Transcriptome analysis reveals molecular anthelmintic effects of procyanidins in *C. elegans*

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Abstract

Worldwide, more than 1 billion people are affected by infestations with soil-transmitted helminths and also in veterinary medicine helminthiases are a severe threat to livestock due to emerging resistances against the common anthelmintics. Proanthocyanidins have been increasingly investigated for their anthelmintic properties, however, except for an interaction with certain proteins of the nematodes, not much is known about their mode of action. To investigate the anthelmintic activity on a molecular level, a transcriptome analysis was performed in *Caenorhabditis elegans* after treatment with purified and fully characterized oligomeric procyanidins (OPC). The OPCs had previously been obtained from a hydro-ethanolic (1:1) extract from the leaves of *Combretum mucronatum*, a plant which is traditionally used in West Africa for the treatment of helminthiasis, therefore, also the crude extract was included in the study. Significant changes in differential gene expression were observed mainly for proteins related to the intestine, many of which were located extracellularly or within cellular membranes. Among the up-regulated genes, several hitherto undescribed orthologues of structural proteins in humans were identified, but also genes that are potentially involved in the worms’ defense against tannins. For example, T22D1.2, an orthologue of human basic salivary proline-rich protein (PRB) 2, and *numr-1* (nuclear localized metal responsive) were found to be strongly up-regulated. Down-regulated genes were mainly associated with lysosomal activity, glycoside hydrolysis or the worms’ innate immune response. No major differences were found between the groups treated with purified OPCs versus the crude extract. Investigations using GFP reporter gene constructs of T22D1.2 and *numr-1* corroborated the intestine as the predominant site of the anthelmintic activity. The current findings support previous hypotheses of OPCs interacting with intestinal surface proteins and provide the first insights into the nematode’s response to OPCs on a molecular level as a base for the identification of future drug targets.
Introduction
Worldwide, more than 1.5 billion people are affected by infestations with intestinal nema-
todes [1,2] and also in veterinary medicine, helminths pose a severe threat to livestock [3–
5]. Commonly used effective drugs against these diseases include the benzimidazoles, pyr-
antel pamoate, ivermectin or levamisole [6–8], however, their number is limited and reports
about emerging resistances are dramatic, especially in veterinary medicine [9,10]. Polyphe-
nols, particularly condensed tannins, are a class of natural compounds that have recently
been extensively investigated in vitro and in vivo for their anthelmintic activities (for re-
views see [11–18]). Condensed and hydrolysable tannins have been reported to cause a vari-
ety of detrimental effects in vitro on different nematode species at different life stages. These
include the inhibition of egg hatch, larval exsheathment, larval migration, feeding and larval
development as well as lethal effects in adults (for review see [12,14,18]). Regarding their
mode of action, tannins are known for their astringent properties and likely interact with
certain proteins, especially those rich in proline or hydroxyproline, mainly via hydrogen
bonding or hydrophobic interaction [19–25]. Several microscopic investigations have indic-
ated that in particular the nematode cuticle, the buccal cavity and the intestine provided
potential binding sites for tannins [12,26–34]. Also flavonoids, another major subgroup of
polyphenols, exert direct anthelmintic effects in some cases [35–39] and no effect in others
[33], depending on the exact flavonoid structure and the test system used [40–43]. More-
over, flavonoids have been shown to enhance the activity of tannins in a synergistic manner
[38]. However, despite the intensive research in this field, there are hardly any investiga-
tions concerning the anthelmintic mode of action of polyphenolic compounds on a molecu-
lar level. Insights into the molecular mechanism would not only contribute to a better
understanding of the anthelmintic effect of polyphenols, but might also lead to potential
new and innovative targets for the development of novel anthelmintic drugs in the future.

Therefore, the current study aims at investigating the molecular effects of a polyphenol rich
extract and isolated procyanidins from the leaves of *Combretum mucronatum*, a plant tradi-
tionally used in West Africa for the treatment of intestinal helminthiasis [44], by transcrip-
tome analysis using the model nematode *Caenorhabditis elegans*.

Materials and methods

Plant material and chemicals
Leaves from *C. mucronatum* were harvested in the Bosomtwi-Atwima-Kwamwoma area in the
Ashanti region of Ghana, located between 0.15–2.25˚W and 5.50–7.46˚N (Campus of the
Kwame Nkrumah University of Science and Technology, Kumasi), by a university technician
between April and May 2011. *C. mucronatum* is a plant species widely distributed within West
Africa. The plant is not endangered and not protected according to relevant international list-
ings. For collection of the plant material no specific permission was required. The plant material
was air dried for two weeks at room temperature after botanical identification and reference
samples were stored at the Institute for Pharmaceutical Biology and Phytochemistry, Münster
(voucher no. IPBP-324). If not stated otherwise, all chemicals were purchased from VWR
(Darmstadt, Germany).

Preparation of plant extracts and fractions
A hydroethanolic extract (1:1 v/v) was prepared in a drug-solvent ratio of 1:10 as described
previously [45]. By bioassay-guided fractionation a fraction was obtained that entirely con-
sisted of oligomeric procyanidins composed of epicatechin building blocks and which has
been characterized previously [45]. Briefly, the crude extract was partitioned between ethyl acetate and water and a methanol soluble fraction of the aqueous partition was subsequently fractionated by MPLC on an RP-18 stationary phase (RP-18, 18–32 μm, 100 Å, 36 × 500 mm (BESTA Technik, Wilhelmsfeld, Germany), flow 4 mL/min, step gradient MeOH 10% (50 min) → MeOH 30% (2 h) → MeOH 50% (2 h) → MeOH (2 h), fraction size 16 mL). Subfraction “H3” (992–1152 mL, yield: 1.29 g) was used in the experiments. The anthelmintic activity of extract and fractions has been assessed using *C. elegans* as described in [45].

**Culture and treatment of *C. elegans***

Cultures of *C. elegans* wildtype (N2 Bristol strain) obtained from the Caenorhabditis Genetics Center, University of Minnesota, were maintained as described by [46] and grown at 20˚C on petri dishes containing Nematode Growth Medium (NGM) supplemented with 800 μL of *Escherichia coli* OP50 strain as a food source [46]. Age synchronous cultures were obtained by alkaline bleaching (600 μL NaOCl solution (Sigma-Aldrich, Germany), 100 μL 10 M NaOH solution, 1300 μL H₂O) for 7 minutes. Eggs were allowed to hatch overnight in M9 buffer [47], L1 larvae were transferred to fresh NGM plates seeded with *E. coli* OP50 and incubated at 20˚C until the development to young adults. Test solutions were prepared in concentrations of 2 mg/mL, corresponding to the LC₅₀ after 6 h of incubation, and its dilutions to 0.2 mg/mL and 0.02 mg/mL of fraction H3 and 0.2 mg/mL of the EtOH-H₂O (1:1) extract in 10 mL M9. 100 μL DMSO were used as a solubilizer, therefore a solution of 1% DMSO was used as a negative control. The solutions were centrifuged for 1 min at 2000 × g and the supernatant was poured into a 75 cm² tissue culture flask. Treatment of the worms for the qPCR experiment was performed accordingly, using 4 mL of test solution in a 25 cm² tissue culture flask. Prior to treatment, worms were rinsed off the NGM plates and the worm suspension was washed three times with M9 buffer to remove remaining bacteria. Approximately 500 to 1000 worms were transferred to each tissue culture flask, and incubated for 6 h at 20˚C. After the incubation time, worms were poured into a fresh 15 mL Falcon tube for centrifugation at 1500 × g for 2 min. The supernatant was removed, worms were washed once in M9, transferred to a 1.5 mL reaction tube and after removal of the supernatant, the tube containing the worm pellet was snap frozen in liquid nitrogen. The procedure was repeated twice to obtain three independent replicates. Both, the microarray as well as the qPCR experiments were conducted with three biological replicates per sample.

**RNA isolation**

RNA was isolated using the RNeasy® Mini Kit (Qiagen, Germany). In the first step, lysis of the worms was achieved by grinding the pellet in a total of 600 μL lysis buffer under repeated freezing in liquid nitrogen. Further isolation, including an on-column DNAse I digest (Qiagen, Germany), was performed according to the manufacturer’s instructions. For the qPCR samples, the on-column DNAse I digest was replaced by digestion of the eluted RNA using the Invitrogen™ Ambion™ TURBO DNA-free Kit (Thermo Scientific, USA) according to the manufacturer’s instructions.

**Transcriptome analysis**

RNA concentration and purity were measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). RNA integrity was checked using an Agilent Bioanalyzer (software version B.02.07.SI532) on a RNA Nano Chip (both Agilent Technologies Inc., Santa Clara, CA, USA). Labelling and hybridization of the RNA were performed as described in the manufacturer’s Affymetrix GeneChip® WT PLUS Reagent Kit. Fragmented and labeled
library was hybridized to an Affymetrix Gene 1.0 ST C. elegans Array containing 28,305 genes and a total of 638,442 probes (24 probes per gene). Following hybridization, the arrays were washed and stained using the Affymetrix GeneChip Fluidics Station 450 and scanned using the Affymetrix GeneChip Scanner 3000 7G. After scanning, first quality analyses were made using the Affymetrix Expression Console (Affymetrix) and Partek Genomics Suite software (Partek Inc., St. Louis, MO, USA) to check the spike-in controls. Microarray data quality was checked as recommended by the manufacturer and by the quality metrics in the Partek Genomics Suite software (Partek Inc., St. Louis, MO, USA). Statistical analyses of microarray data were performed using the Partek Genomics Suite. CEL-files (containing raw expression measurements) were imported to Partek GS. The robust multi-array average (RMA) algorithm was used for normalization. The array data were quantile-normalized and log-2 transformed. For each probe, a one-way analysis of variance was performed: \( Y_{ij} = \mu + \text{Group}_i + \varepsilon_{ij} \), where \( Y_{ij} \) represents the \( j^{th} \) observation on the \( i^{th} \) Group and \( \mu \) is the common effect for the whole experiment. \( \varepsilon_{ij} \) represents the random error present in the \( j^{th} \) observation on the \( i^{th} \) Group. The errors \( \varepsilon_{ij} \) are assumed to be normally and independently distributed with mean 0 and standard deviation \( \delta \) for all measurements [48]. For each probe, Fisher’s least significant difference was tested to statistically compare the difference between the means of the groups’ expression measurements. A false-discovery-rate of \( \leq 0.05 \) was used as a threshold for significance [49]. Only genes with a fold-change of \( \pm 2.0 \) were regarded as significantly changed in expression. Raw data are available from Gene Expression Omnibus (accession number: GSE101680), processed data are given as supplementary file (S1 Table).

Gene ontology (GO) enrichment analysis and functional classification of genes was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID; 6.8 Beta) [50,51].

WormBase (wormbase.org) was referred to regarding information about single genes and corresponding protein sequences.

qPCR

First strand cDNA was synthesized from 1 \( \mu \)g of the digested RNA samples using oligo (dT) primers according to the manufacturer’s instructions (Transcriptor First Strand cDNA Synthesis Kit; Roche Applied Science, Germany). The relative gene expression was quantified for two of the most down-regulated genes, \( \text{pud-1} \) and \( \text{ugt-44} \), and for two of the most up-regulated genes, \( \text{T22D1.2} \) and \( \text{F13E9.4} \). Primers were designed using the open software PerlPrimer v1.1.20 [52], double checked using the online tool Primer 3 v. 0.4.0 [53,54] and purchased from Eurofins Genomics (Ebersberg, Germany). \( \text{pmp-3} \) was used as the housekeeping gene and primer sequences were taken from [55]. A list of all primer sequences is given in Table 1.

RT-PCR was performed in a CFX96 Real-Time System (BioRad, Germany) using the iTaq™ Universal SYBR® Green Supermix (BioRad, Germany) and Hard-Shell® 96-Well PCR Plates (BioRad, Germany). Each well contained a total volume of 20 \( \mu \)L, including 1 \( \mu \)L forward and reverse primers at a final concentration of 400 nmol/L and 2 \( \mu \)L DNA template containing 10 ng cDNA or 2 \( \mu \)L of the respective -RT control. All samples were quantified in duplicate, -RT controls were applied once for every gene and sample. Additionally, no template controls containing 2 \( \mu \)L nuclease free \( \text{H}_2\text{O} \) instead of the DNA template were applied once per gene. Cycling conditions were set as follows: Initial denaturation for 30 s at 95°C, followed by 40 cycles of 5 s at 95°C and 30 s at 60°C. After further 31 s at 65°C, the temperature was increased for 60 cycles by 0.5°C per 5 s cycle for melting curve analysis. The quantitative gene expression of treated versus untreated \( C. \text{elegans} \) was evaluated with the CFX Manager™ software (Bio-Rad, Germany)
based on the comparative Ct method and levels of gene expression were compared to the samples of the negative control (1% DMSO).

GFP reporter gene construct and transgenic C. elegans

pT22D1.2+SP::GFP. A fragment of 2000 bp of T22D1.2 including the promoter region and the signal peptide (SP) which was determined using the online tool SignalP 4.1 [56] was amplified from C. elegans genomic DNA using the Finnzymes Phusion® High-Fidelity DNA Polymerase Kit (Thermo Scientific, USA). 95T22D1.2pSPBamS was used as forward primer (5’-CGACTCTAGAGGATCCTTTTTTTACCAGAATGTTCA-3’), 95T22D1.2pSPKpnA (5’-TCATTTTTTTCTACCGGTACCTGTGATAC-3’) was used as reverse primer. The amplicon was cloned into pPD95.77, which is part of the Fire Lab C. elegans Vector Kit and was kindly provided by A. Fire. A solution of the pT22D1.2::GFP construct (80 μg/mL) and the pRF4 plasmid encoding rol-6 (50 μg/mL) as a marker gene were co-injected into the germline of young adult C. elegans [57,58].

pT22D1.2::GFP. A fragment of 2000 bp including only the promoter region of T22D1.2 was amplified from C. elegans genomic DNA as described above using primers 95T22D1.2pSPBamS (forward; 5’-CGACTCTAGAGGATCCTTTTTTTACCAGAATGTTCA-3’) and 95T22D1.2PrKpnAS (reverse; 5’-TCATTTTTTTCTACCGGTACCTGTGATAC-3’). Strain JF88 mtEx63 [numr-1p::numr-1::GFP + rol-6(su1006)] was obtained from the Caenorhabditis Genetics Center at the University of Minnesota. The transgenic strains were treated as follows: Approximately 10 worms per well were incubated for 6 h in a 24-well-microtiter plate containing 500 μL of the crude hydro-ethanolic extract at concentrations of 0.2 mg/mL and 2 mg/mL or the negative control (1% DMSO in M9 buffer). Each treatment was performed in four replicates. GFP expression was analysed by fluorescence microscopy (Zeiss LSM 510, Zeiss, Germany).

Results

Transcriptome analysis

From the leaves of Combretum mucronatum, a herbal remedy which is widely used as anthelmintic remedy in West Africa [44], an extract was obtained which was strongly enriched with oligomeric procyanidins (OPC). By bioassay-guided fractionation of this extract, fraction H3
was obtained, which contained a mixture of oligomeric procyanidins, which recently have been characterized in detail [45]. All microarray results were conducted with three biological replicates per sample. As shown in Fig 1, treatment with the OPC enriched fractions or the crude hydro-ethanolic extract caused a considerable change in the transcriptome of all treated groups. The number of differentially regulated genes increased with an increase in the concentration of the respective treatment: While “only” 157 genes were up- or down-regulated vs the negative control at 0.02 mg/mL of fraction H3, at 0.2 mg/mL the number increased to 263 and finally to 347 genes at 2 mg/mL. 231 genes were differentially regulated in the group treated with crude extract (RE) at 0.2 mg/mL.

To identify the worm’s key responses to the tannin treatment, further analysis of the data focused on the 134 genes that were differentially expressed in all treatment groups compared to the control (S2 Table). Annotation clustering was performed for all of the 134 key genes as well as for the down-regulated or up-regulated genes respectively, using DAVID; 6.8 beta [50,51]. As shown in Table 2, Cluster 1 (enrichment score 3.47) comprised genes associated with hydrolase function and lipid metabolism, followed by Cluster 2 (enrichment score 3.28) containing lysosome associated genes and Cluster 3 (enrichment score 2.48) containing genes related to saposin-like proteins. Annotation Cluster 4 (enrichment score 2.37) was predominantly composed of cysteine peptidase associated genes. Further Clusters were not considered as they did not contain GO terms of a p-value < 0.05. However, 21 out of the 134 genes were associated with the UniProt Keyword “Membrane/Transmembrane” and GO term “integral component of membrane”.

Interestingly, regarding the non-clustered functional annotation (S3 Table), 53 of the 134 genes were related to the UniProt keyword “Signal” (p-value 1.2·10^{-9}) suggesting them to be either secreted proteins or part of a membrane. The number of genes associated with this keyword was more or less equally distributed between the up- and down-regulated genes (20 vs. 33 genes, respectively). Further enriched terms mainly included the innate immune response, hydrolases, lipid metabolism, unspecified “metabolic processes” and the lysosome.

Several genes out of the above mentioned clusters (cpr-3; spp-1, spp-2, spp-3 and ugt-44 respectively) were additionally identified to be part of the worm’s innate immune response among other differentially expressed genes, such as C-type lectins (clec-166, clec-4), the

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**Fig 1.** Venn diagram showing the number of differentially regulated genes per treatment vs. the untreated negative control (DMSO 1%). H3: OPC enriched fraction “H3”; RE: crude extract (ethanol: water 1:1). 0.02, 0.2 and 2: concentrations of 0.02 mg/mL, 0.2 mg/mL and 2 mg/mL resp., per treatment. Numbers in brackets indicate the number of differentially regulated genes per group.

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Table 2. Functional annotation clusters including up- and down-regulated genes obtained by DAVID.

| Annotation Cluster 1 | Enrichment Score: 3.47 |
|----------------------|-------------------------|
| Category             | Term                    | Count | PValue  | Benjamini |
| UP_KEYWORDS          | Hydrolase               | 15    | 1.3E-4  | 3.3E-3    |
| GOTERM_MF_DIRECT     | hydrolase activity      | 15    | 2.3E-4  | 1.7E-2    |
| GOTERM_BP_DIRECT     | lipid metabolic process | 7     | 1.3E-3  | 3.5E-2    |

| Annotation Cluster 2 | Enrichment Score: 3.28 |
|----------------------|-------------------------|
| Category             | Term                    | Count | PValue  | Benjamini |
| GOTERM_CC_DIRECT     | lysosome                | 6     | 6.4E-6  | 1.2E-4    |
| KEGG_PATHWAY         | Lysosome                | 5     | 2.4E-3  | 6.7E-3    |
| UP_SEQ_FEATURE       | signal peptide          | 4     | 9.6E-2  | 9.1E-1    |

| Annotation Cluster 3 | Enrichment Score: 2.48 |
|----------------------|-------------------------|
| Category             | Term                    | Count | PValue  | Benjamini |
| INTERPRO             | Saposin B               | 4     | 5.6E-4  | 3.6E-2    |
| INTERPRO             | Saposin-like            | 4     | 6.1E-4  | 2.7E-2    |
| SMART                | SapB                    | 3     | 4.0E-3  | 3.7E-2    |
| UP_KEYWORDS          | Disulfide bond          | 7     | 9.1E-2  | 7.0E-1    |

| Annotation Cluster 4 | Enrichment Score: 2.37 |
|----------------------|-------------------------|
| Category             | Term                    | Count | PValue  | Benjamini |
| GOTERM_CC_DIRECT     | lysosome                | 6     | 6.4E-6  | 1.2E-4    |
| SMART                | Pept_C1                 | 4     | 1.5E-4  | 2.8E-3    |
| INTERPRO             | Peptidase C1A, papain C-terminal | 4 | 5.6E-4  | 3.6E-2    |
| INTERPRO             | Peptidase C1A, papain   | 4     | 5.6E-4  | 3.6E-2    |
| GOTERM_MF_DIRECT     | cysteine-type endopeptidase activity | 4 | 1.8E-3  | 4.4E-2    |
| INTERPRO             | Cysteine peptidase, histidine active site | 3 | 2.7E-3  | 6.9E-2    |
| GOTERM_BP_DIRECT     | proteolysis involved in cellular protein catabolic process | 4 | 4.5E-3  | 8.7E-2    |
| INTERPRO             | Cysteine peptidase, cysteine active site | 3 | 5.0E-3  | 1.0E-1    |
| GOTERM_MF_DIRECT     | cysteine-type peptidase activity | 4 | 5.8E-3  | 1.0E-1    |
| GOTERM_CC_DIRECT     | extracellular space    | 5     | 1.4E-2  | 8.3E-2    |
| GOTERM_BP_DIRECT     | proteolysis            | 4     | 3.6E-1  | 9.5E-1    |
| UP_KEYWORDS          | Protease               | 3     | 3.7E-1  | 9.8E-1    |
| GOTERM_MF_DIRECT     | peptidase activity     | 3     | 4.0E-1  | 9.9E-1    |

| Annotation Cluster 5 | Enrichment Score: 1.20 |
|----------------------|-------------------------|
| Category             | Term                    | Count | PValue  | Benjamini |
| INTERPRO             | Glycoside hydrolase, catalytic domain | 3 | 5.7E-2  | 6.2E-1    |
| GOTERM_BP_DIRECT     | carbohydrate metabolic process | 4 | 6.2E-2  | 4.8E-1    |
| INTERPRO             | Glycoside hydrolase, superfamily | 3 | 6.9E-2  | 6.1E-1    |

| Annotation Cluster 6 | Enrichment Score: 1.06 |
|----------------------|-------------------------|
| Category             | Term                    | Count | PValue  | Benjamini |
| GOTERM_CC_DIRECT     | intracellular membrane-bounded organelle | 4 | 8.3E-3  | 7.6E-2    |
| GOTERM_MF_DIRECT     | glucuronosyltransferase activity | 3 | 6.2E-2  | 6.1E-1    |
| GOTERM_BP_DIRECT     | flavonoid biosynthetic process | 3 | 6.3E-2  | 4.4E-1    |
| GOTERM_BP_DIRECT     | flavonoid glucuronidation | 3 | 6.3E-2  | 4.4E-1    |
| INTERPRO             | UDP-glucuronosyl/UDP-glucosyltransferase | 3 | 6.6E-2  | 6.3E-1    |
| GOTERM_MF_DIRECT     | transferase activity, transferring hexosyl groups | 3 | 9.1E-2  | 6.9E-1    |
| UP_KEYWORDS          | Transferase             | 7     | 4.7E-1  | 9.9E-1    |
| GOTERM_MF_DIRECT     | transferase activity    | 6     | 5.9E-1  | 1.0E0     |

| Annotation Cluster 7 | Enrichment Score: 0.64 |
|----------------------|-------------------------|
| Category             | Term                    | Count | PValue  | Benjamini |

(Continued)
caenacin cnc-11, the infection response gene irg-2, lysozyme lys-2, a glutathione-S-transferase (gst-24), invertebrate lysozyme ilys-2, and dod-17 ("Downstream Of DAF-16").

Separate annotation clustering of the down-regulated genes led to an enrichment of very similar terms as obtained for the complete set of differentially regulated genes (S4 Table). Again, genes were related to the lysosome (Cluster 1, ES: 4.95), saposin-like proteins (Cluster 2, ES: 3.29) and cysteine peptidases (Cluster 3, ES: 2.12). Also, the InterPro terms “glycoside hydrolysis” as well as “UDP-glucuronosyl/UDPglucosyltransferase” were significantly enriched. Among the down-regulated genes with the highest fold-changes three “proteins up-regulated in Daf-2” (pud-1, pud 1, and pud-3) were pinpointed. These pud proteins seem to be specific to Caenorhabditis as there are no reports of potential homologues in other species. Due to their association with daf-2 they have been suspected to be somehow involved in aging and lifespan associated processes, but despite intense functional investigations their exact role remains unclear [59].

Regarding the up-regulated genes, no specific function or process was significantly enriched, however, 11 out of 58 genes in total were related to the GO term “integral component of membrane” or the UniProt keywords “Membrane” / “Transmembrane”. Among the genes showing the highest up-regulation were: T22D1.2, an orthologue of human PRB2 and PRB3 (proline-rich protein subfamilies 2 and 3), F13E9.4, which shares 55.7% homology with human filagrin-2, and Y46H3D.8, homologue (88%) to “human keratin associated protein 9–1” (wormbase.org). Further, clx-1 ("collagen sequence x–hybridizing"), numr-1 ("nuclear localized metal responsive") and several heat shock proteins (hsp-16.1, hsp-16.48 and hsp-16.11) that are indicative of a general stress response. Also, homologues to human keratins (K01A6.8; F13E9.14, C28C12.4) and dermokine (H12D21.6) were detected, although their function in C. elegans remains unclear.
Up-regulated genes also seemed to be involved in the immune response and in defense mechanisms to certain toxins, such as heavy metals, drugs or other chemicals. Interestingly, some of the up-regulated genes, e.g. Y46H3D.8, F13E9.4, namr-1, clx-1, C28C12.4 and T22D1.2, had previously been found to be differentially regulated upon treatment with hydrolyzable tannins ([60], wormbase.org).

As the number of differentially regulated genes increased with the concentration of the test solutions (Fig 1), the 347 genes that were exclusively up-regulated in the samples treated with 2 mg/mL of fraction H3 were again cross-checked via WormBase and annotation data to reveal possible functions affected by the tannin treatment that might have not been detected within the groups’ intersection of 134 genes. However, beside some hints pointing towards a general stress response, nothing could be rationally linked to an activity specific for OPCs.

On the other hand, some of the hypothetical proteins corresponding to genes with increased expression, e.g. C50F7.5, clx-1 and T22D1.2, were found to be strikingly rich in repetitive sequences containing proline (Table 3). The most impressive example was that of T22D1.2 for which BLASTp analysis revealed a 91% similarity to the human basic salivary proline-rich protein 2 (PRB2) (wormbase.org). The protein consists of a signal peptide (56) followed by repetitive proline sequences and asparagin−alanine−serine−(NAS) triplets possibly indicating N-glycosylation sites [61].

Seven genes (Y17D7B.10, tag-38, F35E8.19, F08A7.1, T16H12.9, itr-36 and Y106G6D.8) seemed to be differentially regulated exclusively in the group treated with crude extract “RE”.

### Table 3. Predicted protein sequences of T22D1.2, clx-1 and C50F7.5.

| T22D1.2          | CLX-1          | C50F7.5       |
|------------------|----------------|--------------|
| MRTFQLTLLFTALAVTSALAPFSVGETASRPQPPPKGKGTGTPPPPPTEGPQDLSEGEQNA | MFYKALGVVLVLVHADPSDLPAPSSPPAPPSGSQQPQPSLPDGPSSAGGGPH | MQISLTLILAVGATAAPSDLSLGRGGHHHHHHHHTKKAPRTSRIATTTFAPTSDDLPIA |
| PSSPPPPPKGKGTGTPPPPPTEGPQDLSEGEQNA | PSSPPPPPKGKGTGTPPPPPTEGPQDLSEGEQNA | GSASSAPVIASSADPTLSSVQPQNEPSPGTVAPSDPSGSGPSGPPGNPSEDQPSG |
| 60               | 120            | 60           |
| PSSPPPPPKGKGTGTPPPPPTEGPQDLSEGEQNA | PSSPPPPPKGKGTGTPPPPPTEGPQDLSEGEQNA | PSSPPPPPKGKGTGTPPPPPTEGPQDLSEGEQNA |
| 120              | 240            | 180          |
| PSSPPPPPKGKGTGTPPPPPTEGPQDLSEGEQNA | PSSPPPPPKGKGTGTPPPPPTEGPQDLSEGEQNA | PSSPPPPPKGKGTGTPPPPPTEGPQDLSEGEQNA |
| 240              | 300            | 240          |
| PSSPPPPPKGKGTGTPPPPPTEGPQDLSEGEQNA | PSSPPPPPKGKGTGTPPPPPTEGPQDLSEGEQNA | PSSPPPPPKGKGTGTPPPPPTEGPQDLSEGEQNA |
| 300              | 240            | 240          |
| PSSPPPPPKGKGTGTPPPPPTEGPQDLSEGEQNA | PSSPPPPPKGKGTGTPPPPPTEGPQDLSEGEQNA | PSSPPPPPKGKGTGTPPPPPTEGPQDLSEGEQNA |
| 300              | 80             | 80           |
| PSSPPPPPKGKGTGTPPPPPTEGPQDLSEGEQNA | PSSPPPPPKGKGTGTPPPPPTEGPQDLSEGEQNA | PSSPPPPPKGKGTGTPPPPPTEGPQDLSEGEQNA |
| 360              | 540            | 558          |
| PSSPPPPPKGKGTGTPPPPPTEGPQDLSEGEQNA | PSSPPPPPKGKGTGTPPPPPTEGPQDLSEGEQNA | PSSPPPPPKGKGTGTPPPPPTEGPQDLSEGEQNA |
| 540              | 558            | 558          |

P: Proline, NAS: Potential N-glycosylation site. Repetitive sequences are displayed in italics and the complete site of repetition is underlined.

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However, this was most likely due to the cut-off set at a fold-change of 2.0 and not to additional effects caused by other components of the extract than procyanidins, as the values for groups “H3” were slightly below and those for “RE” were slightly above the threshold of 2.0.

Quantitative RT-PCR

To confirm and cross-validate the results of the microarray, qPCR was performed to monitor the expression of selected genes in three independent experiments, receiving the same treatment as the samples prepared for the microarray. F13E9.4 and T22D1.2 were selected as representatives among the up-regulated genes, pud-1.1 and ugt-44 were chosen from the down-regulated genes (for primer sequences see Materials and Methods). Normalized levels of mRNA expression for each gene are shown in Fig 2. In general, the results obtained by qPCR support the findings of the microarray experiment. A clear concentration-dependent regulation was observed for pud-1.1 (down) and T22D1.2 (up) in the groups treated with fraction H3. Again, the increase in the expression of T22D1.2 was extraordinarily strong during qPCR analysis. The expression of ugt-44 was almost exactly in the same range for all treated groups as in the array. F13E9.4 was rather strongly up-regulated in both experiments, but markedly below the expression of T22D1.2.

Similar to the array results, the differential expression in the groups treated with the crude extract (0.2 mg/mL) exceeded that of the groups treated with H3 at the same concentration, and for pud-1.1, ugt-44 and F13E9.4 even the groups treated at 2 mg/mL.

![Quantitative RT-PCR](https://doi.org/10.1371/journal.pone.0184656.g002)

**Fig 2.** Quantitative RT-PCR. Relative normalized expression of ugt-44, pud-1.1, F13E9.4 and T22D1.2 mRNA following the treatment with OPC enriched fraction “H3” or *C. mucronatum* extract (ethanol: water 1:1; “RE”): 0.02, 0.2 and 2: concentrations of 0.02 mg/mL, 0.2 mg/mL and 2 mg/mL respectively. Data are presented on a log2 scale, the line at 1.0 indicates the expression level of the negative control (DMSO 1%).
GFP reporter gene constructs

Since the function of T22D1.2 has not been described so far, its site of expression and secretion was attempted to be revealed by reporter gene constructs using the gene promoter fused to GFP. In order to localize the secretion site, worms containing a GFP construct of the promoter sequence followed by the signal peptide, were treated with the hydro-ethanolic extract at two concentrations (0.2 mg/mL and 2 mg/mL). Unfortunately, the GFP signal in the treated samples was extremely low and hardly detectable (S1 Fig). Possibly, the gene was expressed at a very low rate, somehow contradicting the array results, or the low fluorescence was caused by secretion of GFP into the intestine due to the signal peptide, followed by quick degradation by proteases.

Therefore, the promotor sequence was subsequently fused to GFP without the signal peptide. In this case a strong, concentration dependent GFP signal was observed particularly in the group treated with 2 mg/mL of the extract, but also at 0.2 mg/mL (Fig 3). The main expression was observed in the intestine, particularly the part posterior to the pharynx, extending to the entire intestine with increasing concentration. No expression at all was seen in the negative controls.

Numr-1, which was found to be strongly increased by the procyanidins during the transcriptome analysis, has previously been reported as an indicator exclusively for metal induced stress [62]. A C. elegans strain containing a numr-1 promoter::GFP construct was treated with the extract, in order to assess whether the transcription could also be induced by stressors.

![Representative images of C. elegans expressing pT22D1.2::GFP depending on treatment with hydroethanolic extract from C. mucronatum. A: negative control (DMSO 1%); B: treatment with 0.2 mg/mL extract; C: treatment with 2 mg/mL extract. Scale bars = 100 μm.](https://doi.org/10.1371/journal.pone.0184656.g003)
other than metals. As displayed in Fig 4, basal gene expression was mainly observed in tail and head, but in some cases also in the intestine of the negative controls. Treatment with the extract at a concentration of 0.2 mg/mL slightly increased the expression of numr-1 in head and tail compared to the negative control whereas a concentration of 2 mg/mL led to a strong fluorescence of the nuclei throughout the worms’ body, mainly in the intestine.

In summary, both genes, T22D1.2 and numr-1 showed a concentration-dependent increase in the expression as well as an extension of the expression site after contact of the worm with oligomeric procyanidins, visually confirming the microarray results. Taken together, it can be proposed that the main site of action of proanthocyanidins is the worms’ intestine.

Discussion

Condensed tannins are known for their ability to interact with certain proteins mainly via hydrogen bonding [19,21,22], therefore, it is very likely that the observed differential gene expression is based on an interaction of the proanthocyanidins with proteins of the nematode in a more or less specific way. Particularly proline- or histidine-rich proteins are suspected to be possible interaction partners due to the high affinity of tannins in general to these proteins [20,25,63].

As previous microscopic studies revealed structural alterations in the cuticle of different parasitic nematodes [14,29] and C. elegans [26], tannins have been suspected to bind to sheath or cuticle which are composed of collagen-like and non-collagen-like proteins (cuticlins) that are both rich in proline and hydroxyproline [64,65]. Also, the hypodermis, that seemed to be detached from the muscle tissue below, the muscle tissue itself and particularly the intestine
which showed severe signs of damage and digestion, were strongly affected by tannin treatment [28,31,33,34,66].

The results from the current study suggest that an alteration of gene expression most frequently occurs for intestinal proteins and enzymes as the intestine is the tissue mainly exposed to tannins. However, this finding could also be due to the short incubation time with the substances and other tissues might also be affected following a longer exposure.

A common feature of many differentially regulated genes within this study is their localization to membranes. However, regarding the sequences of the respective corresponding proteins, none of these proteins “integral to membrane” is particularly enriched in either proline or histidine and might therefore not act as a direct binding partner of PAC. More likely, PACs exert membrane destabilizing effects that could then affect membrane-bound enzymes or transporters, similar to the activity recently described for a trimeric A-type procyanidin against Bacillus cereus [67]. Moreover, flavonoids and tannins are also capable of chelating a variety of polyvalent metal cations via their o-dihydroxy function in the B-ring or involving the 4-oxo function of flavones [68–72] and the chelating activity seems to increase with the molecular size of condensed tannins [72]. Therefore, an interference of the tannins (and flavonoids) with certain ions within the worm (e.g. iron or zinc as part of metalloproteases), similar to previously described deleterious effects in mammals (reviewed by [63]) and bacteria [67,73], is also feasible.

The (mainly down-regulated) enzymes clustered according to their structure or functions as hydrolases, part of the lysosome, saposin-like proteins, cysteine peptidases or proteins associated with the worms’ immune response, also share the feature of possessing a signal peptide, indicating their extracellular location. Therefore, these enzymes are possibly affected by the treatment due to their accessibility for PAC and general detrimental effects on membranes rather than due to their specific function or structure. In line with this hypothesis is the absence of any receptor mediated response or of specific signal pathways activated or inactivated by the treatment.

Finally, three members of the hitherto uncharacterized PUD family (proteins up-regulated in daf-2), pud-1.1, pud-1.2 and pud-3 were among the strongest down-regulated genes. Due to their association with daf-2 they have been suspected to be somehow involved in aging and lifespan associated processes and were suggested to act as transcription regulators for collagen genes, but despite intense functional investigations their exact role remains unclear [59].

T22D1.2 was the gene that was by far up-regulated the strongest with a fold-change > 900 at the highest concentration of H3. As mentioned above, it is an orthologue of human basic salivary proline-rich protein 2, however, not much is known about its function in C. elegans, except for one knockdown study and subsequent treatment with mercury chloride revealing a phenotype hypersensitive to chemicals [74]. As shown in Fig 3, the intestine is the main expression site and the expression increases with the amount of test substance. Following translation of T22D1.2 in the enterocytes, the protein enters the secretory pathway as indicated by the presence of a signal peptide [56]. Unfortunately, the final localization of the protein could not be unambiguously determined as the construct containing the signal peptide only led to an extremely weak fluorescence (S1 Fig). Possibly, the protein was immediately degraded after secretion to the intestinal lumen, the fluorescence signal has been quenched or the protein was rapidly excreted due to the very frequent defection of the worm [75]. Regarding the role of T22D1.2, it can be seen as a defense mechanism against tannins in the soil-inhabiting C. elegans, similar to salivary proteins in mammals (reviewed by [76]). Tannins can interact with proteins, calcium and iron absorption or digestive enzymes and lead to growth impairment and weight loss in animals. On the other hand, proline-rich salivary proteins are an effective mechanism in many animals to bind tannins and to prevent these detrimental effects.
In some species, i.e. rats and mice, proline-rich proteins are not constitutively expressed, but are induced by tannins [63,79]. Toxic effects of tannins in invertebrates have frequently been described for different species of plant-herbivore insects. In sensitive species, severe lesions of the midgut have been detected whereas other species remained unaffected (reviewed by [80]). The underlying mechanism is still unclear, a direct effect of tannins binding to epithelial membranes has been discussed as well as an indirect effect by oxidative stress, as insect guts contain iron (Fe$^{3+}$) as a potential oxidant [80]. However, in cases where oxidative stress was involved in the toxicity against C. elegans, typical detoxifying enzymes, such as glutathione-S-transferases, should be expected to be up-regulated more pronouncedly [81,82]. Concerning defense mechanisms in insects, the presence of surfactants and possibly hydrolases as well as several ways of inhibiting a possible oxidation of tannins have been described. On the other hand, the presence of tannin-binding proteins, similar to salivary proteins, has been suspected, but has not been identified until now [80]. Possibly, T22D1.2 could provide such a detoxification mechanism in C. elegans, but further studies are ongoing to confirm the function of this gene and the related protein.

Not only proline-rich salivary proteins, but also histidine-rich proteins, such as histatins can interact with tannins [63,83]. Therefore, also the histidine rich NUMR-1 [62] is potentially involved in the defense as a binding protein. However, as it is localized within the nucleus, a direct interaction with tannins seems very unlikely. Numr-1 (nuclear localized metal responsive) which showed a strongly increased expression, has also been shown to be strongly up-regulated upon treatment with tannic acid, but not after treatment with quercetin [60]. The corresponding protein NUMR-1 was localized in the nuclei of head neurons, egg-laying muscles of the vulva and cells in the tail [62] and was found to be induced under metal stress, particularly by cadmium and copper. Other stressors such as juglone, tunicamycin, heat shock, starvation or infections with pathogens did not cause an increased transcription [62]. The expression pattern of NUMR-1::GFP induced at 100 μM cadmium [62] resembled that of 2 mg/mL EtOH-H$_2$O (1:1) extract from C. mucronatum (Fig 4), although slightly more nuclei seemed to be fluorescing upon extract treatment. As it is unlikely that the result observed for the extract and the respective fractions obtained from this extract is caused by heavy metals (e.g. as a contamination) and also commercially available purified tannic acid caused an up-regulation of this gene [60], numr-1 could be part of a more or less specific stress response.

**Conclusion**

The current study provides first insights into transcriptomic changes in C. elegans upon treatment with a proanthocyanidin-enriched extract from C. mucronatum at different concentrations. Despite the lack of distinct signal pathways or receptors affected, the findings strongly point towards proteins within the intestinal membrane as well as membrane bound and secreted enzymes and peptides to be the main target for PACs. Most likely, the observed alterations in gene expression are caused indirectly via detrimental effects of the PACs on membranes, nevertheless, the current results provide an insight into the molecular processes affected by tannin treatment as a potential base for future drug development. Further, T22D1.2 which was massively up-regulated was identified as a possible detoxification mechanism.

**Supporting information**

S1 Table. Processed data of the microarray experiment. (XLSX)
S2 Table. Intersection of differentially expressed genes in the treated samples versus the negative control showing at least a 2-fold difference and a p-value < 0.05. H3 0.02 / 0.2 / 2: samples treated with fraction H3 in concentrations of 0.02, 0.2 or 2 mg/mL resp.; RE 0.2: samples treated with 0.2 mg/mL crude extract.

(S3X)

S3 Table. Non-clustered functional annotation table of the differentially regulated genes among all treated groups.

(S3X)

S4 Table. Annotation Clusters of the down-regulated genes among all treated groups versus the negative control.

(S4X)

S1 Fig. Representative images of C. elegans expressing pT22D1.2::GFP including the signal peptide. A: negative control (DMSO 1%); B: treatment with 2 mg/mL extract. Scale bars = 100 μm.

(DOCX)

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