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Tryptophan metabolism in *C. elegans* links aggregation behavior to nutritional status

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Abstract

Caenorhabditis elegans uses aggregation pheromones to communicate its nutritional status and recruit fellow members of its species to food sources. These aggregation pheromones include the IC-ascariosides, ascariosides modified with an indole-3-carbonyl (IC) group on the 4'-position of the ascarylose sugar. Nothing is known about the biosynthesis of the IC modification beyond the fact that it is derived from tryptophan. Here, we show that *C. elegans* produces endogenously several indole-containing metabolites, including indole-3-pyruvic acid (IPA), indole-3-acetic acid (IAA; auxin), and indole-3-carboxylic acid (ICA), and that these metabolites are intermediates in the biosynthetic pathway from tryptophan to the IC group. Stable isotope-labeled derivatives of IPA and IAA are incorporated into the IC-ascariosides. Importantly, we show that flux through the biosynthetic pathway is affected by the activity of the pyruvate dehydrogenase complex (PDC). Knockdown of the PDC by RNA interference leads to the accumulation of upstream metabolites and a reduction in downstream metabolites in the pathway. Our results show that production of aggregation pheromones is linked to PDC activity and that aggregation behavior may reflect a favorable metabolic state in the worm. Lastly, we show that treatment of *C. elegans* with indole-containing metabolites in the pathway induces the biosynthesis of the IC-ascariosides. Because the natural environment of *C. elegans* is rotting plant material, indole-containing metabolites in this environment could potentially stimulate pheromone biosynthesis and aggregation behavior in the worm. Thus, there may be important links between tryptophan metabolism in *C. elegans* and in plants and bacteria that enable interkingdom signaling.

INTRODUCTION

In the natural environment, the model organism *C. elegans* can be found proliferating on decomposing plant material, such as rotting stems and fruit¹ The worm primarily eats the bacteria in these microbially rich sites and undergoes rapid reproductive growth until its population density overwhelms available resources. *C. elegans* then enters a dispersal larval stage known as the dauer that enables it to seek food sources, often by catching a ride on a passing invertebrate carrier such as a slug or snail. *C. elegans* communicates using a family of pheromones known as the ascariosides that enable it to engage in many types of cooperative behavior that improve the survival of the population.²⁻⁹ Many of these ascariosides have modifications on the 4'-position of the ascarylose sugar (Figure 1A). It has

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Supporting Information Available: This material is available free of charge via the internet at <http://pubs.acs.org>.

been hypothesized that when *C. elegans* encounters favorable environments, such as food-rich ones, it secretes aggregation pheromones that are used to recruit other *C. elegans* to the food source.⁶ These aggregation pheromones include ascarosides modified on the 4'-position with an IC group (Figure 1A). Conversely, it has been hypothesized that when *C. elegans* encounters unfavorable environments, such as food-poor ones, it secretes avoidance pheromones, which include ascarosides modified on the 4'-position with the octopamine succinyl (OS) group (Figure 1A).⁸ In addition to its behavior, *C. elegans* also cooperatively regulates its development. *C. elegans* secretes certain ascarosides, collectively known as the dauer pheromone, that accumulate at high population densities and enable the worm population to enter the dauer stage just as resources are running out.^{2-5,10}

Environmental conditions, such as nutrition and temperature, and other factors, such as larval stage, sex, and larval history, have been shown to influence the composition of ascaroside pheromones that *C. elegans* produces.^{7,11,12} However, the specific mechanisms by which these factors affect ascaroside biosynthesis are poorly understood. Ascaroside pheromones are biosynthesized through peroxisomal β -oxidation, which shortens the side chains of long-chain ascarosides to generate short- and medium-chain ascaroside pheromones.^{7, 11, 13, 14} This β -oxidation pathway must be coordinated with the attachment of various modifications, such as the IC group.^{15,16} We previously showed that only ascarosides with medium-length side chains can be modified with the IC group at the 4'-position of the ascarylose sugar to yield aggregation pheromones such as IC-asc-C9 (icas#3) (Figure 1B),¹⁶ but that, in response to declining environmental conditions, these IC-ascarosides can be further processed through β -oxidation to make the dauer pheromone IC-asc-C5 (icas#9) (Figure 1A).¹⁶ The indole portion of the IC group in these IC-ascarosides is derived from L-tryptophan (L-Trp), since feeding experiments with deuterium-labeled L-Trp (L-[²H₃]-Trp) in an axenic, defined medium have shown label incorporation into the IC-ascarosides.⁶ However, it is not known how L-Trp is converted to the IC group.

Unlike in nematodes, metabolism of tryptophan to various indole-containing metabolites has been studied extensively in plants. Auxins, such as indole-3-acetic acid (IAA), are phytohormones that are found in plants as well as in some bacteria species. IAA controls several developmental processes and affects the response to pathogens and other stresses in plants.¹⁷ IAA is made from L-Trp through several pathways in plants, including the indole-3-pyruvic acid (IPA) pathway, the indole-3-acetamide (IAM) pathway, the tryptamine (TAM) pathway, and the indole-3-aldoxamine (IAOx) pathway (Figure S1).¹⁸ The IPA pathway, which is thought to be the main IAA biosynthetic pathway in the majority of plant species, involves a flavin monooxygenase, *YUCCA* (*YUC*) that oxidizes IPA to IAA (Figure S1).^{19, 20} Indole-3-carboxylic acid (ICA), a metabolite that accumulates upon pathogen exposure in *Arabidopsis*, has been shown to be biosynthesized through the IAOx pathway (Figure S1).^{21, 22} Only limited analysis of the indole-containing metabolites in nematodes has been performed.²³

Here, we establish a pathway for the biosynthesis of the IC group in the IC-ascarosides and show how nutritional status and environmental conditions might influence this pathway. By supplementing *C. elegans* cultures with unlabeled and labeled indole-containing metabolites, we identified candidate metabolites in the biosynthesis of the IC group. We determined that

C. elegans produces several of these metabolites endogenously, including IPA, IAA, and ICA. By screening by RNA interference (RNAi) for candidate genes involved in IC-ascaroside biosynthesis, we showed that production of the IC-ascarosides and the levels of key indole-containing metabolites are affected by the activity of the pyruvate dehydrogenase complex (PDC). Our work shows that nutritional conditions, as well as the levels of various indole-containing signaling molecules in the environment, could potentially influence the production of aggregation pheromones in *C. elegans* and thus affect social behavior in the worm.

RESULTS AND DISCUSSION

Indole-containing metabolites induce IC-ascarosides.

In our efforts to identify intermediates in the biosynthetic pathway of the IC-ascarosides, we made the serendipitous discovery that supplementation of worm cultures with certain indole-containing metabolites leads to increased production of the IC-ascarosides. We tested a panel of indole-containing metabolites chosen based on their involvement in L-Trp metabolism in plants and bacteria (Figure S1).¹⁸ Supplementation of worms grown in the semi-defined, axenic medium CeHR²⁴ with IPA, IAA, indole-3-glycolic acid (IGA), ICA, indole-3-acetonitrile (IAN), and indole-3-acetaldehyde (IAAld) leads to increased production of the IC-ascarosides (Figure 2, Figure S2). Conversely, IAM and TAM do not lead to increased production suggesting that the plant pathways involving these metabolites are not relevant to IC-ascaroside biosynthesis in *C. elegans* (Figure 2, Figure S1). To determine the concentration dependence, we supplemented worm cultures with increasing concentrations of IAA and analyzed ascaroside production (Figure S3). IC-ascaroside production increases dramatically at low μM concentrations of IAA (Figure S3). Similar results were obtained when we supplemented cultures with increasing concentrations of ICA. Thus, low μM concentrations of specific indole-containing metabolites in the natural environment of *C. elegans* could potentially influence IC-ascaroside production in the worm. In addition to the IC-ascarosides IC-asc-C5 and IC-asc-C9, we also detected IC-asc-C6-MK (icas#2), especially when supplementing with high concentrations of indole-containing metabolites that induced IC-ascaroside biosynthesis (Figure S2, Figure S3). IC-asc-C6-MK has been detected in several other *Caenorhabditis* species, but has not yet been described in *C. elegans*.²⁵

To provide further evidence that specific indole-containing metabolites are potential intermediates in the biosynthesis of the IC group, we supplied stable isotope-labeled indole metabolites, L-[$U\text{-}^{13}\text{C}_{11}$]-Trp, L-[$^2\text{H}_5$]-Trp, [$^2\text{H}_5$]-IAA, and [$^2\text{H}_5$]-IPA²⁶ to worms grown in CeHR (Figure 3)²⁴ We then calculated the amount of isotope enrichment in the IC-ascarosides (see Figure 3 legend). We also performed the experiment with [$^2\text{H}_5$]-IAM, which we did not expect to be incorporated into the IC-ascarosides, as a negative control. It has been demonstrated that the indole part of the IC group is derived from L-Trp by showing that the deuterium labels in L-[$^2\text{H}_5$]-Trp are incorporated into the IC-ascarosides when worms are cultured in the fully defined medium CeMM.⁶ Our data confirm this result, although the isotope enrichment in our experiment is low ($\sim 20\%$) relative to the previously published experiment (Figure 2). This low enrichment is likely due to the fact that we are

using the partially defined medium CeHR², which contains cow's milk (and thus non-labeled L-Trp) to enable healthy worm growth.

Although it has been established that the indole portion of the IC group is derived from L-Trp, it has not been determined whether the carbonyl carbon of the IC group comes from the β -carbon of L-Trp. We supplied worms with $U\text{-}^{13}\text{C}_{11}$ -Trp and determined that the labeled β -carbon is, in fact, incorporated into the IC-ascarosides (Figure 3). The deuterium labels from [$^2\text{H}_5$]-IPA and [$^2\text{H}_5$]-IAA were efficiently incorporated into the IC-ascarosides, suggesting that IPA and IAA are in the biosynthetic pathway for the IC group, or can otherwise be salvaged for incorporation into the IC group. Given that IPA degrades to IAA at room temperature,^{27, 28} it is also possible that IAA, but not IPA, is a biosynthetic precursor to the IC group. The greater incorporation of [$^2\text{H}_5$]-IAA and [$^2\text{H}_5$]-IPA into the IC-ascarosides relative to L-[$^2\text{H}_5$]-Trp and L-[$U\text{-}^{13}\text{C}_{11}$]-Trp may be due to IPA and/or IAA lying downstream of the rate-determining step in the pathway. As expected, given that IAM did not stimulate IC-ascaroside biosynthesis, [$^2\text{H}_5$]-IAM did not show incorporation into the IC-ascarosides (Figure 3).

RNAi screen for IC-ascaroside biosynthesis genes.

To implicate genes in IC-ascaroside biosynthesis, we developed a small-scale (5 mL) liquid culture method of RNAi that enables us to analyze pheromone secretion by liquid chromatography-mass spectrometry (LC-MS) after knocking down expression of candidate biosynthetic genes. We hypothesized that the biosynthesis of the IC, (*E*)-2-methyl-2-butenoyl (MB), 4-hydroxybenzoyl (HB), and OS modifications that occur on the 4'-position of some ascarosides (as shown in Figure 1A) might require one of the three mitochondrial α -ketoacid dehydrogenases: the branched-chain α -ketoacid dehydrogenase complex (BCKDC), the α -ketoglutarate dehydrogenase complex (KGDC), and PDC. These complexes are very large and include E1 α , E1 β , E2, and E3 subunits, with the E3 subunit being shared between all three complexes. The BCKDC is involved in the catabolism of branched chain amino acids and is likely required for the biosynthesis of the MB group from L-Ile (Figure S4A). The KGDC is involved in the generation of succinyl-CoA from α -ketoglutarate, and this succinyl-CoA could potentially be incorporated into the ascarosides modified with the OS group (Figure S4B). The PDC catalyzes the decarboxylation of pyruvate to produce acetyl-CoA, which then enters the TCA cycle. We were surprised to find that when we knocked down the expression of the E1 α (*pdha-1*), E1 α *pdhb-1*, or E2 (*dlat-1*) subunits of the PDC, the biosynthesis of the IC-ascarosides, but not other ascarosides, was inhibited. As expected, RNAi directed against the E3 subunit of the PDC complex was lethal. RNAi screening against the E1 α subunit of the BCKDC (Y39E4A.3) or the E1 α subunit of the KGDC (*ogdh-1*) had no effect. In order to identify additional genes involved in IC-ascaroside biosynthesis, we screened by RNAi the *C. elegans* homologs of plant enzymes involved in L-Trp metabolism, including AMI1 from the plant IAM pathway and NIT1, AAO1, and cytochrome P450 enzymes from the plant IAOx and ICA pathways (Table S1; Figure S1). We also screened *C. elegans* mutants in genes that were homologous to plant AMI1 and YUCCA (Table S2). None of these homologs appeared to play a role in the biosynthesis, although the possibility remains that the RNAi was ineffective or that some of these genes may act redundantly such that knock down of single genes has no effect.

Growth on RNAi(*pdha-1*) bacteria made the worms sterile. Thus, we determined that diluting the RNAi(*pdha-1*) bacteria by 10-fold with control bacteria gave optimal growth, while still reducing the production of the IC-ascarosides. To obtain a more complete analysis of the effect of the PDC on ascaroside biosynthesis, we cultured the worms in large-scale (150 mL) liquid cultures fed with control or diluted RNAi(*pdha-1*) bacteria and extracted the culture medium. These results again show that knocking down expression of the PDC specifically inhibits the production of the IC-ascarosides (Figure 4A) and were further confirmed using LC-MS/MS in precursor ion scanning mode.⁷

Effect of different metabolic inhibitors.

Through its effects on primary metabolism, the PDC could indirectly influence the activity of the biosynthetic enzymes that directly biosynthesize the IC moiety of the IC-ascarosides. In order to test this hypothesis, we analyzed the effect of various metabolic inhibitors, including 2-deoxyglucose (2-DG),²⁹ dichloroacetate (DCA),³⁰ oligomycin,²⁹ and rotenone,²⁹ on ascaroside production. The concentrations used were based on previous studies in *C. elegans*.^{29,30} None of these inhibitors dramatically affected production of the IC-ascarosides, suggesting that the role of the PDC in ascaroside biosynthesis is not simply an indirect effect of its control of carbon flux (Figure 4B). Although there was some reduction of the IC-ascarosides in response to 2-DG, this reduction was much less than that induced by RNAi(*pdha-1*).

Production of indole-containing metabolites in *C. elegans*.

A previous study suggested that *C. elegans* and certain plant parasitic nematodes could produce IAA and certain IAA-conjugates.²³ In order to determine whether *C. elegans* produces endogenous indole-containing metabolites, we adapted published methods using gas chromatography-mass spectrometry (GC-MS)³¹ to analyze the production of IAA and ICA in *C. elegans*. Lysates of worms that were grown on *E. coli* food were acidified, extracted, and derivatized for GC-MS analysis. IAA and ICA were detected in the worm extracts, but not in extracts of the *E. coli* food alone (Figure 5A, B). IAA and ICA could also be detected in worms grown in the axenic medium CeHR, further suggesting that bacteria are not required for the production of any of these metabolites. IAN could not be detected. As IPA is a labile metabolite that is difficult to detect analytically, we adapted a specific protocol for detecting IPA in plants using LC-MS/MS.²⁸ Using this method, we were able to detect very small amounts of IPA in worms (Figure 5C, Figure S5).

To determine whether PDC activity was affecting IC-ascaroside levels by influencing the biosynthesis of the IC group, we analyzed the levels of these indole-containing metabolites in *C. elegans* upon partial RNAi knockdown of *pdha-1*. We grew large-scale cultures of worms treated with control RNAi bacteria or diluted RNAi(*pdha-1*) bacteria and extracted them for analysis of IAA and ICA by GC-MS or of IPA by LC-MS/MS. Reduced *pdha-1* expression led to a dramatic decrease in the amount of IAA and a less dramatic decrease in the amount of ICA. These data suggest that IAA levels are coupled strongly to PDC activity in the worm. The fact that ICA levels were less affected suggests that there may be multiple pathways leading to the production of ICA, some that are affected by PDC activity and some that are not. On the other hand, reduced *pdha-1* expression led to increased levels of IPA in

the worm (Figure 5A). This result suggests that the PDC may influence a step that lies downstream of the IPA intermediate in the pathway. This result also suggests that our earlier data showing incorporation of the deuterium label from [2H_5]-IPA into the IC group is likely not due to degradation of IPA into IAA, but due to IPA being an endogenous compound in *C. elegans* that is an intermediate in the biosynthetic pathway to the IC group. Based on these results, we have proposed a biosynthetic pathway to the IC group (Figure S6).

Conclusions.

The IC modification has a dramatic effect on the activity of the ascarosides, with unmodified ascarosides, such as asc- C9, inducing avoidance and IC-modified ascarosides inducing aggregation.⁶ *C. elegans* is thought to aggregate on food to improve the survival of conspecifics⁶ and thus likely coordinates the production of aggregation pheromones with the presence of favorable nutritional conditions. Here, we show that the biosynthesis of the IC group and the IC-ascarosides is closely tied to the activity of the PDC. The PDC is a central enzyme in carbon metabolism with high PDC activity reflecting the presence of plenty of glucose.³² Thus, we show how production of the IC group of the IC-ascarosides reflects the nutritional availability in the environment and thus enables the communication of this information to other worms in the population.

Our work shows that *C. elegans* biosynthesizes IAA and many other indole-containing metabolites found in plants and that these metabolites are intermediates in the biosynthesis of the IC-ascarosides. Plants utilize several parallel pathways to biosynthesize IAA and ICA. The main IAA biosynthetic pathway, the IPA pathway, requires *YUCCA* (*YUC*) that oxidizes IPA to IAA.^{19, 20} We demonstrate that in *C. elegans* the levels of IPA, IAA, and ICA are closely tied to PDC activity, although the nature of the PDC's role is unclear. In *Arabidopsis*, a homolog of the PDC E1 α subunit, *iar4*, was isolated in a screen for mutants with reduced sensitivity to IAA-amino acid conjugates.³³ Although it was speculated that this gene might be involved in the conversion of IPA to IAA-CoA, later work showed that the *iar4* did not have defects in converting stable isotope-labeled L-Trp to free IAA and suggested that the reduced IAA levels that were seen in the mutant were likely due to an inability to hydrolyze IAA-amino acid conjugates.³⁴ Thus, it is likely that the PDC indirectly influences IC-ascaroside biosynthesis, but regardless, our data reveals an intriguing connection between the PDC and IAA metabolism in *C. elegans*.

The metabolism of L-Trp to IAA in plants is a complex area that has undergone multiple revisions in the past few decades. This complexity is in part due to the fact that different pathways can produce IAA, depending on the plant species and type of tissue.¹⁸ Our data implicate indole-containing metabolites from the IPA/IAA, IAOx/IAAld, and IAOx/IAN branches of L-Trp metabolism, suggesting that perhaps the IC group in the IC-ascarosides can be made through multiple pathways. Consistent with the hypothesis of multiple pathways to the IC group, knockdown of PDC leads to a dramatic decrease in IAA, but a less dramatic decrease in ICA. In the future, it will be of interest to investigate the relevance of IAAld and IAN as intermediates in the biosynthesis of the IC group. The pathway from tryptophan to IAA, ICA, and other indole-containing metabolites in *C. elegans* may prove to be as complex as it is in plants.

Interestingly, we demonstrated that supplementation of *C. elegans* cultures with many indole-containing metabolites, including IAA, ICA, IAAld, and IAN leads to increased production of the IC-ascarosides. *C. elegans* lives in the wild on rotting plant material where they could potentially encounter indole-containing metabolites such as IAA and ICA produced by the plants or the bacteria in their environment.³⁵ Our data show that biologically relevant concentrations of IAA in the low micromolar range that are found in plants^{36, 37} are able to induce the production of IC-ascarosides in *C. elegans*. Thus, it is possible that the IAA in rotting plant material could affect nematode chemical communication. This type of interspecies signaling would enable *C. elegans* to respond to changing conditions in its natural environment.

METHODS

Strains and materials.

C. elegans strain N2 (Bristol), *rrf-3(pk1426)*, *fmo-1(ok405)*, *fmo-2(ok2147)*, *fmo-3(ok354)*, *fmo-4(ok294)*, *fmo-5(gk225532)*, *F15E6.6(ok1816)*, *F55B11.1(ok3234)* and *gad-3(gk481139)* were provided by the Caenorhabditis Genetics Center. Stable isotope-labeled compounds L-[U - $^{13}C_{11}$]-Trp, L-[2H_5]-Trp, [2H_5]-IAA, and [2H_5]-IAM were purchased from Cambridge Isotope Laboratories. dsRNA expressing *E. coli* were from either the Ahringer library or the Vidal library (Table S1).^{38,39} Cow's milk (ultra-pasteurized, fat free) for CeHR medium was purchased from a local grocery store.

Supplementation with indole-containing compounds.

500 μ M of L-Trp, IAA, ICA, IPA, IGA, IAM, TAM or IAN or 255 μ M IAAld⁴⁰ was added to CeHR medium²⁴ to make the supplemented CeHR medium. For each condition, 0.5 mL of wild-type worm pre-culture was transferred to 4.5 mL of supplemented CeHR medium with 0.5 mL skim milk. After growing at 22.5°C for 4 d, 3 mL medium was collected and directly loaded onto a 200 mg C18 Sep-Pak column (Waters) (prepared according to the manufacturer's protocol). The column was washed once with 3 mL of water and eluted with 3 mL of methanol. The elution was dried in a SpeedVac (Thermo) for about 2.5 h without heating, and then dissolved in 100 μ L of 50% (v/v) aqueous methanol for LC-MS analysis.

Supplementation with isotope-labeled compounds.

The [2H_5]-IPA synthesis was adapted from a previously published method, with NMR data consistent with a previous report.²⁶ Reaction started with 102.0 mg L-[2H_5]-Trp generated 52.0 mg of final product, giving a 51.2% yield. L-[U - $^{13}C_{11}$]-Trp, L-[2H_5]-Trp, [2H_5]-IAA-, [2H_5]-IAM, or [2H_5]-IPA at a final concentration of 376.5 μ M was added to CeHR medium without L-Trp. The concentration of stable isotope-labeled indole-containing metabolites added was chosen based on the fact that it is 2 times the amount of L-Trp in milk.⁴¹ To make CeHR medium without L-Trp, the MEM amino acid solution was made without L-Trp (Table S3). Culture and sample preparation were the same as for the supplementation of indole-containing compounds in CeHR medium experiments.

Small-scale RNAi screen.

dsRNA expressing *E. coli*^{38,39} from a -80°C stock was streaked onto LB-agar plates containing 150 µg mL⁻¹ ampicillin, and allowed to grow at 37°C for 16 h. The RNAi bacteria were then inoculated into 5 mL of LB medium (containing 150 µg mL⁻¹ ampicillin), shaken at 37°C for 6 h, and then induced with 4 mM IPTG for 1 h. 100 µL of induced RNAi bacteria were placed on each of the 6 cm NGM agar plates (containing 1 mM IPTG and 25 µg mL⁻¹ carbenicillin), and allowed to dry and grow overnight. On the next day, 20 L4 *rrf-3(pk1426)* worms were placed on each of the 6 cm plates to lay eggs and were removed from the plate after 48 h. After 4 d from the initial seeding of L4 worms, all worms on the plates were washed off using 5 mL of S medium and transferred to a culture tube that contained a pellet of RNAi bacteria. This pellet was obtained from a 5 mL fresh RNAi bacteria culture that had been grown as described above for plate seeding, but was then centrifuged at 3,500 rpm so that the LB medium could be removed. Worms were fed one more time after 4 d of growing in liquid culture, and 3 d later, the medium was collected and processed with a Sep-Pak column as described above. All bacterial cultures were grown at 37°C, and all worm cultures at 20°C. Ascaroside analysis by LC-MS was done as previously described.¹⁴

Large-scale RNAi cultures.

The large-scale RNAi cultures were fed in the following manner: For the experimental group, 1:10 *pdha-1* RNAi bacteria (induced with IPTG) to control bacteria (with empty L4440 plasmid, not induced with IPTG) was used. For the control group, 1:10 control bacteria (with empty L4440 plasmid, induced with IPTG) to control bacteria (with empty L4440 plasmid, not induced with IPTG) was used. The ratio of the different bacteria (*pdha-1* and L4440) was determined by OD₆₀₀. An amount equal to 40 mL of OD₆₀₀ 1.0 RNAi bacteria was pelleted, re-suspended in 1 mL of S medium, seeded onto a 10 cm NGM plate (containing 1 mM IPTG and 25 µg mL⁻¹ carbenicillin), spread, and allowed to dry overnight. 20 L4 worms were placed on each plate the next day. Adults were not removed for large-scale cultures, because the number of initial worms is much less than the ultimate number of RNAi-affected worms after several generations. Worms from four 10 cm plates were transferred to 200 mL of S medium when they were running out of food (designated culture day 1). An amount equal to 200 mL of OD₆₀₀ 1.0 1:10 diluted RNAi/control bacteria was prepared freshly, pelleted, re-suspended in 3 mL of S medium, and fed to each of the 200 mL cultures on culture day 1, and every day from day 3 to day 6. Cultures were harvested on day 7 for analysis.

Addition of metabolic inhibitors.

Wild-type worms from one 6 cm plate were washed off into 5 mL of S medium containing 10 µM of 2-DG, 50 µg mL⁻¹ DC A, 50 µM oligomycin, or 5 µM rotenone (final concentrations),^{29, 30} and the liquid cultures were fed with 1 mL of concentrated (25X) OP50. The culture medium was collected after 5 d, and processed with a Sep-Pak column as described above.

GC-MS analysis of IAA and ICA.

Worms collected from 200 mL of RNAi cultures were freeze-dried, weighed for dry mass, and then ground with 0.1 g of sand. The worm powder, together with 1 μg [$^2\text{H}_5$]-IAA as the internal standard, was extracted with 10 mL of methanol 3 times, and the extracts were combined and dried with a rotary evaporator. The extract was resuspended with 5 mL of water, and the pH was lowered to 2.5 with 4 M HCl. The sample was then extracted three times with an equal volume of ethyl acetate. The ethyl acetate extractions were combined and dried with a rotary evaporator. Residues were suspended with 100 μL of 50% (v/v) aqueous methanol and purified by HPLC (Discovery HS C18 column 25 cm $\times$ 10 mm, 10 μm using a solvent gradient: 5% B holding for 2 min, followed by gradually increasing to 50% B over 33 min; solvent A: water with 0.1% (v/v) formic acid; B: acetonitrile with 0.1% (v/v) formic acid; flow rate 4 mL min⁻¹). The relevant HPLC fractions were dried with a rotary evaporator. The residues were suspended with methanol, transferred to a 2 mL-vial, and dried. Samples were derivatized with methoxylamine and silylated with *N*-methyl-*N*-*tert*-butyldimethylsilyl trifluoroacetamide / 1% *tert*-butyldimethylchlorosilane (Thermo) according to a previously published method³¹ Samples were analyzed on a Thermo Scientific DSQ GC-MS with 0.5 μL splitless (0.5 min) injection at 300°C, temperature gradient elution (1 min hold at 100°C, followed by 5°C min⁻¹ increasing to 310°C) on a 30 m Rxi-5ms column (0.25 mm ID, 0.25 μm df) with 1 mL min⁻¹ constant flow of helium carrier gas, electron ionization at 70 eV, and qualifier ions for the analytic target being 289.2>232.1/188.1/130.1 for IAA and 389.2>332.2/288.2/258.2 for ICA.

LC-MS and LC-MS/MS analysis of IPA.

Worms collected from six 200 mL cultures were distributed evenly between two falcon tubes. The worms in one tube were freeze-dried and weighed for dry mass. The worms in the other tube were frozen as droplets in liquid nitrogen immediately after collection, and ground to a fine powder with a mortar and pestle that had been pre-chilled with liquid nitrogen. The frozen worm powder was then suspended on ice with 10 mL of ice cold worm extraction buffer (35% (v/v) 200 mM imidazole, pH 7.0, 65% (v/v) acetonitrile, 45 $\mu\text{g mL}^{-1}$ butylated hydroxytoluene and 0.2 mg mL⁻¹ diethyldithiocarbamic acid) with 1 μg of [$^2\text{H}_5$]-IPA as an internal standard. After adding 4 mL of hydroxylamine hydrochloride (50 mg mL⁻¹, in anhydrous pyridine), the reaction was stirred on ice for 60 min. The product was purified as described in a previously published method for IPA analysis.²⁸ Quantification was obtained by LC-MS (Agilent 6130 single quad mass spectrometer with a Phenomenex Luna 5 μm C18 (2) 100 Å (100 $\times$ 4.6 mm) column) using a solvent gradient: 15% B to 50% B over 60 min (solvent A: water with 0.1% (v/v) formic acid; solvent B: acetonitrile with 0.1% (v/v) formic acid; flow rate: 0.7 mL min⁻¹; fragmentor voltage of 85V). The IPA-oxime was identified based on $[\text{M}-\text{H}]^-$, $[\text{M}+\text{H}]^+$, and the retention time relative to the internal standard [$^2\text{H}_5$]-IPA-oxime, and it was quantified based on the peak area of $[\text{M}-\text{H}]^-$. Products and retention times were further verified by monitoring the precursor and the corresponding product ions with a Thermo Scientific LCQ DECA Ion Trap mass spectrometer (Figure S5).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Structures and biological roles of ascarosides modified with various head groups. a) Structures and biological roles of ascarosides modified on the 4'-position of the ascarylose sugar with the indole-3-carbonyl (IC), 4-hydroxybenzoyl (HB), (*E*)-2-methyl-2-butenoyl (MB), and octopamine succinyl (OS) head groups. b) The IC group, which is biosynthesized from tryptophan, is attached to the 4'-position of asc- C9. Ascarosides that are not modified with the IC group, such as asc- C9, tend to induce avoidance while ascarosides that are modified with the IC group, such as IC-asc- C9, tend to induce aggregation.

Figure 2.

Ratio of ascarosides in worm cultures using supplemented CeHR medium versus standard CeHR medium. Supplements included L-Trp, indole-3-pyruvic acid (IPA), indole-3-acetic acid (IAA), indole-3-glyoxylic acid (IGA), indole-3-carboxylic acid (ICA), indole-3-acetamide (IAM), tryptamine (TAM), indole-3-acetonitrile (IAN), and indole-3-acetaldehyde (IAAlD). Data represent the mean $\pm$ SD of three independent experiments.

Figure 3.

Isotope enrichment in IC-asc-C5 (and other IC-ascarosides), after feeding worm cultures in CeHR medium with deuterium-labeled and ¹³C-labeled indole-containing metabolites. Isotope enrichment = (¹³C₉-IC-asc-C5 or [²H₅]-IC-asc-C5) / total IC-asc-C5 * 100%. Data represent the mean ± SD of three independent experiments.

Figure 4.

Effect of PDC RNAi and metabolic inhibitors on ascaroside production. a) Ratio of ascarosides produced by worms fed RNAi (*pdha-1*) bacteria (1:10 RNAi (*pdha-1*) bacteria diluted with control bacteria) versus control bacteria. b) Ratio of ascarosides produced by worms treated with the metabolic inhibitors dichloroacetate (DCA), 2-deoxyglucose (2-DG), oligomycin, and rotenone versus control worms. Data in (a) and (b) represent the mean $\pm$ SD of three independent experiments.

Figure 5.

Effect of partial RNAi knockdown of *pdha-1* on indole-containing metabolites. a) Partial RNAi (*pdha-1*) strongly reduces IAA levels, moderately reduces ICA levels, and strongly increases IPA levels in the worm. [$^2\text{H}_5$]-IAA for GC-MS and [$^2\text{H}_5$]-IPA for LC-MS was spiked into samples to control for differences in extraction efficiencies across samples. Data represent the mean $\pm$ SD of five independent experiments. Examples are shown of GC-MS traces used in the analysis of IAA (b) and ICA (c) and LC-MS traces used in the analysis of IPA (d). These traces are representative of those traces that were analyzed to generate the graph in (a).