz-2*; *rsf-1*) (Table 1).

Validation of the microarray data using real-time qPCR

The microarray data were validated by quantifying selected DEGs using qPCR. As shown in Fig. 2, the mRNA expression level from *msp-77*, *msp81*, *msp-56*, *ins-2* and *flp-15* genes was increased by TYR, while B0024.4 mRNA was under-expressed in response to this treatment. The expression patterns obtained through RT-qPCR analysis were consistent with those obtained from the microarray, indicating that the microarray results give an accurate report of transcript levels in our experimental model.

Functional enrichment analysis

Gene ontology (GO) analysis of the differentially expressed mRNAs in response to TYR treatment is shown in Fig. 3. The data are presented according to the following categories: *biological processes* (A), *molecular functions* (B) and *cellular components* (C). The most represented biological processes identified by GO enrichment analysis were regulation of growth, body morphogenesis and regulation of transcription, among others (Supplementary Material 2-BP). The same analysis showed that the main molecular functions affected by TYR treatment were DNA binding and structural molecule activity (Supplementary Material 2-MF). The most represented cellular components detected by our analysis were pseudopodium, cell projection, cytoskeleton and non-membrane-bound organelle (Supplementary Material 2-CC). We noticed that most of the genes grouped in the *cellular components* category appear also included into the group named *structural molecule activity* under the *molecular functions* category. Many of these genes belong to the family of major sperm proteins (MSPs), which is conserved in nematodes and consists of closely related, small, basic proteins that make up 15 % of sperm protein.

In order to identify possible protein–protein interaction (PPI) patterns from our data, we used the STRING Web engine to build the PPI network of the differentially expressed gene products. This analysis grouped TYR-induced genes mainly in two independent network components as shown in the figure in Supplementary Material 3. These results indicate that TYR treatment is able to up-regulate two separated groups of transcripts; one of them corresponds mainly to MSPs with structural function and some other genes involved in lipid storage (K07F5.3, C35D10.11), body morphogenesis (*nspd-4*, *nspd-5*) and protein phosphorylation (C39H7.1); the main network component, more heterogeneous, includes genes related to the regulation of growth and larval development (*eya-1*, *noah-1*, *ceh-43*, *lpr-3*, *sqt-3*), transcription (F23F12.9, F49E12.6, *ces-2*), metabolism (*cht-1*, *skr-21*, W04A8.4, *hch-1*), lipid storage (*igcm-3*), signal transduction (*cfz-2*, *cav-1*) and neuron migration (*ham-1*, *unc-39*).

Table 1 Identification, direction of change and description of selected DEGs in response to treatment with 250 μM TYR, grouped by the main gene ontology biological processes

Transcript ID	CGC name	Description	FC
Reproduction/embryo development			
NM_069339	*msp-79*	Major sperm protein	4.45
NM_069358	*msp-81*	Major sperm protein	4.21
NM_068310	*msp-51*	Major sperm protein	3.88
NM_068359	*msp-19*	Major sperm protein	3.82
NM_062497	*msp-31*	Major sperm protein	3.80
NM_068312	*msp-57*	Major sperm protein	3.42
NM_068310	*msp-113*	Major sperm protein	2.98
NM_062464	*msp-40*	Major sperm protein	2.95
NM_068379	*msp-65*	Major sperm protein	2.90
NM_068377	*msp-59*	Major sperm protein	2.89
NM_062500	*msp-152*	Major sperm protein	2.86
NM_062558	*msp-45*	Major sperm protein	2.72
NM_001026080	*tir-1*	TIR (toll and interleukin-1 receptor) domain protein	2.31
NM_062487	*msp-33*	Major sperm protein	2.31
NM_171725	*sox-2*	SOX (mammalian SRY box) family	2.27
NM_062748	*msp-64*	Major sperm protein	2.26
Lipid storage and metabolism			
NM_069361	*msp-56*	Major sperm protein	3.87
NM_069359	*msp-10*	Major sperm protein	3.82
NM_069341	*msp-78*	Major sperm protein	3.80
NM_069433	*msp-36*	Major sperm protein	3.63
NM_069321	*msp-76*	Major sperm protein	3.54
NM_001129703	*igcm-3*	Immunoglobulin-like cell adhesion molecule family	1.74
NM_066599	*nac-2*	NADC (Na^+-coupled dicarboxylate transporter) family	1.73
NM_074468	*acs-2*	Fatty acid CoA synthetase family	1.69
Larval development			
NM_076414	*mls-2*	Mesodermal lineage specification	1.95
NM_075592	*mlt-11*	Molting defective	2.24
NM_075289	*ptr-22*	Member of the sterol-sensing domain (SSD) proteins	2.05
NM_065477	*mnp-1*	Matrix non-peptidase homolog	1.91
NM_059557	*dpy-14*	Type III (alpha 1) collagen	1.90
NM_170870	*noah-1*	Drosophila (nompA) homolog	1.82
NM_073557	*ceh-32*	Homeobox	1.78
NM_001025884	*eya-1*	EYA (drosophila eyes absent) homolog	1.75
NM_073694	*sqt-3*	Squat, cuticle collagen	1.71
Transcription			
NM_060185	*ceh-5*	Homeobox	2.61
NM_061209	*ces-2*	Basic region leucine-zipper (bZIP) transcription factor	2.20
NM_066025	*zip-8*	bZIP transcription factor family	1.97
NM_062834	*fkh-8*	Forkhead transcription factor family	1.93
NM_063370	*efl-3*	Protein with two E2F domains (involved in apoptosis)	1.71
NM_001047598	*nhr-11*	Nuclear hormone receptor family	1.71
NM_001027384	*ceh-20*	Homeobox	1.68
Transport			
NM_063571	*aqp-2*	Aquaporin or aquaglyceroporin related	2.08
NM_069583	*ncx-3*	Na/Ca exchangers	1.84
NM_061794	C40A11.4	Hypothetical protein	1.84

Table 1 continued

Transcript ID	CGC name	Description	FC
NM_001038246	*mps-2*	MiRP K channel accessory subunit	1.73
NM_001047552	Y11D7A.3	Hypothetical protein	1.67
Body morphogenesis and positive regulation of growth rate			
NM_062474	*nspd-5*	Nematode-specific peptide family, group D	2.62
NM_062470	*nspd-4*	Nematode-specific peptide family, group D	2.12
NM_076934	*zig-4*	2 (Zwei) IG-domain protein	1.86
NM_062965	*dpy-10*	Cuticle collagen protein	1.66
NM_073247	B0024.4	Hypothetical protein	−2.03
Signal transduction			
NM_071563	*cfz-2*	Signal transducer activity/receptor activity	2.01
NM_001027190	*rsf-1*	Homolog of the Ras-association domain family protein 1	1.57
NM_069462	*ptp-4*	Protein tyrosine phosphatase	1.65
NM_068436	C39H7.1	Serine/threonine kinase/casein kinase	1.63
NM_069342	*cav-1*	Caveolin	1.61
Carbohydrate/protein/nucleotides metabolism			
NM_061021	W04A8.4	Nucleic acid binding/3′–5′ exonuclease activity	2.27
NM_078039	*hch-1*	Metalloendopeptidase activity	2.16
NM_077793	*skr-21*	SKp1 related (ubiquitin ligase complex component)	2.06
NM_060959	W05H12.2	Nucleic acid binding/3′–5′ exonuclease activity	1.90
NM_076187	*cht-1*	Chitinase	1.67
NM_001026095	*unc-44*	Ankyrin-like protein	1.60
NM_072351	*odc-1*	Ornithine decarboxylase	1.57
Locomotion/cytokinesis			
NM_063296	*ltd-1*	Protein with LIM and transglutaminase domains	2.06
NM_001026798	*cdc-14*	Dual-specificity phosphatase	1.68
NM_063179	*wrt-10*	Hedgehog-like protein	1.67
NM_077184	*tbb-4*	Nucleotide binding/GTPase activity	1.67
NM_070000	*ham-1*	Protein with a winged helix DNA-binding motif	1.65
NM_001027968	*klp-11*	Kinesin motor protein	1.56
NM_069165	*unc-129*	Member of the TGF-beta family	1.83
Other functions			
NM_062793	*ins-2*	Insulin-like peptide	2.58
NM_001083195	*nspd-6*	Nematode-specific peptide family, group D	2.45
NM_067419	*flp-15*	FMRFamide-related short peptide neurotransmitter	2.32
NM_066620	*ced-11*	Predicted transmembrane ion channel	2.01
NM_066226	*numr-1*	Nuclear localized metal responsive	1.84
NM_171615	*ncam-1*	NCAM (neural cell adhesion molecule) homolog	1.80
NM_072953	*hsp-16.1*	Heat shock protein	1.76
NM_071727	*irg-2*	Hypothetical protein	1.68

Comparison of global transcriptional patterns in TYR longevity to selected datasets in the literature

As a result of comparing our microarray data with selected published datasets [12, 25–28], we found that out of the 206 genes significantly over-expressed in response to 250 μM TYR treatment, 45 of them were also up-regulated in response to quercetin (50, 100 and 200 μM) and 24 of them were up-regulated in response to tannic acid treatment (100, 200 and 300 μM) (Fig. 4). We also found that 19 TYR-induced genes were up-regulated in the long-lived *daf-12* (*rh273*) mutants. Moreover, a total of seven genes were found to be commonly up-regulated in these four experimental conditions (Fig. 4, underlined genes). Interestingly, most of these overlapping genes belong to the MSPs family which, as mentioned before, constitutes one of the most represented expression mountains in response to TYR treatment. In contrast, we did not find overlapping

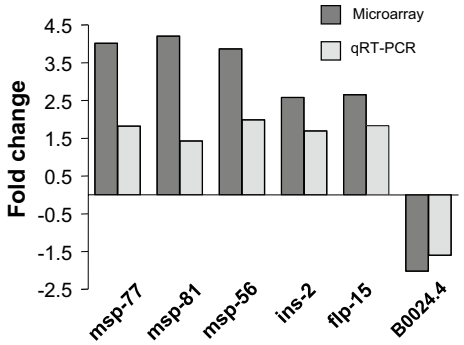

Fig. 2 Microarray validation by qPCR analysis of five over-expressed and two under-expressed selected genes. *Graph bars* represent mean FC values from normalized mRNA relative levels of target genes next to mean FC values from the same genes obtained in the microarray analysis in response to 250 μM TYR treatment

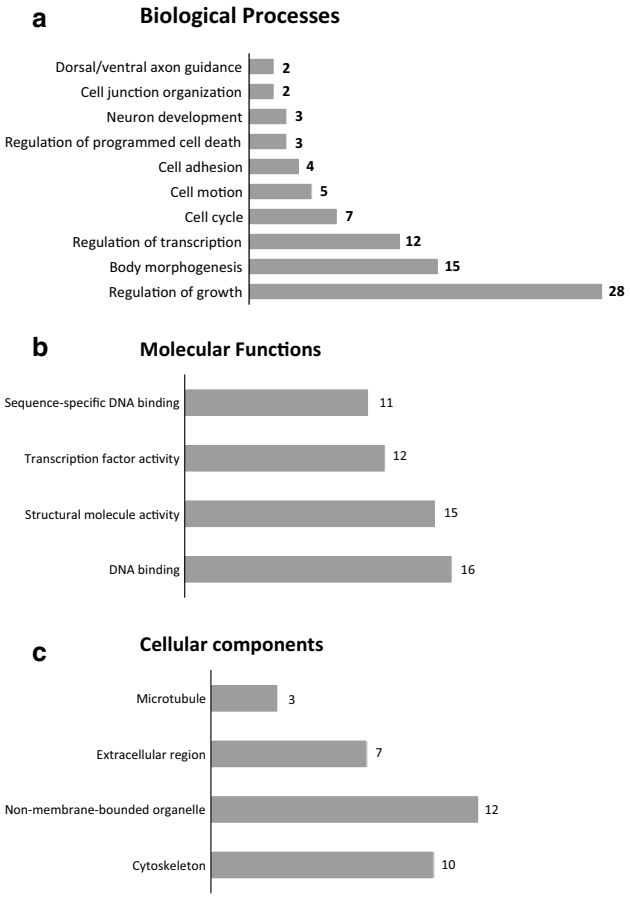

Fig. 3 Most representative significant GO terms for 250 μM TYR treatment grouped under the categories of "biological process" (**a**), "molecular function" (**b**) and "cellular component" (**c**). A complete list with all the GO terms and their correspondent *p* values can be found in the *Supplementary Table 2*

genes when we compared our data to published lists of DEGs from TGF-beta and *daf-2* mutants (results not shown). In order to further examine the possibility that this cluster of genes is regulated by the transcription factor DAF-12, we used the *C. elegans* database WormBase to look for regulatory elements in all of the TYR-up-regulated *msp* DNA sequences. Indeed, we found that some of these genes (*msp-51*, *msp-55*, *msp-113* and *sss-1*) have binding regions for the transcription factor DAF-12. Interestingly, we also found that the TYR-up-regulated genes *msp-31*, *msp-36*, *msp-38*, *msp-40*, *msp-152*, *msp-3*, *msp-49 and msp-64* have binding regions for the transcription factor DAF-16 (Fig. 5).

Discussion

In recent years, there has been an increasing body of evidence showing that many plant polyphenols are able to affect the cellular and physiological status of an animal [11, 29–32]. In the current study, we have analyzed for the first time the gene expression profile of *C. elegans* in response to TYR, whose lifespan-extending properties in this model organism have been recently reported by us [13]. These results, combined with data obtained from a previous study from our group to identify differentially expressed proteins in the same experimental conditions [22], might provide useful insights into the global mechanisms and cellular processes modulated by TYR in vivo.

TYR effect on reproduction signals and lipid metabolism

Our analysis revealed that the 25 most significantly up-regulated DEGs in response to TYR treatment corresponded to a numerous group of genes encoding MSPs. This multigene family consists of over fifty genes, including many pseudogenes, resulting in MSP isoforms which assemble into fibrous networks whose function is to drive movement of the *C. elegans* sperm and which are also involved in extracellular signaling during reproduction [33–35]. In this sense, it has also been described that MSP antagonizes Eph/ephrin signaling, in part, by binding VAB-1 Eph receptor tyrosine kinase on oocytes and sheath cells to promote oocyte maturation via MAPK activation [36]. The majority of these TYR-over-expressed MSP genes also appear grouped in one of the two main network components shown in our PPI analysis, together with a smaller number of genes involved in lipid storage, body morphogenesis and protein phosphorylation. Although the role of MSPs in *C. elegans* reproduction has been described, being the sperm signal promoting oocyte maturation in fertile worms, we have not found many studies analyzing in depth the possible relationship between MSPs and longevity. Interestingly, Pietsch et al. [12] also reported a significant over-expression of MSP genes in response to treatments with tannic acid and quercetin, both

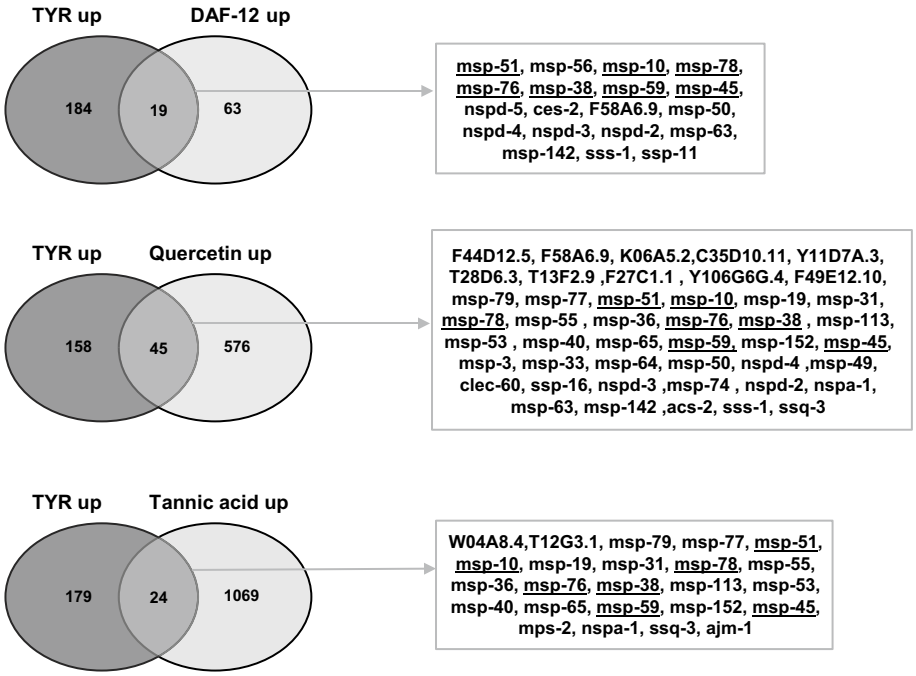

Fig. 4 Venn diagrams of DEGs. Comparison of DEGs (FC > 1.5) derived from TYR treatment (250 μM) and **a** *daf-12* (rh273) mutants [25], **b** quercetin treatment (50, 100 and 200 μM) and **c** tannic acid treatment (100, 200 and 300 μM) [12]. Shown is the overlap of significantly over-expressed genes from each pair of experimental conditions compared. Genes shown *underlined* are those over-expressed in all four experimental groups

of which are phenolic compounds that had been previously shown to extend the lifespan of *C. elegans* [11, 31, 37]. A different microarray study also revealed a significant up-regulation of numerous MSP genes in the long-lived DAF-12 mutant *rh273* [25]. By performing a thorough processing of global transcription expression values from several microarray datasets in this model organism, Pietsch et al. [12] were able to propose a model by which both polyphenols would affect lifespan, in part, through modulation of the transcription factor DAF-12. This steroid hormone receptor has been shown to influence, together with DAF-16, the mechanism of gonad-dependent adult longevity in *C. elegans* [38–40]. Our comparison with these and other datasets revealed that many of the MSP genes up-regulated in response to TYR are overlapped with MSP genes over-expressed in long-lived DAF-12 mutants and, also, with many MSP genes over-expressed in response to quercetin and tannic acid. The fact that at least some of these genes (*msp-51*, *msp-55*, *msp-113* and *sss-1*) have specific binding regions described for DAF-12 corroborates that this transcription factor might be a conserved regulator of this gene cluster. Also, we found that another group of TYR-up-regulated MSP genes have binding regions for the transcription factor DAF-16 which, as we had previously reported, could be partially involved in TYR effect on longevity and stress resistance. These results support evidence suggesting a role of TYR, and possibly other phenolic compounds, in modulating *C. elegans* longevity by affecting MSPs expression through the DAF-12 pathway.

Another remarkable result we can extract from this study is that out of the 208 differentially expressed genes in our analysis, only two were significantly under-expressed in response to TYR, suggesting that the main actions of this phenol could be related to the modulation of specific pathways that result primarily in promoting gene expression. Interestingly, one of the down-regulated genes, B0024.4, encoding a hypothetical protein of unknown function (predicted positive regulation of growth rate), has been related to longevity, as reported by Oliveira et al. [41]. Thus, microarray analysis in *C. elegans* mutants for the Nrf ortholog SKN-1, which promotes defense mechanisms and delays aging under stress, revealed that the gene B0024.4 was significantly down-regulated by SKN-1 under normal conditions, while RNAi knockdown of this gene in *wild-type* nematodes promoted a lifespan increase of a 15 %. The fact that B0024.4 is one of the two TYR-under-expressed genes in our analysis may be somehow related to the lifespan extension observed in TYR-treated nematodes by our previous study [13].

The other TYR-down-regulated gene is F49E12.10, which encodes an uncharacterized protein involved in fatty acids biosynthesis that seems to correspond to either a fatty acid hydroxylase or a sterol desaturase. In this sense, TYR induced also the over-expression of genes related to lipid metabolism, such as *acs-2*, an acyl-CoA synthetase involved in mitochondrial fatty acid β-oxidation. These results seem to indicate that TYR is capable of down-regulating steps involved in fatty acid biosynthesis while up-regulating genes that function in fatty acid degradation, pathways that when combined would lead to decreased fat storage in these animals. It has been shown that fat mobilization from its storage tissues promotes longevity in *C.*

Fig. 5 List of TYR-up-regulated sperm genes indicating the presence of DAF-12 and DAF-16 binding regions in their DNA sequence as it appears in www.wormbase.org database. Also shown is the comparison of these genes with up-regulated genes in response to tannic acid (TA), quercetin (Q) and in long-lived DAF-12 mutants (*rh273*) in previous studies [12, 25]

TYR –UP sperm gene	TA-UP	Q-UP	*daf-12 (rh273)*-UP	DAF-12 binding regions	DAF-16 binding regions
msp-79	+	+			
msp-81					
msp-77	+	+			
msp-51	+	+	+	+	
msp-56			+		
msp-10	+	+	+		
msp-19	+	+			
msp-31	+	+			+
msp-78	+	+	+		
msp-55	+	+		+	
msp-36	+	+			+
msp-76	+	+	+		
msp-38	+	+	+		+
msp-57					
msp-113	+	+		+	
msp-53	+	+			
msp-40	+	+			+
msp-65	+	+			
msp-59	+	+	+		
msp-74		+			
msp-152	+	+			+
msp-45	+	+	+		
ssp-16		+			+
msp-3		+			
msp-33		+			
msp-64		+			+
msp-50		+	+		
msp-49		+			+
msp-63		+	+		
msp-142		+	+		
sss-1		+	+	+	
ssp-11			+		
ssq-3	+	+			

elegans, and this mechanism would be regulated by germline stem cells [42]. According to our results, TYR may exert an effect on lipid metabolism by shifting the balance toward fat mobilization processes, which could explain, at least in part, the observed lifespan-extending properties of this compound. In this sense, our previous data from a proteomic approach also reported lipid metabolism as one of the cellular processes mainly affected by TYR [22]. Taken together, these results suggest that TYR might be able to influence fat storage mechanisms and also signals from the reproductive system, both of which are closely connected biological processes that have been shown to play a key role in regulating *C. elegans* lifespan.

Transcription factors

Among the significant TYR-up-regulated genes, we found that 12 of them were involved in the regulation of transcription. Interestingly, although this biological process appears as the fourth most represented in our gene ontology analysis behind other processes like regulation of growth and body morphogenesis, this group of genes is included in around a 50 % of the most represented molecular functions shown in our analysis, suggesting that the regulation of transcription is a key cellular mechanism affected by TYR treatment. Most of these genes encode transcription factors of different families and include F23F12.9 and *ces-2* (basic leucine-zipper transcriptional factors), F49E12.6 (an E2F-like transcription factor), *ceh-5*, *ceh-20*, *ceh-32*, *ceh-43* and *unc-39* (homeodomain transcription factors) and *nhr-11*, a member of the superfamily of nuclear receptors that respond to fat-soluble hormones, such as steroids and fatty acids and also one of the most abundant class of transcriptional regulators. Maybe it could be of interest to investigate in more detail the role of *nhr-11* since it belongs to the same family as the nuclear receptor DAF-12 and, as discussed before, there are specific genes in common affected by TYR response and by DAF-12 signaling pathway, suggesting a possible

connection between both mechanisms in terms of longevity. Moreover, it has been recently suggested that nuclear hormone receptors (NHRs) may mediate metabolic states associated with dietary restriction and could play an important role in mediating dietary restriction-induced metabolic and longevity responses in *C. elegans* [43].

Other biological processes affected by TYR

Other important biological processes affected by TYR treatment according to our analysis are body morphogenesis, regulation of growth rate, cell motion and adhesion, cell cycle, programmed cell death and neuron development. These results suggest that TYR is able to induce the expression of different genes involved in the regulation of larval development and growth, two basic physiological processes directly related to adult longevity. Nevertheless, in our previous study, we assessed larval development rate, adult growth and progeny production in the *C. elegans* strain used and we did not find significant changes in these parameters in response to the same TYR concentration [13]. Thus, it is possible that TYR impact on these genes may not be sufficient to result in significant effects on the overall developmental rate or growth in the nematodes. In this sense, although we cannot exclude a slight impact of this expression pattern on longevity, based on our previous results, it does not seem likely that these biological processes are among the candidate ones responsible for TYR-dependent lifespan increase.

Heat shock response

Heat shock proteins (HSPs) are involved in reparation of misfolded or damaged proteins and are essential for recovery of cells after heat treatment, indicating that protein misfolding and aggregation are important factors in aging [44]. In our previous work [13], we observed an important increase in thermal stress resistance of *C. elegans* induced by TYR and we reported a role of the HSF-1 transcription factor in TYR effects on thermotolerance and longevity. Interestingly, microarray analysis shows that *hsp-16.11* is one of the significantly up-regulated genes in response to TYR. This gene encodes a 16-kD HSP that is induced in response to heat shock or other environmental stresses and is likely to function as a passive ligand temporarily preventing unfolded proteins from aggregating. It has been reported that over-expression of this gene, which in turn is regulated by DAF-16 and HSF-1 transcription factors, increases longevity in *C. elegans* [45].

In a recent work published by our group analyzing the proteomic changes induced by TYR treatment in *C. elegans*, we also found a TYR-induced up-regulation of two chaperone proteins (HSP-4 and ENPL-1) [22]. In addition, we had reported the up-regulation of *hsp-12.6* mRNA, another small chaperone from the same family as *hsp-16.11*, under this TYR treatment [13]. The same study also reported the requirement of DAF-16 and HSF-1 in TYR effects on longevity, which could explain chaperone up-regulation observed both at mRNA and protein levels. In this context, our results confirm that TYR effects on thermotolerance and longevity in *C. elegans* may also be related to the up-regulation of specific chaperones.

Future directions

Taken together, the results presented here suggest that one of the candidate mechanisms worth to explore next could be that relating TYR (and may be other dietary polyphenols) to lifespan regulation through germline signaling, since this phenol seems to partially mimic the effects of DAF-12 pathway down-regulation. This pathway has been reported to connect longevity with germline and steroid hormones signaling [46–48], although the precise mechanisms underlying this regulation are not fully understood. In this sense, it would be very attractive to analyze in depth how TYR may affect DAF-12-dependent longevity in order to seek for possible common regulatory mechanisms to those of other phenolic compounds.

Conclusions

In the current study, we have identified a group of genes that are induced by TYR in C. *elegans*. Microarray analysis combined with some of our previous results using this same treatment and animal model has contributed importantly to gain insight into the molecular and cellular functions that may underlie TYR effects on *C. elegans* lifespan and stress resistance. Although these results do not show a specific mechanism to explain TYR effect on longevity, they pinpoint several candidate processes and signaling pathways that seem to be more affected by this compound. Therefore, this study will allow us and other researchers to focus further efforts when addressing the role of dietary polyphenols on animal health and longevity.

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Conflict of interest Ana Cañuelo, Francisco J. Esteban and Juan Peragón declare that they have no conflict of interest.

Ethical standard All institutional and national guidelines for the care and use of laboratory animals were followed.

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