Transgenerational lipid-reducing activity of benzylisoquinoline alkaloids in *Caenorhabditis elegans*

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Abstract

Epigenetic mechanisms allow for transgenerational memory of an ancestor's environment and can affect the gene expression, physiology and phenotype of that ancestor's descendants, independent of DNA sequence alteration. Among many model organisms, *Caenorhabditis elegans* has been instrumental in studies of transgenerational inheritance, most of which have focused on the effects of external stressors of the parent worm on the lifespan and stress resistance of future generations. In this work, we used Nile red staining of accumulated lipids in *C. elegans* to investigate the transgenerational effect of two benzylisoquinoline alkaloids, namely, berberine and sanguinarine. Our results showed that a reduction in Nile red fluorescence can be propagated to subsequent worm generations. Using mutant worms, we found that the transgenerational effect requires the ASH-2 component of the histone H3K4me3 complex and the HRDE-1 worm Argonaute protein. *Ash-2* is also required for transgenerational inheritance of the xenobiotic response in the worm. Our study offers new insights into transmissible drug effects across multiple generations and suggests the importance of such analyses in the drug development process.

1 INTRODUCTION

Sublethal stress pretreatments in diverse combinations of stressors and recipient organisms have been shown to increase germination in plants, animal cell proliferation in culture, protein turnover, subsequent stress resistance, and lifespan extension. An increased lifespan has been reported to result from diverse types of stressors, such as heat, cold, oxidative stress, radiation, electrical stimulation, exercise, and fasting (Ristow et al., 2011; Lagisz et al., 2013; Matsuyama et al., 2014; Chuang et al., 2016; Rechavi et al., 2014). However, the biological mechanisms of these stress treatment effects are not fully understood. Advances in molecular genetics research have revealed that environmental stimuli and exogenous stressors on an organism can induce responses that are passed on to subsequent generations. This phenomenon is called epigenetic inheritance, and epigenetic changes are defined as heritable changes in gene expression that are not due to alterations in the DNA sequence (Holliday, 1987).

Many studies on epigenetic inheritance have been carried out using the model organism *Caenorhabditis elegans* due to its numerous physiological advantages, such as a rapid reproductive cycle, small size and short lifespan, as well as its genetic homology with mammalian models. Some of the earliest work in *C. elegans* showed that sublethal heat treatment of the worms affected their heat resistance and lifespan (Lithgow et al., 1994; 1995). In recent years, studies have revealed that those responses are transmitted to subsequent generations through epigenetic modifications such as changes in chromatin

structure, including DNA methylation, histone modification and variant incorporation, and noncoding RNAs (Egger et al., 2004; Greer et al., 2011, Houri-Ze'evi et al., 2016; Klosin et al., 2017). As these studies have reported, transgenerational inheritance often involves external stimuli or stressors, which result in the increased resistance and survival of the exposed organisms.

In this work, we investigated whether the effects of two benzylisoquinoline alkaloids, namely, berberine and sanguinarine, on lipid reduction in *C. elegans* (Chow & Sato, 2013) are propagated to its descendants. Our results indicated that the effects of alkaloids are transgenerational.

2 RESULTS

2.1 Treatment with berberine and sanguinarine reduced Nile red fluorescence of accumulated lipids in *C. elegans*, and the effect was transmitted to subsequent generations

Previously, we reported that berberine and sanguinarine reduced lipid accumulation in *C. elegans* using Oil red O staining. Such activity was consistently observed with 400 to 500 μM berberine and 10 to 25 μM sanguinarine in the worms. A similar reduction was also observed with Nile red staining in an evaluation of lipid accumulation in *C. elegans* (Chow and Sato, 2013). Although several reports have claimed that the measurement of lipid

droplets in *C. elegans* using Nile red staining is invalid (Brooks et al., 2009; O'Rourke et al., 2009; Zhang et al., 2010), our results from assessments using both Oil red O and Nile red staining methods are concordant. Thus, we used Nile red vital dye in this study as it allows for rapid and live in vivo observation of lipid accumulation in worms. Nile red is widely used for the analysis of lipid droplets in *C. elegans* and has led to the identification of numerous evolutionarily conserved fat regulatory genes and small molecules that affect fat metabolism (Van Gilst et al., 2005; Srinivasan et al., 2008; Chen et al., 2009; Cohen et al., 2009; Mullaney et al., 2010; Lemieux et al., 2011; Pathare et al., 2012). However, to clarify our interpretation of lipid accumulation in *C. elegans* in this study, we used the difference in Nile red fluorescence intensity between treated and control worm groups as the indicator of in vivo lipid-level changes.

As Figure 1a shows, treatment of L4 larval stage wild-type N2 worms with 500 μM berberine and 10 μM sanguinarine for 48 hours significantly reduced Nile red fluorescence. The treated parent worms (P0) were then transferred to culture plates containing fresh nematode growth medium (NGM) without alkaloids and allowed to lay eggs. Adults of the next generation, F1, were then stained with Nile red to observe their fluorescence, and a portion of the worms were transferred to new NGM plates to lay eggs that hatched into F2 worms. This procedure was repeated until the F3 worms were evaluated. Nile red staining showed that reduced fluorescence in berberine- and sanguinarine-treated P0 worms was also exhibited by their F1, F2, and F3 offspring (Figure 1b, c, d). These results imply that the

memory of an ancestor's environment (e.g., alkaloid treatment of the P0 generation) is transmissible and can affect the gene expression and physiology of future generations.

2.2 The transgenerational effect of lipid reduction by berberine and sanguinarine treatment of parent worms was abolished in the F1 to F3 progeny of *ash-2* (*tm1726*) and *hrde-1* (*tm1200*) mutants

Epigenetic modifications are reported to be involved in the transgenerational inheritance of parental phenotypes (Lim and Brunet, 2013; Heard and Martienssen, 2014). Thus, we hypothesized that xenobiotics, i.e., alkaloid treatment of the *C. elegans* parent generation, would induce epigenetic alterations, which could be maintained and transmitted to future generations that are unexposed to alkaloids. To test this hypothesis, we used mutant strains, namely, *ash-2* and *hrde-1*. ASH-2 is one of the histone H3 lysine 4 trimethylase (H3K4me3) regulatory components of chromatin modification (Greer et al., 2011), and HRDE-1 is one of several *C. elegans* WAGO Argonaute proteins that bind RNAi-associated 22G-RNAs in the germline. HRDE-1 localizes to the nucleus to affect transcriptional gene silencing and transgenerational RNAi (Buckley et al., 2012; Luteijn et al., 2012; Shirayama et al., 2012). Both *ash-2* and *hrde-1* deletion mutants are homozygous knockouts generated by the National BioResource Project of Japan (The *C. elegans* Deletion Mutant Consortium, 2012).

When we treated the wild-type N2, *ash-2* and *hrde-1* worms with berberine and sanguinarine, all the P0 generations showed reduced Nile red fluorescence (Figures 2a, 3a). This result

indicates that *ash-2* and *hrde-1* did not abrogate the lipid-reduction effect of alkaloid treatment in the parent worms. However, we observed that the deletion mutants of ASH-2 and HRDE-1 failed to reduce the Nile red fluorescence in the F1 to F3 descendants (Figure 2b-d, 3b-d). These results suggest that the inheritance of lipid-reduction effects requires specific histone modification factors, such as the H3K4 trimethylation complex or possibly additional epigenetic mediators, and may involve the nuclear Argonaute protein bound to PIWI-interacting RNAs for transgenerational silencing (Ashe et al., 2012).

A previous study using *C. elegans* reported that exposure to heavy metal stressors in the parent generation induced epigenetic alterations that could be maintained and transmitted to the descendants (Kishimoto et al., 2017). The environmental stressor increased the lifespan of the exposed parent worms and subsequently increased the resistance of the descendants to oxidative stress. However, knockdown of the H3K4me3 complex components (*wdr-5.1*, *ash-2* and *set-2*) in the P0 generations was found to have no effect on the increased stress resistance of the parents, whereas it failed to increase the resistance of the F1 progeny. These results suggest that the parent worms acquire stress-induced effects independently of these histone modifiers.

In our experiments, the worms were exposed to alkaloids, which mimicked a xenobiotic stressor. The lipid-reduction effect of berberine and sanguinarine was transmissible to the F1 to F3 descendants of wild-type N2 worms. However, this effect was abolished in *ash-2* and *hrde-1* offspring. Assuming that alkaloids are exogenous stressors to the worms, we

hypothesized that the lipid reduction activities of berberine and sanguinarine were the result of xenobiotic effects. Thus, we next investigated the xenobiotic response of the worms and the correlation between this response and the lipid metabolism of the worms in the P0 to F3 generations.

2.3 Xenobiotic response of *C. elegans* to berberine and sanguinarine treatment

Plant alkaloids have been suggested to function in the defense against herbivores and pathogens. Therefore, these compounds are likely to induce a xenobiotic response in *C. elegans* to metabolize and consequently eliminate the chemical toxins from their bodies. The isoquinoline alkaloids berberine and sanguinarine were reported to exhibit cytotoxicity and inhibit mitochondrial respiratory complex I (Adhami et al., 2003; Turner et al., 2008). As a result of such inhibition, berberine and sanguinarine were found to activate the cellular energy sensor AMP-activated protein kinase (AMPK) due to an increase in cellular AMP levels and reduced ATP levels. AMPK activation leads to inhibition of acetyl-coenzyme A carboxylase (ACC) in the cells, which in turn inhibits triglyceride synthesis. Therefore, we expected that berberine and sanguinarine would induce a xenobiotic response in worms. *C. elegans* has many genes encoding enzymes for their xenobiotic responses, including four main classes of detoxification enzymes: cytochrome P450 (CYP), short-chain dehydrogenases (SDR), UDP-glucuronosyl or glycosyl transferases (UGT), and glutathione-*S*-transferases (GST) (Lindblom and Dodd, 2006). Previously, we investigated the genome-wide response of the worms to berberine and sanguinarine treatment by microarray analysis.

176 The results were subsequently verified by quantitative reverse transcription-polymerase
177 chain reaction (qRT-PCR) and indicated that defense response and detoxification genes,
178 including a CUB-like domain-containing protein gene (*F08G5.6*) affecting innate immune
179 response, a cytochrome P450 family gene (*cyp-35C1*) affecting xenobiotic response, a
180 glutathione *S*-transferase gene (*gst-5*), and UDP-glucuronosyltransferase gene (*ugt-21* and
181 *ugt-25*) affecting detoxification, were the most significantly upregulated (Chow et al., 2014).
182
183 In this study, we evaluated the xenobiotic response of *C. elegans* to berberine and
184 sanguinarine treatment by quantifying the expression of these genes in P0 parents and their
185 untreated F1, F2, and F3 offspring by qRT-PCR. The results showed that the detoxification
186 response genes *cyp-35C1*, *gst-5*, *ugt-21*, *ugt-25* and *F08G5.6* were significantly upregulated
187 in treated wild-type N2 parent worms (Figure 4a). The induction of these genes in alkaloid-
188 treated worms was still observed in the F1 worms, although the levels were diminished,
189 especially in the offspring of sanguinarine-treated worms. The detoxification response genes
190 were also found to be significantly upregulated in the F2 worms, and some genes remained
191 significantly induced in the F3 offspring of sanguinarine-treated P0 worms (Figure 4b-d).
192
193 However, stress-response genes, such as a superoxide dismutase gene (*sod-5*) and a heat
194 shock protein gene (*hsp-16.2*), that are induced in response to osmotic, heat shock or other
195 environmental stresses were repressed in P0 worms treated with an alkaloid. However, the
196 expression of these genes was upregulated in F2 worms, and the *hsp-16.2* gene remained
197 upregulated in the F3 offspring of sanguinarine-treated P0 worms. This result suggests that

198 xenobiotic stimulation could increase stress resistance in the descendants of affected
199 ancestor worms. This phenomenon supports the hypothesis that transgenerational epigenetic
200 inheritance acts to protect future progeny from the effects of external stress (Kaati et al.,
201 2007; Baugh, 2013; Schott et al., 2014).
202
203 **2.4 The transgenerational effect of the xenobiotic response was abolished in the F1 to**
204 **F3 progeny of *ash-2* but not *hrde-1* worms**
205
206 When we treated the *ash-2* (*tm1726*) and *hrde-1* (*tm1200*) worms with berberine and
207 sanguinarine in the parent generation, the detoxification response genes were upregulated, as
208 was also observed for wild-type N2 worms (Figures 5 & 6). However, this response was
209 reduced or abolished in *ash-2* F1 to F3 progeny, especially in F3 progeny of sanguinarine-
210 treated P0 (Figure 5d).
211
212 In contrast, the induction of detoxification response genes in *hrde-1* was reduced but still
213 detected in the F1 to F3 descendants of alkaloid-treated P0 worms. This trend was similar to
214 that of wild-type worms. The stress-response gene *hsp-16.2* was upregulated in the P0 and
215 F3 generations of the *hrde-1* worms (Figure 6) and remained upregulated in the F1 to F3
216 offspring of the sanguinarine-treated ancestor.
217
218 These results suggest that alkaloid treatment possibly acts as a stressor, inducing
219 transgenerational effects and subsequently increasing stress resistance in worms. As was

reported in other studies using *C. elegans*, the epigenetic memory of such stress exposure requires ASH-2, which is a component of the histone H3K4me3 regulatory complex (Greer et al., 2011; Kishimoto et al., 2017). Our results also show that *ash-2* is involved in the transgenerational inheritance of detoxification response in the worms, but *hrde-1* is not.

2.5 The upregulated xenobiotic response might reduce fertility in the F3 generation of wild-type and *hrde-1* mutant worms

The cytotoxicity of berberine and sanguinarine has been previously reported (Chow et al., 2016; 2017). Furthermore, these alkaloids induce detoxification-responsive genes, indicating their toxicity to worms. The xenobiotic response was still observed in the F3 offspring that were unexposed to alkaloids. Therefore, we wanted to determine whether this xenobiotic effect would alter reproduction in future generations of alkaloid-treated P0 worms. However, after 24 hours, the mean number of offspring that hatched from the F3 generation of berberine and sanguinarine-treated worms was lower than that of the untreated N2 control, although the difference was statistically nonsignificant. This trend was also observed in the *hrde-1* and *ash-2* mutants (Figure 7).

Although the mechanisms controlling the transmission and duration of epigenetic changes across generations are still unknown, the number of generations where epigenetic inheritance was found varied from as few as one to more than forty. A switch between a transitory, reversible effect and a stable, inherited effect was observed when only one

generation of worms was exposed to volatile odorants compared to exposure of four generations continuously (Remy, 2010). Our results showed that berberine and sanguinarine induced a relatively strong detoxification response in the parent generation that ingested the alkaloids, followed by a transient shift close to the basal level in the immediate F1 progeny, but the memory of the xenobiotic response was then inherited in the generations thereafter.

Exposures of *C. elegans* to various stressors, e.g., oxidative and osmotic stress, electrical pulses, UV light, radiation, and caloric restriction, have been reported to increase the lifespan of the worms. It has also been suggested that the number of progeny decreases as energy is conserved for lifespan extension. The induction of xenobiotic response genes other than those in the CYP-35 family that have a direct impact on worms' reproduction remains to be elucidated.

3 DISCUSSION

In this study, we investigated the transgenerational effect of two benzylisoquinoline alkaloids, namely, berberine and sanguinarine, in *C. elegans*. Our results suggested that these alkaloids affected the worms' lipid metabolism and xenobiotic responses transgenerationally (Figures 1 & 4). Through epigenetic modifications, an ancestor's experience can affect its gene expression, physiology, and lifespan, which are transmissible to its descendants. Through quantification of Nile red fluorescence in *C. elegans*, we found that the lipid-reduction effects of alkaloids were inherited by the offspring of wild-type

worms but diminished in *ash-2* and *hrde-1* worms (Figures 1-3). This result indicates that the ASH-2 component of the histone H3K4me3 complex and the HRDE-1 worm Argonaute protein are involved in transgenerational inheritance of the lipid-reduction phenotype.

We hypothesized that the lipid reduction activities of alkaloid treatment are the result of the xenobiotic response of the worms and the changes in gene expression in the P0 and F1 to F3 generations. Our results showed that the upregulation of detoxification response genes in P0 worms was inherited by F3 wild-type offspring, whereas such effects were decreased in *hrde-1* worms and diminished in *ash-2* worms (Figures 4-6). This finding suggests that *ash-2* is required for transgenerational inheritance of the xenobiotic response, whereas *hrde-1* is not. We also observed that alkaloid treatment of the P0 worms resulted in reduced fertility in their F3 offspring (Figure 7). Of the various detoxification genes, *cyp-35C1* was noticeably highly induced in alkaloid-treated P0 worms, and its enhanced expression was maintained at a diminished level in the F1 to F3 offspring of these worms. Incidentally, a reduction in the expression of *cyp-35C1* seemed to restore the fertility of F3 *ash-2* and *hrde-1* worms with sanguinarine-treated ancestors but not that of berberine-treated worms. This result supports the finding of a previous report that the *cyp-35A/C* gene reduces the reproduction of *C. elegans* exposed to xenobiotics (Menzel et al., 2005). This result also suggests that *cyp-35C1* is a useful indicator of the worm's xenobiotic response for transgenerational inheritance experiments.

Since berberine and sanguinarine induced transgenerational lipid-reducing activity in *C. elegans*, we also analyzed the expression of several genes involved in the worm's lipid metabolism (Supplementary Figures S1 and S2). AAK-1 and AAK-2 are the *C. elegans* homologs of the catalytic α-subunits of AMPK that regulate triglyceride synthesis. Our qRT-PCR results show enhanced *aak-1* expression in alkaloid-treated P0 wild-type, *ash-2* and *hrde-1* worms but a gradual reduction in *aak-1* expression in the F3 generations (Supplementary Figure S1). Although the role of AAK-1 in the transmissible lipid-reduction effect is unclear, a similar change in *aak-1* expression found in all three worm strains suggests that it is not directly involved in the transgenerational effect on lipid accumulation. Additionally, the qRT-PCR results showed that *nhr-49*, the key regulator of fat consumption, and its downstream target genes were downregulated in F3 wild-type, *ash-2* and *hrde-1* worms (Supplementary Figure S2). Since the *nhr-49* mutant exhibits a high-fat phenotype due to repression of fatty-acid β-oxidation genes, the reduction in lipid accumulation in F3 wild-type worms treated with alkaloids also suggests that the effect is independent of fatty-acid β-oxidation. These results indicate that further investigation is necessary to identify the mechanisms involved.

Our results show that *hrde-1* is not required for transgenerational inheritance of the xenobiotic response. However, HRDE-1 is involved in the transgenerational inheritance of the lipid-reduction effect of alkaloid treatment in P0 worms (Figure 3). HRDE-1 is essential for transgenerational shuttling of heritable small RNAs. In a transcriptome-sequencing experiment using starvation as a stressor in *C. elegans*, Rechavi et al. (2014) reported that

96.8% of the heritable small RNAs were not inherited in *hrde-1* mutants. Future small RNA transcriptome analysis could help identify the heritable small RNAs and their target genes that are responsible for the transgenerational lipid-reducing effect in alkaloid-treated worms.

Investigation of transgenerational inheritance of drug effects is pertinent to the fields of pharmacology and toxicology since these drug effects impact the offspring in future generations. Our study reveals that the biological activity of a xenobiotic in *C. elegans* is transmissible to descendants. Although the exact mechanisms are largely unknown, we found that the epigenetic memory of the lipid-reduction effect of berberine and sanguinarine requires *ash-2* and *hrde-1*. qRT-PCR results of detoxification response and lipid metabolism gene expression show that sanguinarine treatment has a stronger effect in F3 offspring than berberine treatment, suggesting that the impact of transgenerational inheritance is specific to each stimulus and is not a generalized epigenetic memory of the ancestor's exposure to xenobiotics. In this study, we evaluated the transgenerational inheritance of alkaloids' lipid-reducing effect in *C. elegans*. Taki et al. (2013) reported that nicotine exposure induced heritable behavioral changes in the F1 and F2 offspring of *C. elegans*. These results offer new insights into transgenerational drug effects on the descendants of treated subjects and suggest the importance of such analysis beyond pharmacokinetics in drug development.

4 EXPERIMENTAL PROCEDURES

329 **4.1 Chemicals and reagents**

330 Berberine sulfate was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo,
331 Japan), and sanguinarine chloride and Nile red were purchased from Sigma-Aldrich (St.
332 Louis, U.S.A.). All other reagents were purchased from Wako Pure Chemicals (Osaka,
333 Japan) unless otherwise stated. Berberine and sanguinarine were dissolved in distilled
334 water. Nile red stock solution was prepared with acetone at 250 mg/ml and diluted in
335 phosphate buffer solution to 0.5 μg/ml.

336

337 **4.2 Nematode strains**

338 The following worm strains were used: the wild-type N2 (Bristol) and two knockout
339 mutants classified as homozygous viable by the National BioResource Project of Japan
340 (The *C. elegans* Deletion Mutant Consortium, 2012), namely, *hrde-1* (*tm1200*; a mutant
341 with a deletion of 374 bp at 32391-32764, chromosome III) and *ash-2* (*tm1726*; a
342 mutant with a deletion of 469 bp at 12372-12840, chromosome II). All worms were
343 maintained on NGM at 20°C according to standard culture methods (Stiernagle, 2006).

344

345 **4.3 Nematode treatment**

346 L4 larval stage worms were treated with berberine, sanguinarine or distilled water (as a
347 control) and 50 ng/ml Nile red in 24-well plates for 48 hours at 20°C under 180 rpm (for
348 aeration) in liquid S-medium with *Escherichia coli* OP50 as a food source.
349 Approximately 10-12 worms were randomly chosen from two different plates for Nile
350 red fluorescence measurement, and the rest of the treated parent worms (P0) were rinsed

in M9 buffer and transferred to fresh NGM plates with OP50 without alkaloids and allowed to lay eggs. Adult worms of the next generation, F1, were then stained with Nile red to observe their lipid accumulation, and a portion of these worms was then transferred to new NGM plates to lay eggs that hatched into F2 worms. The procedure was repeated until F3 worms were evaluated.

4.4 Nile red fluorescence quantification

P0-treated worms after 48 hours and adult worms of the F1, F2, and F3 generations were sampled randomly from two plates for microscopic observation. Nile red fluorescence images were acquired at the same exposure time using the Keyence Biorevo BZ-9000 imaging system (Keyence Co., Osaka, Japan) after the same staining treatment. Fluorescence images of ten to twelve nematodes were used to quantify lipid droplets using ImageJ software (http://rsbweb.nih.gov/ij/). The most anterior four pairs of intestinal cells were selected, and the fluorescence intensity per unit area for each sample was quantified (Lemiuex, 2011). The results were verified by reproducibility in two independent experiments.

4.5 Egg laying assay

Five adult worms were randomly chosen from the F3 generation and transferred to new NGM plates with OP50 and allowed to lay eggs for 24 hours at 20°C, after which the adult worms were removed from the plates. Eggs were incubated at 20°C for another 24 hours, and the number of eggs that hatched into larvae was counted.

373

374 **4.6 Quantitative RT-PCR (qRT-PCR)**

375 Worms were rinsed three times with M9 buffer. Total RNA was extracted from the
376 worm pellet (> 1000 worms) with Sepasol-RNA I Super G (Nacalai Tesque, Kyoto,
377 Japan), purified with an RNeasy Mini Kit (Qiagen, Tokyo, Japan), and reverse-
378 transcribed into cDNA using the PrimeScript RT-PCR Kit (Takara Bio, Kusatsu, Japan)
379 with oligo(dT) primer. cDNA (at a final concentration of 500 pg/μl) was subjected to
380 qRT-PCR analysis using a CFX96 Real-Time PCR system (Bio-Rad Laboratories, Inc.,
381 Tokyo, Japan) with IQ SYBR Green Super Mix (Bio-Rad). The conditions for PCR
382 were as follows: 95°C for 15 min, followed by 40 cycles of 95°C for 10 s, 60°C for 20 s
383 and 72°C for 20 s. Melting curve analysis was performed after each run at 72°C to 95°C
384 to check the specificity of amplification. Data were analyzed using Bio-Rad CFX
385 Manager (Bio-Rad). The number of transcripts in a sample was determined by
386 comparing the number of cycles (c) required for the reaction to reach a common
387 threshold (t). The mRNA abundance values are plotted as the average of triplicate
388 cDNA templates applied to run qRT-PCR, and the results were normalized by the
389 amplification of *cdc-42* as an internal control.

390

391 The sequences of forward and reverse primers used in the quantitative RT-PCR analysis
392 were as follows:
393 *sod-5* forward, 5'-TTGGCTTACCCAGAAAGCCGAAGGT-3'; reverse, 5'-
394 GACGTACATCCATCGGTTGAGTCTC-3'

hsp-16.2 forward, 5'-CTCCAGTCTGCAGAATCTCTCCAT-3'; reverse, 5'-GTGAGACGTTGAGATTGATGGCA-3'

cyp-35C1 forward, 5'-AATTGGAGGACATCCTGTCG-3'; reverse, 5'-AAATACAGCTCGGCTCTTGC-3';

gst-5 forward, 5'-TCAAGCTCAACGGAAAAACC-3'; reverse, 5'-CCGAAGCCTTCAAGAAGTTG -3';

ugt-21 forward, 5'-AGGGAGAAATGCACAAATGC-3'; reverse, 5'-CTTGCTGCAAATTCCACGTA-3';

ugt-25 forward, 5'-AAATCCGAGCCAAATGTCAC-3'; reverse, 5'-TGCAAGCATATTCGCATTTC-3';

F08G5.6 forward, 5'-GTCCCACTGTCACAAGCTCA-3'; reverse, 5'-GTTTCGACCGAGAAATCGAG-3';

aak-1 forward, 5'-TGGTTCCGTATCGATCTTCC-3'; reverse, 5'-TCCCAAAAATCTTCCATTGC-3'

aak-2 forward, 5'-CCGGAATTCGTGGAACTAGA-3'; reverse, 5'-AACGAGCCAGTGTTCCAATC-3'

nhr-49 forward, 5'-TTAAATCCAGCCGGATCAGT-3'; reverse, 5'-CTGCTCACTGTTCAAAAATGGAA-3'

acs-2 forward, 5'-TGACGTGCTCAAGTCTCCAC-3'; reverse, 5'-CTTCACCATCTTCTCGCACA-3';

cpt-5 forward, 5'-TGCGATGGAGCTGAGTTAGA-3'; reverse, 5'-GTGACAGTCGCAATCTCCAA-3';

ech-1 forward, 5'-GAGGCTAAGGCATTTGGTGA-3'; reverse, 5'-CGATTTCATTGACCGGAAGT-3';

gei-7 forward, 5'-GGAAATCCTTTCGCTCACCGCCCAA-3'; reverse, 5'-ATATCAGCCTGAACTTGGTTGCGCT-3'

cdc-42 forward, 5'-AGCTTCATTCGAGAATGTCC-3'; reverse, 5'-CTCGAGCATTCCTGGATCAT-3'.

4.7 Statistical analysis

Data are expressed as the mean ± standard deviation (SD) unless otherwise noted and were analyzed for significance using Student's *t*-test or ANOVA for multiple-comparison tests. A probability value of $p < 0.05$ indicates statistical significance.

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Author Contributions

Y-LC designed and performed the experiments; Y-LC and FS analyzed the data; and Y-LC and FS wrote the manuscript. Both authors discussed and approved the final version of the manuscript.

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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617

618 **Figure Legends**

619

620 **Figure 1.** Nile red staining in wild-type N2 *C. elegans* in the P0, F1, F2 and F3 generations.
621 Worms were treated without alkaloids (control) or with alkaloids (B500: 500 μM berberine,
622 S10: 10 μM sanguinarine) at the L4 larval stage in the P0 generation for 48 hours; then,
623 offspring were obtained on fresh NGM medium without alkaloids. Relative Nile red
624 fluorescence intensity (Relative Intensity) was determined based on the value of the control
625 sample (P0, 0.022; F1, 0.018; F2, 0.016; and F3, 0.015) measured using ImageJ software.
626 Panels (a) to (d) refer to parent worms P0, and F1, F2, F3 untreated offspring, respectively.
627 The scale bar indicates 100 μm. Error bar = standard deviation (SD). * indicates statistical
628 significance; *$p < 0.05$, **$p < 0.005$, and ***$p < 0.001$ vs control; two-tailed Student's *t*-test.
629 The fluorescence data are averages from 10 to 12 worms, and the experiments were
630 validated by duplication.

631

632 **Figure 2.** Nile red staining in *ash-2* mutant worms in the P0, F1, F2 and F3 generations.
633 Worms were treated without alkaloids (control) or with alkaloids (B500: 500 μM berberine,
634 S10: 10 μM sanguinarine) at the L4 larval stage in the P0 generation for 48 hours; then,
635 offspring were obtained on fresh NGM medium without alkaloids. Relative Nile red
636 fluorescence intensity was determined based on the value of the control sample (P0, 0.018;
637 F1, 0.021; F2, 0.014; and F3, 0.016) measured using ImageJ software. Panels (a) to (d) refer
638 to parent worms P0, and F1, F2, F3 untreated offspring, respectively. The scale bar indicates
639 100 μm. Error bar = standard deviation (SD). * indicates statistical significance; *$p < 0.05$,

640 $^{**}p < 0.005$, and $^{***}p < 0.001$ vs control; two-tailed Student's *t*-test. The fluorescence data
641 are averages from 10 to 12 worms, and the experiments were validated by duplication.
642
643 **Figure 3.** Nile red staining in *hrde-1* mutant worms in the P0, F1, F2 and F3 generations.
644 Worms were treated without alkaloids (control) or with alkaloids (B500: 500 μM berberine,
645 S10: 10 μM sanguinarine) at the L4 larval stage in the P0 generation for 48 hours; then,
646 offspring were obtained on fresh NGM medium without alkaloids. Relative Nile red
647 fluorescence intensity was determined based on the value of the control sample (P0, 0.019;
648 F1, 0.020; F2, 0.012; and F3, 0.015) measured using ImageJ software. Panels (a) to (d) refer
649 to parent worms P0, and F1, F2, F3 untreated offspring, respectively. The scale bar indicates
650 100 μm. Error bar = standard deviation (SD). * indicates statistical significance; $^{*}p < 0.05$,
651 $^{**}p < 0.005$, and $^{***}p < 0.001$ vs control; two-tailed Student's *t*-test. The fluorescence data
652 are averages from 10 to 12 worms, and the experiments were validated by duplication.
653
654 **Figure 4.** Quantitative RT-PCR of xenobiotic response genes in wild-type N2 worms in the
655 P0 (a), F1 (b), F2 (c), and F3 (d) generations. C (black bar), control; B (white bar), 500 μM
656 berberine treatment; S (gray bar), 10 μM sanguinarine treatment. n = 3; Error bar = SD. *
657 indicates statistical significance; $^{*}p < 0.05$, $^{**}p < 0.005$, and $^{***}p < 0.001$ vs control; two-
658 tailed Student's *t*-test.
659
660 **Figure 5.** Quantitative RT-PCR of xenobiotic response genes in *ash-2* worms in the P0 (a),
661 F1 (b), F2 (c), and F3 (d) generations. C (black bar), control; B (white bar), 500 μM

berberine treatment; S (gray bar), 10 μM sanguinarine treatment. n = 3; error bar = SD. * indicates statistical significance; *$p < 0.05$, **$p < 0.005$, and ***$p < 0.001$ vs control; two-tailed Student's *t*-test.

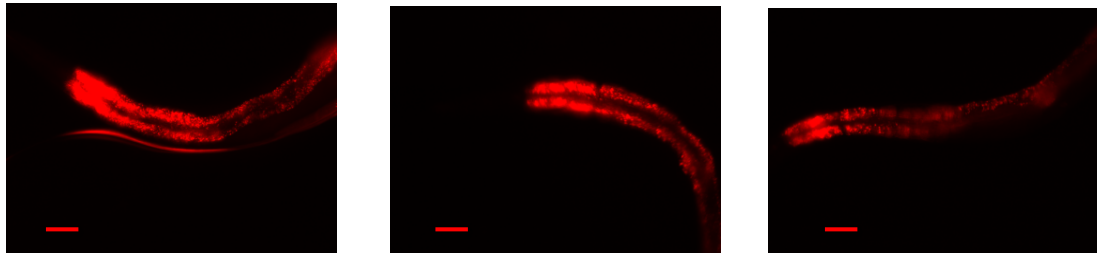
Figure 6. Quantitative RT-PCR of xenobiotic response genes in *hrde-1* worms in the P0 (a), F1 (b), F2 (c), and F3 (d) generations. C (black bar), control; B (white bar), 500 μM berberine treatment; S (gray bar), 10 μM sanguinarine treatment. n = 3; error bar = SD. * indicates statistical significance; *$p < 0.05$, **$p < 0.005$, and ***$p < 0.001$ vs control; two-tailed Student's *t*-test.

Figure 7. Box plot showing the number of offspring hatched from F3 worms after 24 hours of egg laying. N2 wild-type, *ash-2* and *hrde-1* mutants were treated without alkaloids (control) or with alkaloids (B500: 500 μM berberine, S10: 10 μM sanguinarine) in the P0 generation. The offspring of five worms were measured. Different letters indicate statistical significance at $p < 0.05$ (ANOVA followed by Dunnett's multiple comparisons test).

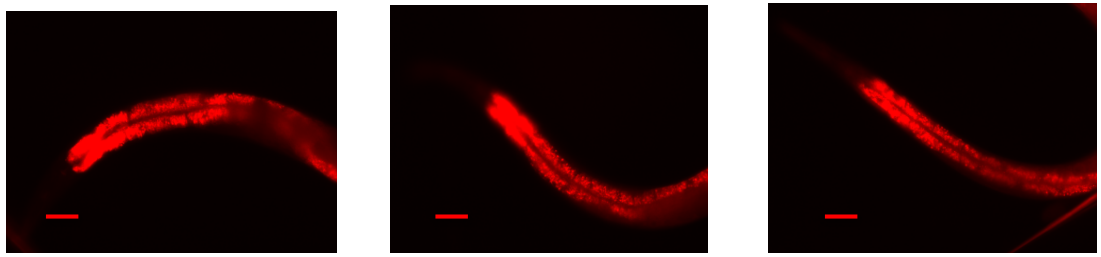
Supplementary Figure S1. Quantitative RT-PCR of AAK genes in P0 (a) and F3 (b) generation worms. Control (C; black bar), 500 μM berberine (B; white bar), 10 μM sanguinarine (S; gray bar) treatments. n = 3; Error bar = SD. * indicates statistical significance; *$p < 0.05$, **$p < 0.005$, and ***$p < 0.001$ vs control; two-tailed Student's *t*-test.

Supplementary Figure S2. Quantitative RT-PCR of lipid metabolism-related genes in P0 (a) and F3 (b) generation worms. Control (C; black bar), 500 μM berberine (B; white bar), 10 μM sanguinarine (S; gray bar) treatments. n = 3; Error bar = SD. * indicates statistical significance; *$p < 0.05$, **$p < 0.005$, and ***$p < 0.001$ vs control; two-tailed Student's *t*-test.

a) N2 P0 treated



b) N2 F1



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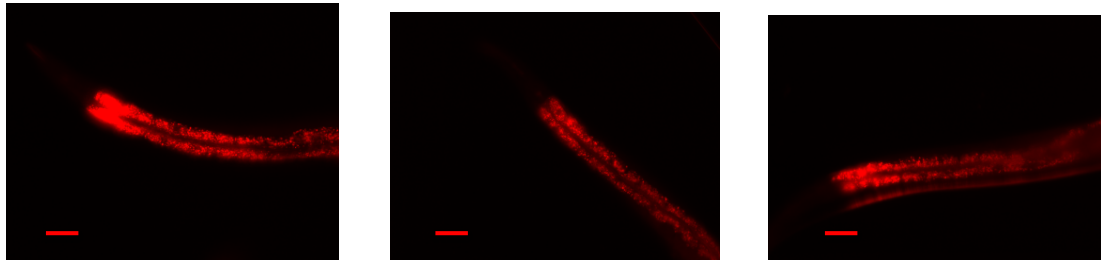
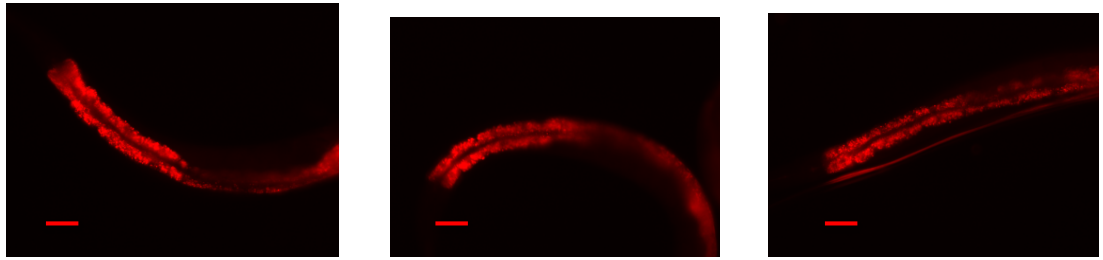
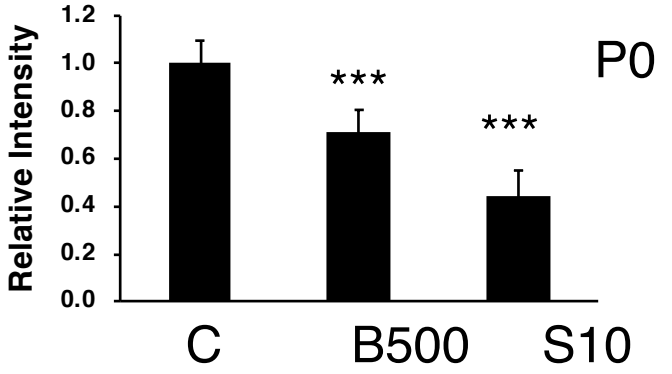
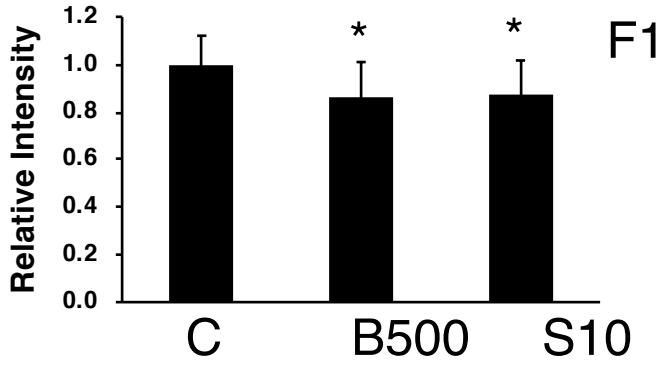


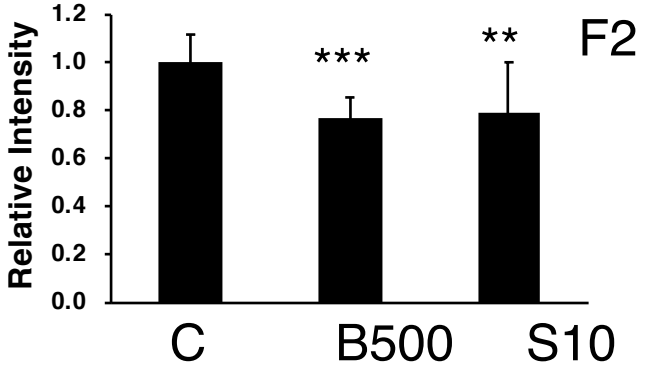
d) N2 F3

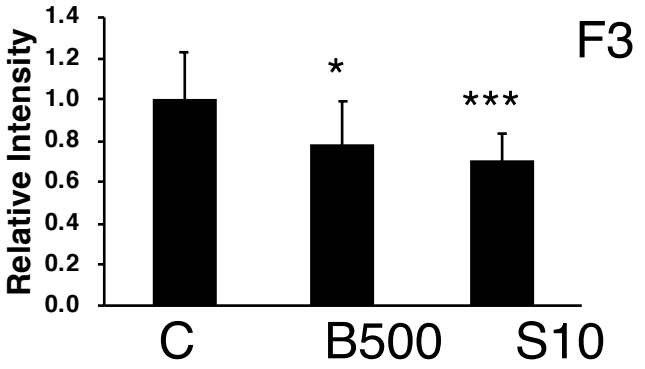


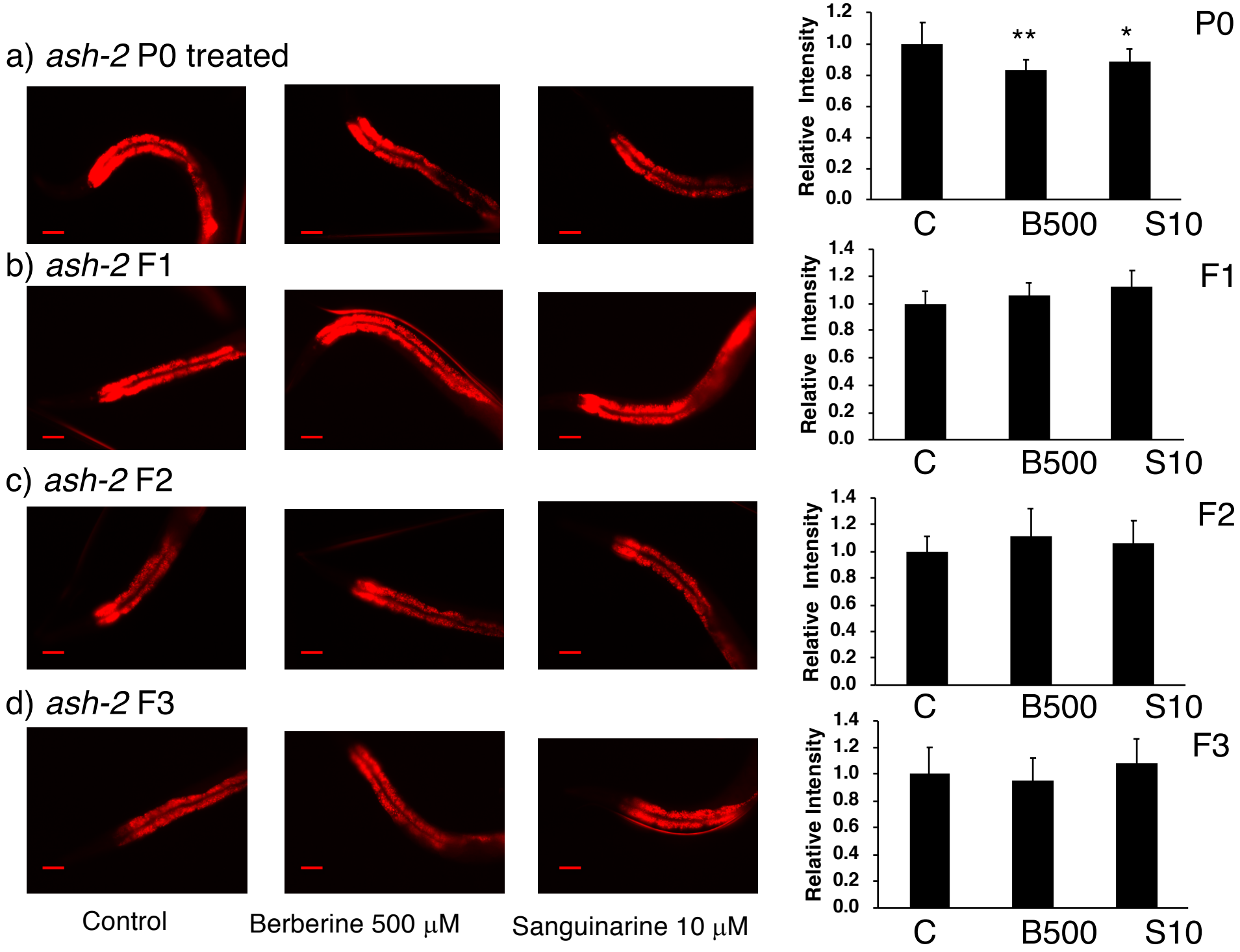
Control | Berberine 500 μM | Sanguinarine 10 μM

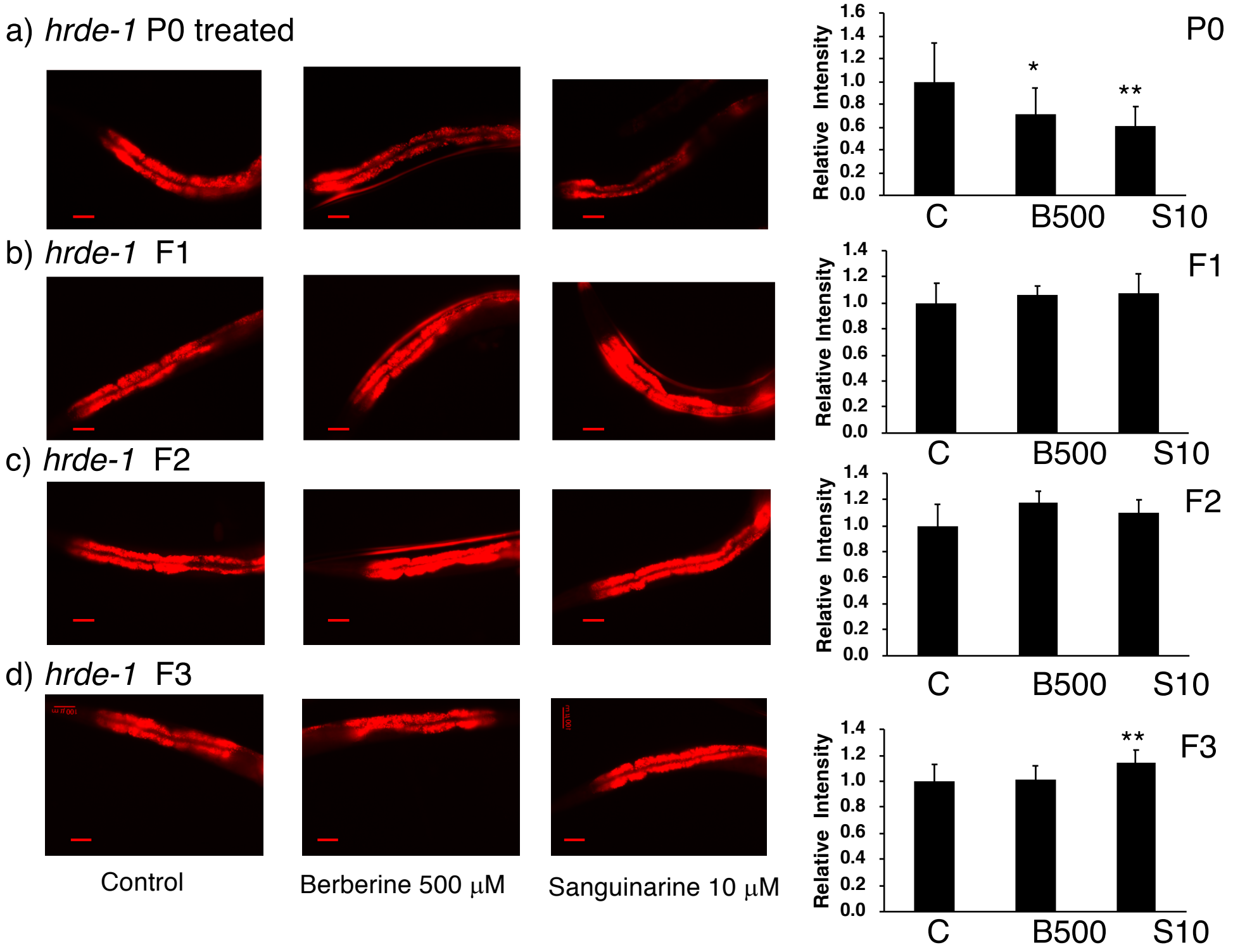


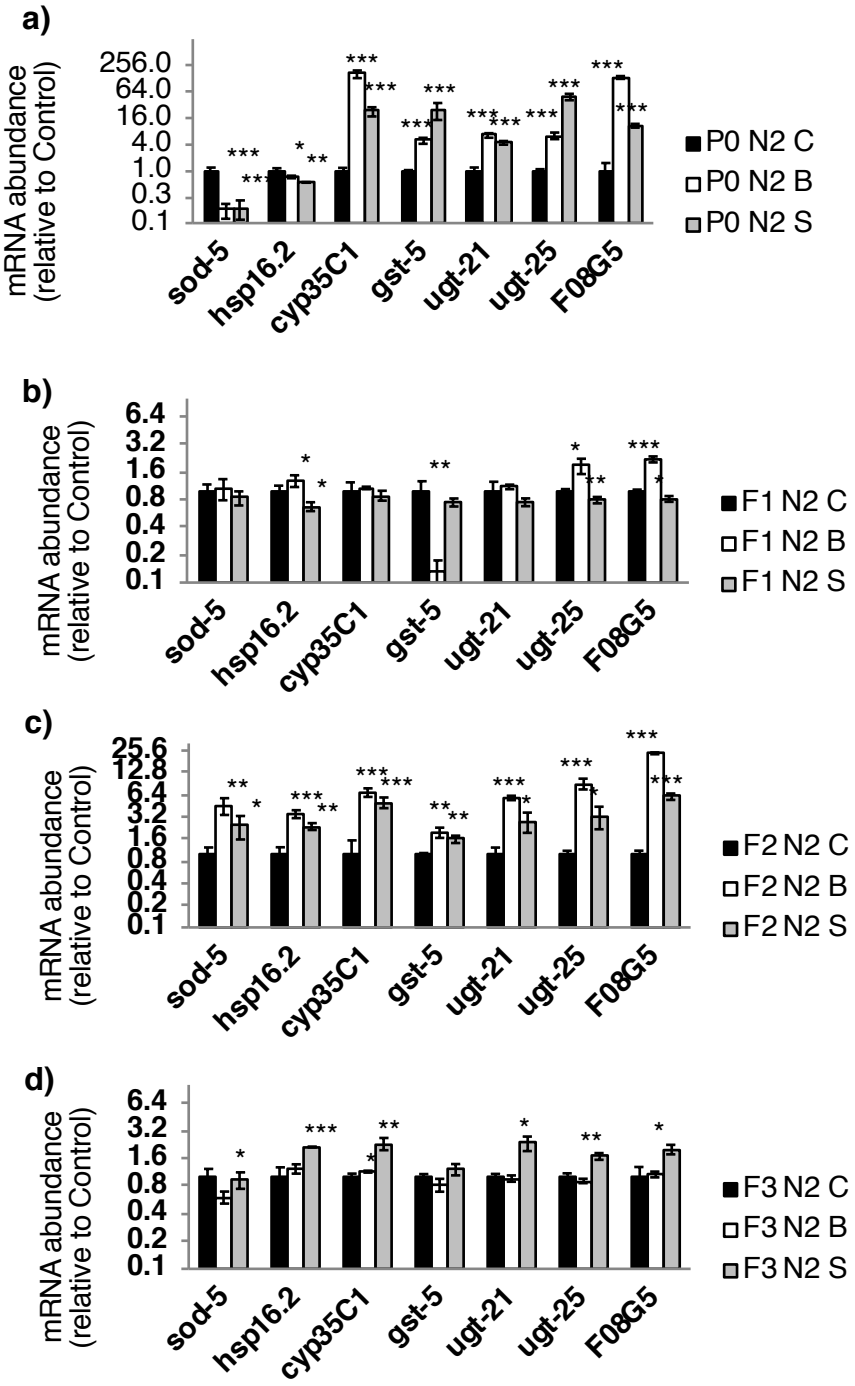


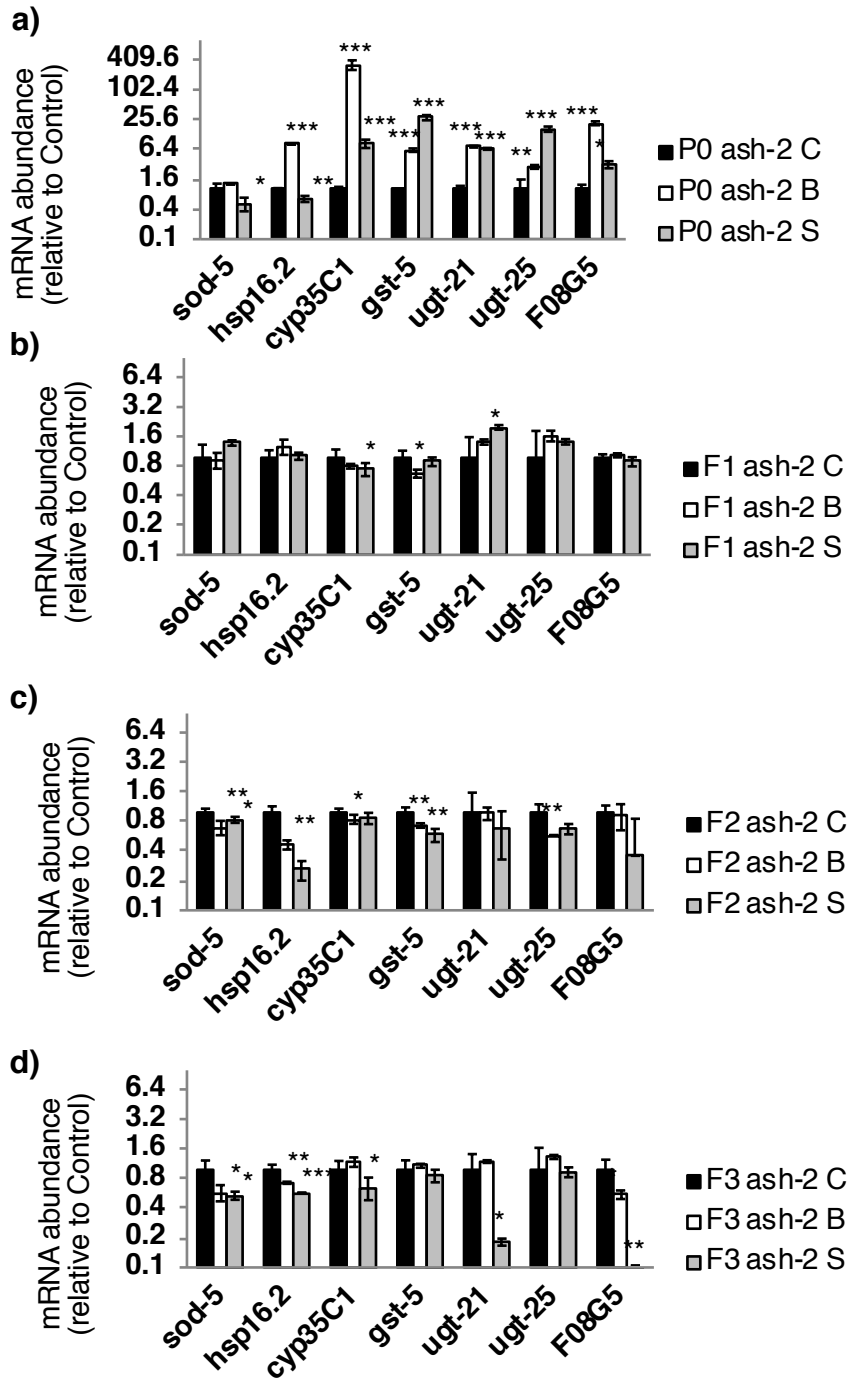


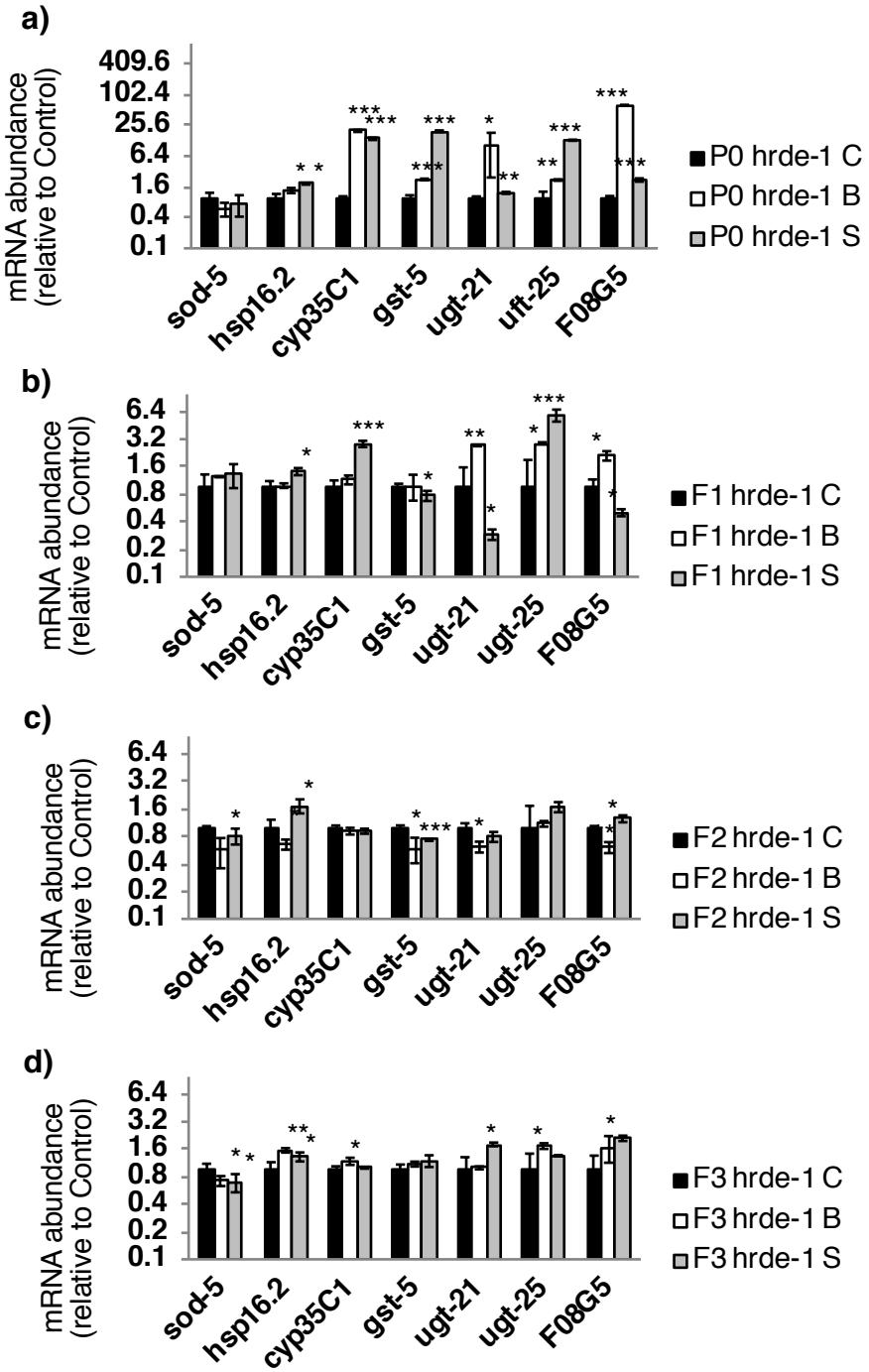


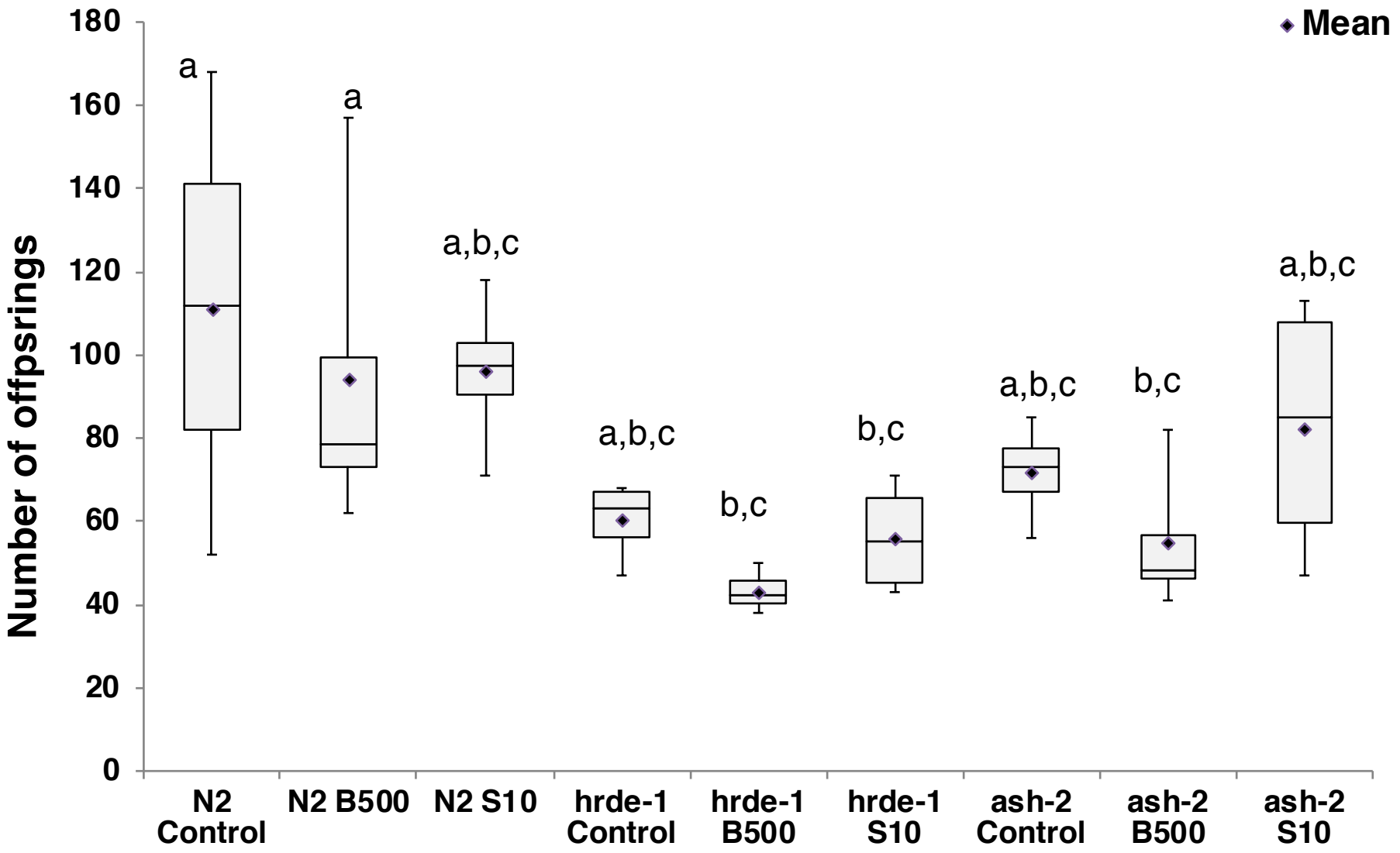

a) ash-2 P0 treated
b) ash-2 F1
c) ash-2 F2
d) ash-2 F3
Control
Berberine 500 μM
Sanguinarine 10 μM
Relative Intensity
P0
F1
F2
F3
C
B500
S10
**
*


a) hrde-1 P0 treated
b) hrde-1 F1
c) hrde-1 F2
d) hrde-1 F3
Control
Berberine 500 μM
Sanguinarine 10 μM
Relative Intensity
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
**
C
B500
S10
P0
F1
F2
F3


a)
mRNA abundance (relative to Control)
256.0
64.0
16.0
4.0
1.0
0.3
0.1
sod-5
hsp16.2
cyp35C1
gst-5
ugt-21
ugt-25
F08G5
P0 N2 C
P0 N2 B
P0 N2 S
b)
mRNA abundance (relative to Control)
6.4
3.2
1.6
0.8
0.4
0.2
0.1
sod-5
hsp16.2
cyp35C1
gst-5
ugt-21
ugt-25
F08G5
F1 N2 C
F1 N2 B
F1 N2 S
c)
mRNA abundance (relative to Control)
25.6
12.8
6.4
3.2
1.6
0.8
0.4
0.2
0.1
sod-5
hsp16.2
cyp35C1
gst-5
ugt-21
ugt-25
F08G5
F2 N2 C
F2 N2 B
F2 N2 S
d)
mRNA abundance (relative to Control)
6.4
3.2
1.6
0.8
0.4
0.2
0.1
sod-5
hsp16.2
cyp35C1
gst-5
ugt-21
ugt-25
F08G5
F3 N2 C
F3 N2 B
F3 N2 S


a)
mRNA abundance (relative to Control)
409.6
102.4
25.6
6.4
1.6
0.4
0.1
P0 ash-2 C
P0 ash-2 B
P0 ash-2 S
sod-5
hsp16.2
cyp35C1
gst-5
ugt-21
ugt-25
F08G5
b)
mRNA abundance (relative to Control)
6.4
3.2
1.6
0.8
0.4
0.2
0.1
F1 ash-2 C
F1 ash-2 B
F1 ash-2 S
sod-5
hsp16.2
cyp35C1
gst-5
ugt-21
ugt-25
F08G5
c)
mRNA abundance (relative to Control)
6.4
3.2
1.6
0.8
0.4
0.2
0.1
F2 ash-2 C
F2 ash-2 B
F2 ash-2 S
sod-5
hsp16.2
cyp35C1
gst-5
ugt-21
ugt-25
F08G5
d)
mRNA abundance (relative to Control)
6.4
3.2
1.6
0.8
0.4
0.2
0.1
F3 ash-2 C
F3 ash-2 B
F3 ash-2 S
sod-5
hsp16.2
cyp35C1
gst-5
ugt-21
ugt-25
F08G5


a)
mRNA abundance (relative to Control)
409.6
102.4
25.6
6.4
1.6
0.4
0.1
sod-5
hsp16.2
cyp35C1
gst-5
ugt-21
uft-25
F08G5
P0 hrde-1 C
P0 hrde-1 B
P0 hrde-1 S
b)
mRNA abundance (relative to Control)
6.4
3.2
1.6
0.8
0.4
0.2
0.1
sod-5
hsp16.2
cyp35C1
gst-5
ugt-21
ugt-25
F08G5
F1 hrde-1 C
F1 hrde-1 B
F1 hrde-1 S
c)
mRNA abundance (relative to Control)
6.4
3.2
1.6
0.8
0.4
0.2
0.1
sod-5
hsp16.2
cyp35C1
gst-5
ugt-21
ugt-25
F08G5
F2 hrde-1 C
F2 hrde-1 B
F2 hrde-1 S
d)
mRNA abundance (relative to Control)
6.4
3.2
1.6
0.8
0.4
0.2
0.1
sod-5
hsp16.2
cyp35C1
gst-5
ugt-21
ugt-25
F08G5
F3 hrde-1 C
F3 hrde-1 B
F3 hrde-1 S


Mean
Number of offpsrings
180
160
140
120
100
80
60
40
20
0
a
a
a,b,c
a,b,c
b,c
b,c
a,b,c
b,c
a,b,c
N2 Control
N2 B500
N2 S10
hrde-1 Control
hrde-1 B500
hrde-1 S10
ash-2 Control
ash-2 B500
ash-2 S10

Transgenerational lipid-reducing activity of benzylisoquinoline alkaloids in *Caenorhabditis elegans*

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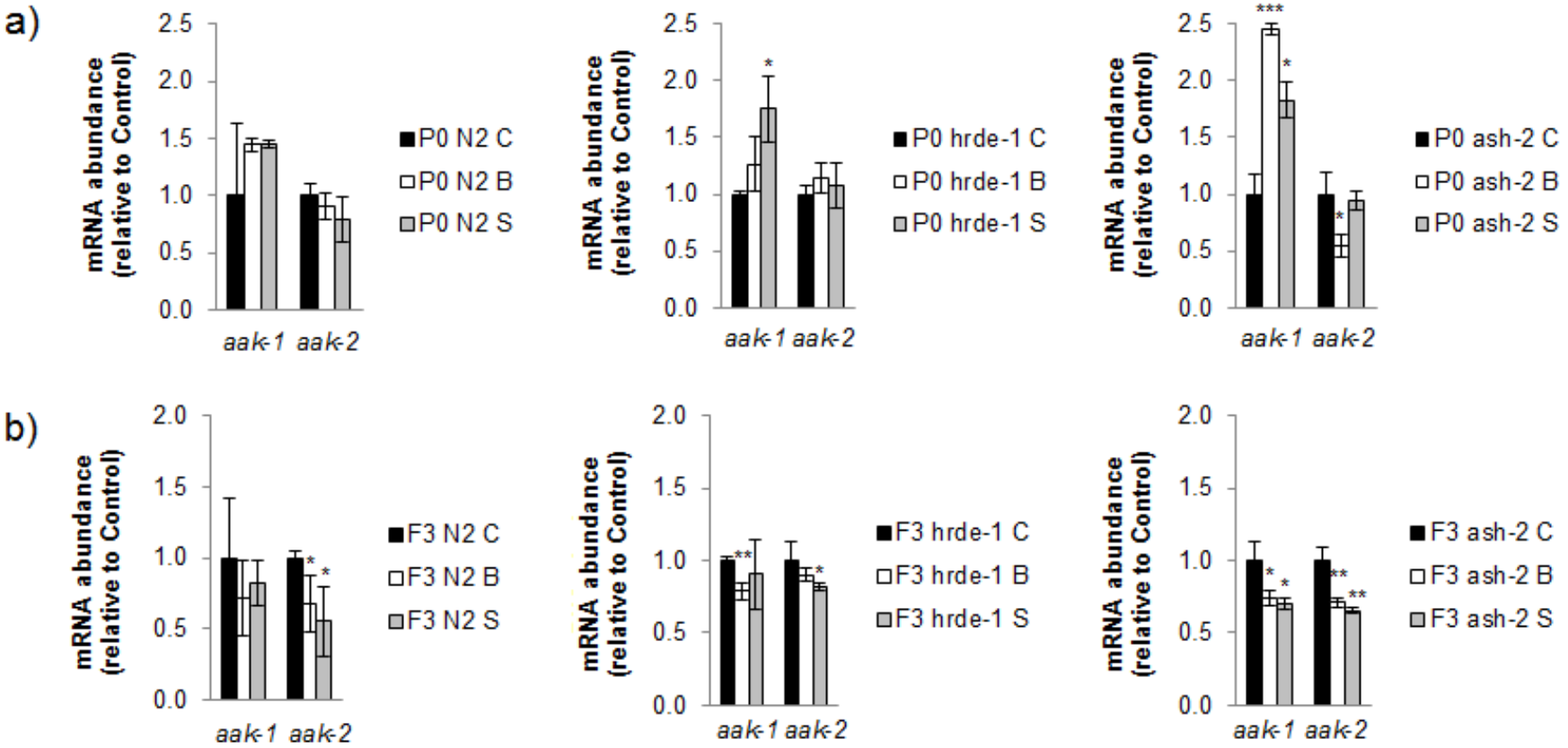
Supplementary Figure S1.

Quantitative RT-PCR of AAK genes in P0 (a) and F3 (b) generation worms.

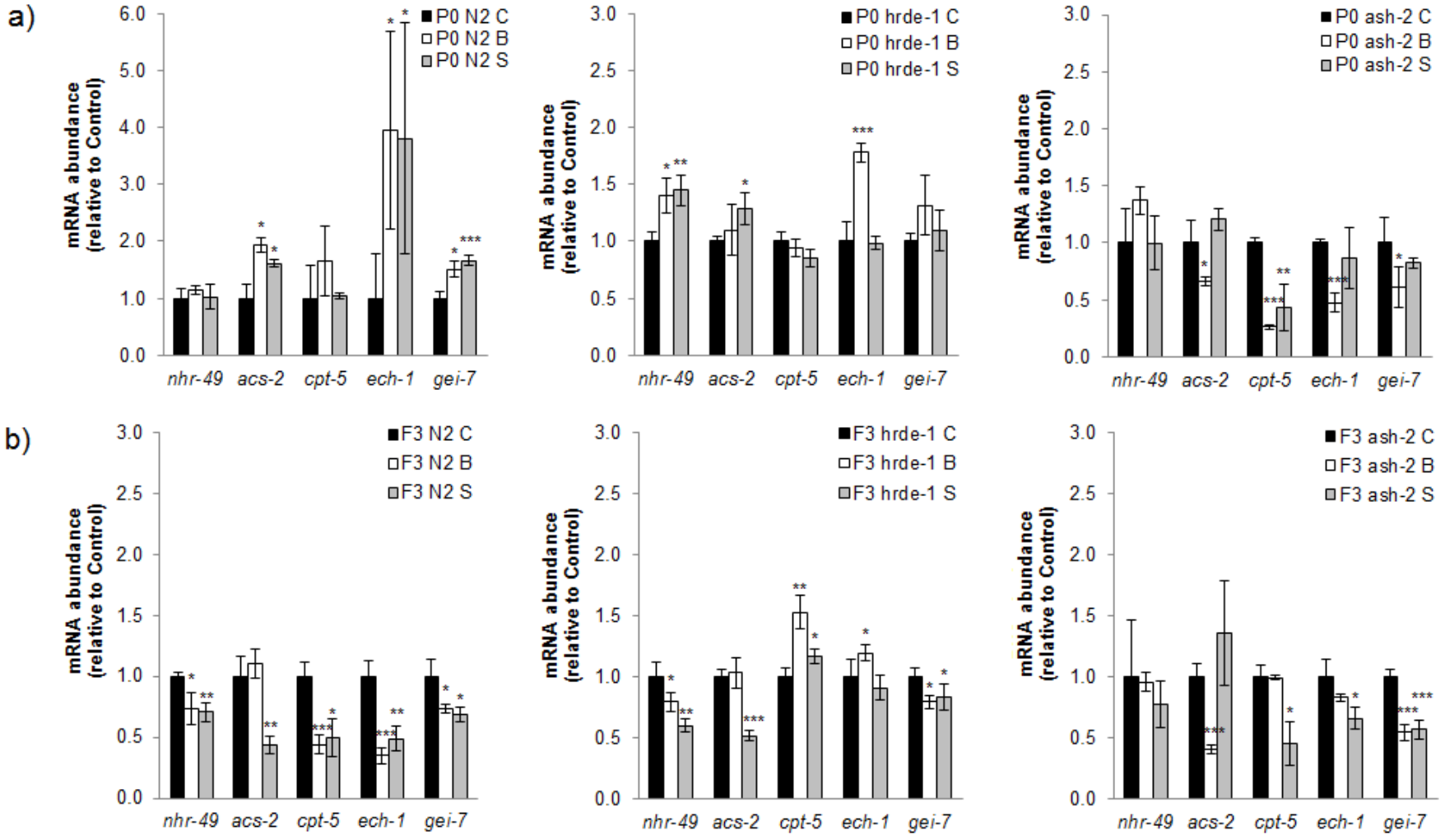
Supplementary Figure S2.

Quantitative RT-PCR of lipid metabolism-related genes in P0 (a) and F3 (b) generation worms.



Supplementary Figure S1.

Quantitative RT-PCR of AAK genes in P0 (a) and F3 (b) generation worms. Control (C; black bar), 500 mM berberine (B; white bar) , 10 mM sanguinarine (S; gray bar) treatments. n = 3; Error bar = SD. * indicates statistical significance; *$p < 0.05$, **$p < 0.005$, and ***$p < 0.001$ vs control; two-tailed Student's *t*-test.



Supplementary Figure S2.

Quantitative RT-PCR of lipid metabolism-related genes in P0 (a) and F3 (b) generation worms. Control (C; black bar), 500 mM berberine (B; white bar) , 10 mM sanguinarine (S; gray bar) treatments. n = 3; Error bar = SD. * indicates statistical significance; *$p < 0.05$, **$p < 0.005$, and ***$p < 0.001$ vs control; two-tailed Student's *t*-test.