Linalool odor stimulation improves heat stress tolerance and decreases fat accumulation in nematodes

Naoko Hirano and Kazuichi Sakamoto*

Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8572, Japan

*Corresponding author. Faculty of Life and Environmental Sciences, University of Tsukuba, Tennodai 1-1-1, Tsukuba Ibaraki 305-8572, Japan. Tel.: +81 2985346761; fax: +81 298534676.

E-mail address: sakamoto@biol.tsukuba.ac.jp

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Abstract

Aromatherapy uses plant essential oils and fragrant ingredients for relaxation, sleep assistance, and improvement of restlessness related to dementia. Certain aromatic substances increase the life span and stress tolerance of nematodes. We investigated effects of exposure to linalool, a linear chain monoterpenic alcohol that is present in the essential oils of many plants, and its optical isomer, L-linalool, in *Caenorhabditis elegans*. Nematodes were repelled by the odor of both linalool and L-linalool; however, linalool odor stimulation decreased fat accumulation and increased motility after thermal stress. Analysis of a gene-deficient mutant revealed that the DAF-16 insulin-signaling pathway, which is involved in heat stress tolerance, was enhanced by linalool treatment. Linalool stimulation increased the expression of downstream genes such as *sod-3* and *hsp-12.6* via DAF-16. We conclude that linalool odor induces a repelling behavior in nematodes, improves heat stress tolerance through the DAF-16 signaling pathway, and affects fat accumulation.

Keywords: Odor; Linalool; Thermo tolerance; Fat accumulation; DAF-16

Introduction

Aromatherapy is a traditional medicinal practice that uses fragrant plant essential oils. This therapy is applied to support relaxation and sleep [1], as well as to improve dementia-related restlessness [2,3]. However, scientific research on aromatherapy and essential oils is relatively new, and scientific evidence of the physiological actions and functional mechanisms is limited.

The nematode *Caenorhabditis elegans* was used as an experimental model organism in this study. The hermaphrodite nematode consists of 959 somatic cells, 302 of which are nerve cells. Individual nerve cells have names as well as known cell body position and lineage [4]. There are twelve sensory nerve cilia in the head of the nematode, and two types of sensory cilia in the tail phasmide sensea. Nematodes respond to various chemical substances in the environment using sensory cilia and can sense more than 100 types of chemical substances to which they develop chemotactic behaviors [5]. Three types of olfactory neurons, AWA, AWB, AWC, and the two sensory neurons, ASH and ADL, can sense volatile substances [6, 7]. AWA and AWC neurons sense attractive substances; AWB, ASH, and ADL neurons sense repellent substances. Previous studies have identified the molecular mechanisms of

55 olfactory receptors in each nerve [8].

56 The research on lifespan and stress tolerance in nematodes is extensive. The
57 insulin/IGF-1 signal (IIS) pathway is a representative pathway that controls both
58 features [9]. This transmission pathway is necessary for various biological functions,
59 such as metabolism, development, and lifespan, and is homologous with the IIS
60 signaling pathway in mammals. In the IIS pathway, when an insulin-like peptide
61 ligand binds to the insulin-like peptide receptor DAF-2 on the cell surface, it activates
62 the PDK-1 and AKT-1/AKT-2/SGK-1 kinase cascades. As a result, phosphorylation
63 of the transcription factor DAF-16 inhibits its nuclear translocation. Conversely,
64 when the IIS pathway signal is lost, DAF-16 can translocate into the nucleus, and
65 transcriptional activity increases. Activated DAF-16 controls the expression of
66 various genes involved in lifespan, heat stress tolerance, oxidative stress tolerance,
67 metabolism, and immunity. In addition, the transcription factor HSF-1, which
68 controls the expression of various genes involved in lifespan extension and heat stress
69 tolerance, is negatively regulated by the IIS pathway [10, 11].

70

71 Previous reports on the effects of isoamyl alcohol and acetic acid on lifespan and
72 stress tolerance of nematodes are examples of the physiological action by odor

73 stimulation [12]. Isoamyl alcohol is perceived by nematode AWC olfactory neuron
74 [5] and can result in increased life span, but acetic acid did not have this effect. In
75 addition, isoamyl alcohol odor stimulation and acetic acid improved thermal stress
76 tolerance in nematodes [12].

77 Linalool is one of the linear-chain monoterpene alcohols that are found in many
78 plant-based essential oils. Linalool is composed of the optical isomers D-linalool and
79 L-linalool. There are also several industrial synthesis methods for producing linalool
80 isomers, which have the fragrance of lily, lavender, or bergamot; therefore, they are
81 popular as perfume additives. Lavender essential oil and linalool odor stimulation
82 can affect autonomic neurotransmission and decrease blood pressure in rats [13].
83 Linalool also has an anti-inflammatory action [14]. Lavender essential oil and
84 linalool steam improved autonomic and hormonal imbalance in menopausal female
85 rat models [15]. These studies indicated that linalool can influence a variety of
86 physiological functions; however, studies on how it may affect lifespan and thermal
87 stress resistance have not been conducted.

88 In this study, we aimed to characterize the physiological effects of linalool and L-
89 linalool on nematode stress tolerance and to define their mechanism of action.

90

Materials and methods

Nematodes and growth

C. elegans nematodes were raised on nematode growth medium (NGM) plates (OP plates) coated with the *E. coli* strain OP50. The breeding temperature in a thermostatic chamber was 20°C. Every five days, three worms were transferred to new OP plates to maintain the line. *C. elegans* wild-type N2 and the *daf-16*(*mgDf50*), *daf-2*(*e1370*), and *odr-3*(*n1605*) mutants were provided by the Caenorhabditis Genetics Center, University of Minnesota, Minneapolis, USA.

Synchronization

Synchronization was performed to align nematode growth stages. Adult worms raised at 20°C were collected in 5 mL of S-basal medium (0.1 M NaCl, 50 mM potassium phosphate buffer pH 6.0). Then, the adult worms were treated with 100% NaClO (Haiter, Kao, Tokyo, Japan) and eggs were collected in a 15-mL tube in S-basal medium. After 18 h, hatched L1 larvae were seeded onto OP plates and used for experiments.

109

110 ***Odor substances and administration***

111 Linalool (referred to as DL-linalool in the text) (Wako Pure Chemical Industries,
112 Osaka, Japan) and L-linalool (Sigma-Aldrich, St. Louis, USA) were diluted in DMSO
113 (Nacalai Tesque, Kyoto, Japan), and 0.1%, 1%, and 10% DL- and L-linalool solutions
114 were prepared. DMSO alone was used for the control treatments in all experiments.
115 Each solution was added to the back of the OP plate lid on which the nematodes were
116 cultured; five 4-μL spots were sufficient to give an odor stimulus (Fig. 1A).

117

118 ***Chemotaxis experiment***

119 Synchronized nematodes were bred for 96 h at 20°C. A 3-cm circle was drawn from
120 the center of a 6-cm NGM plate. Five nematodes were transferred to four points on
121 the outer periphery of the circle for a total of 20 animals per plate. A 2-μL volume of
122 each odor substance was added to the center of the NGM plate, and after 60 min,
123 nematodes outside the circle were considered 'repelled' by the odor. The repelling
124 index was calculated as follows: avoidance index = ((number of worms outside the
125 circle) – (number of worms inside the circle))/total number of worms.

126

127

128 ***Fat accumulation quantification***

129 Synchronized nematodes were bred for 72 h and then given odor stimulation for 24
130 h. Nile red (Wako Pure Chemical Industries, Ltd.) was dissolved in acetone (Kanto
131 Kagaku) to prepare a stock solution of 500 μg/mL, which was then diluted with S-
132 basal to prepare a 1 μg/mL Nile Red solution. The nematodes were treated with 1
133 μg/mL Nile Red and incubated at room temperature for 30 min with agitation. Then,
134 the worms were fixed in 8% ethanol (Wako Pure Chemical Industries) for 5 min and
135 observed with a fluorescence microscope. The fluorescence intensity was analyzed
136 using ImageJ software.

137

138 ***Pharyngeal pumping motion measurements***

139 The pharyngeal pumping motion of the nematodes cultured on OP plates was
140 measured every 15 s in nematodes stimulated with odor substances after 24 h of
141 exposure.

142

143 ***Nematode survival rate under thermal stress***

144 Nematodes were stimulated for 24 h with odor compounds and then transferred to

145 NGM plates containing 5 mg/mL ampicillin (Wako Pure Chemical Industries, Ltd.)
146 and *E. coli* OP50, and cultured at 35°C. Survival rates were measured every 2 h
147 starting 10 h after the heat treatment.
148
149 ***Nematode motility after thermal stress***
150 The nematodes were stimulated for 24 h with odor compounds and then collected in
151 S-basal medium and washed. Then, the worms were transferred to sterile NGM plates
152 and incubated at 35°C or 20°C for 4 h. The worms were then transferred to OP plates
153 and the whole-body movement (i.e., thrashing) of the worms in S-basal medium was
154 measured every 15 s after the heat stress treatment. The rate of restoration of mobility
155 was calculated as follows: motion restoration rate = (number of whole-body
156 movements of nematodes at 35°C)/(number of whole-body movements of nematodes
157 at 20°C) × 100.
158
159 ***Gene expression analysis***
160 RNA was extracted from *C. elegans* treated for 24 h with odor compounds using
161 RNAiso Plus (Takara, Shiga, Japan). Then, cDNA was synthesized by first removing
162 genomic DNA with the PrimeScript™ RT Reagent Kit with gDNA Eraser (Perfect

163 Real Time) (Takara). Quantitative reverse transcription PCR (qRT-PCR) was
164 performed using the THUNDERBIRD SYBR qPCR Mix (Toyobo, Osaka, Japan);
165 the *actin* gene was used as an internal standard. Primers used in this study are shown
166 in Table 1 [Table 1 near here].
167
168 ***Statistical analysis***
169 Statistically significant differences were evaluated using SPSS software (IBM,
170 Armonk, NY). Survival curves were analyzed using a log-rank test, and significant
171 differences between three or more groups were judged by Tukey's honest significant
172 difference test. Values of $p < 0.05$ and $p < 0.01$ are indicated with * and **,
173 respectively.
174
175 **Results**
176 ***Nematodes are repelled by DL-Linalool and L-Linalool***
177 Nematodes respond to external stimuli such as odor, temperature, mechanical
178 stimulation, and light by moving. To evaluate how nematodes behave in response to
179 each odorant, their chemotaxis was observed after exposure to linalool isoforms.
180 Nematodes were repelled by DL-linalool in a concentration-dependent manner; the

10% concentration resulted in the highest repellency index (Fig. 1B (a)). Conversely, L-linalool had the highest repellency index at a 1% concentration, but the nematodes presented evasive behavior at all concentrations (Fig. 1B (b)). These results suggest that nematodes are repelled by linalool odors, but are particularly responsive to L-linalool.

DL-Linalool odor stimulation reduces fat accumulation in nematodes

To investigate the effect of linalool exposure on fat accumulation, nematodes were treated with each isoform for 24 h before body fat was stained with Nile Red reagent. Body fat was observed using a fluorescence microscope and analyzed with ImageJ software. We observed that fat accumulation decreased in nematodes treated with DL-linalool, whereas no change was observed in nematodes treated with L-linalool (Fig. 2A). In addition, we evaluated changes in feeding movements due to the decrease in fat accumulation by measuring the pumping motion of nematodes treated with odor stimulation for 24 h. Neither DL-linalool nor L-linalool affected the pumping rate in treated compared to control nematodes (Fig. 2B). In previous studies, odor stimulation with isoamyl alcohol lengthened the lifespan of nematodes [12]; therefore, the effect of linalool on the lifespan of nematodes was analyzed. However, we did not observe any changes in lifespan after linalool treatment (data not shown).

199

200 ***DL-Linalool or L-Linalool odor stimulation improves nematode motility after heat***

201 ***stress***

202 Next, the effect of linalool odor stimulation on motility after thermal stress was

203 examined. When nematodes were cultured at 35°C for 4 h, whole-body movement

204 decreased significantly, but could be recovered after 3–24 h. However, nematodes

205 stimulated with DL-linalool and L-linalool had higher motility and better restoration

206 after 12 h than the control worms (Fig. 3 (a)).

207 Previous studies have shown that DAF-16 is involved in motility restoration after

208 thermal stress [16]. Therefore, a *daf-16*(*mgDf50*) mutant was used to evaluate the

209 association between DAF-16 and motility improvement with odor stimulation. Wild-

210 type N2 worms treated with each odor stimulus showed higher motility restoration

211 after 12 h of heat stress than the control worms (Fig. 3 (b)). In the *daf-16* mutant,

212 even when odor stimulus was given, it had the same or lower motility than the control

213 (Fig. 3 (b)). Therefore, DAF-16 is involved in improving motility after linalool odor

214 stimulation. DAF-16 is mainly regulated by insulin/IGF-1 signaling, and the insulin-

215 like peptide receptor DAF-2 is involved in this regulation [17]. Therefore, the *daf-*

216 *2*(*e1370*) mutant was used. In wild-type N2 nematodes, the restoration of motility

217 rafter 3 h was greater than that in the control (Fig. 3 (c)); however, the motility was
218 not recovered in the *daf-2* mutant after treatment (Fig. 3 (c)). Therefore, DAF-2 is
219 involved in improving motility after heat stress with linalool odor stimulation.
220 Odorous substances are first recognized by G-protein-coupled receptors expressed
221 on neurons, which open downstream channels via Gα protein activity, and allow
222 signals to be transmitted. We focused on ODR-3, which is a Gα protein. ODR-3 is
223 expressed in five types of neurons; it is expressed in the olfactory neurons AWA,
224 AWB, and AWC, and the sensory neurons ASH and ADF [18]. Therefore, we
225 analyzed the *odr-3*(*n1605*) mutant. In wild-type N2 nematodes, motility was
226 significantly recovered at 12 h after heat stress by odor stimulation (Fig. 3 (d)).
227 However, in the *odr-3* mutant, there was no restoration
228 of motility with either odor stimulus (Fig. 3 (d)). From these results, we suggest
229 that the ODR-3 protein is involved in odor-dependent restoration of motility after
230 thermal stress.
231 We also tested the effect of linalool odor treatment on the nematode survival rate
232 under thermal stress conditions. The survival rate of nematodes treated with L-
233 linalool odor stimulation was lower than that of the control nematodes (Fig. 4A). In
234 addition, although there was no significant difference when we used DL-linalool, the

235 survival rate was lower than that of the control nematodes (Fig. 4A).

236

237 ***DL-Linalool odor stimulation increases the expression of genes regulated by DAF-***

238 ***16***

239 From the above results, it was clear that DL-linalool odor stimulation reduces fat

240 accumulation in nematodes and improves their motility after thermal stress, which is

241 dependent on DAF-16 function. Therefore, the expression of the genes *sod-3* and

242 *hsp-12.6*, which are regulated by DAF-16 [19, 20], was examined using real-time

243 PCR. The expression levels of *sod-3* increased approximately three-fold and the

244 expression level of *hsp-12.6* increased approximately five-fold upon DL-linalool

245 treatment (Fig. 4B). Conversely, with L-linalool treatment, we measured no

246 significant change in the expression levels of *sod-3* and *hsp-12.6*. Next, we focused

247 on the expression of the transcription factor *HSF-1*, which is involved in heat stress

248 tolerance [11, 21]. When the expression levels of the HSF-1-regulated genes *hsp-*

249 *16.2* and *hsp-70* were examined, *hsp-70* was not changed by DL-linalool treatment,

250 but *hsp-16.2* expression was increased (Fig. 4B). With L-linalool treatment, neither

251 of the genes showed expression level changes compared to the DMSO-treated control

252 (Fig. 4B). There are a number of insulin-like peptides, but we focused on INS-7 and

253 DAF-28, which are agonists that activate DAF-2 [22]. We found that DL-linalool
254 treatment did not change the expression level of *daf-28*, while it did increase the
255 expression level of *ins-7* (Fig. 4B). L-Linalool treatment did not induce a significant
256 change in gene expression levels (Fig. 4B).
257
258 **Discussion**
259 This study revealed that odor stimulation with DL-linalool decreased fat
260 accumulation in nematodes, while L-linalool had no such effect. Furthermore,
261 pumping motion in the nematodes did not change, suggesting that this odor
262 stimulation does not affect nematode feeding. However, previous studies in rats
263 reported that lavender oil and linalool could inhibit lipolysis through a histaminergic
264 response and promote appetite and weight gain [23].
265 DL-Linalool and L-linalool odor stimulation improved motility in nematodes after
266 heat stress. When odor stimulation was applied to *daf-16* and *daf-2* mutants, motility
267 was restored to the similar extent as that observed in the control, suggesting the
268 involvement of DAF-16 and DAF-2. In addition, ODR-3 is involved in the heat stress
269 tolerance by odor stimulation, as motility was not recovered in the *odr-3* mutant with
270 or without linalool treatment. DL-Linalool treatment increased the expression of *ins-*

271 *7*, but L-linalool did not. This result contradicts the involvement of DAF-16, but there
272 are approximately 40 insulin-like peptides in *C. elegans* [24] and odor stimulation
273 may affect the expression of other agonists and antagonists. Furthermore, DL-linalool
274 increased the expression of *sod-3* and *hsp-12.6*, but both DL-linalool and L-linalool
275 lowered the survival rate of nematodes under thermal stress, which may be due to
276 changes in the expression of other genes involved in heat stress tolerance as well as
277 tissue-specific *DAF-16* expression. Indeed, in nematodes, the activation of DAF-16
278 in the intestine is important for lifespan extension [25].
279 In this paper, we newly described physiological effects of DL-linalool and L-linalool
280 odor stimulation in nematodes. In addition, we showed that the physiological actions
281 of DL-linalool and L-linalool partly differ. DAF-16 is a factor related to aging and
282 heat stress tolerance and is a homologous protein of the forkhead type transcription
283 factor FOXO [26]. Moreover, the IIS pathway that controls DAF-16 function has
284 homology between nematodes and humans [27]. In this study, nematodes showed
285 repelling behavior against both DL-linalool and L-linalool (Fig.1). However, it is
286 unknown whether there is a clear association between physiological action by each
287 odors and chemotaxis. Previous studies using isoamyl alcohol and acetic acid have
288 shown that there is no particular relationship between chemotaxis and lifespan or

289 stress resistance due to odor stimulation [12].

290 The results of this study have opened up possibilities for application to mammals

291 and humans.

292

293 **Author contribution**

294 NH and KS conceived and designed experiments; NH performed all experiments,

295 KS provided every tools and reagents, NH and KS analyzed data, NH and KS wrote

296 the paper. NH and KS made manuscript revisions. KS supervised the study as a

297 principal investigator. All authors read and approved the final manuscript.

298

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302 or recommendations expressed in this publication are those of the author(s).

303

304 **Conflict of interest**

305 On behalf of all authors, the corresponding author states that there are no conflicts of

306 interest.

307

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Figure Legends

Figure 1. Nematode chemotaxis with odorous substance treatments

A: L1 synchronized wild type N2 nematodes were raised on OP plates for 96 h, after which 2 μL of odorous linalool substance was dropped in the center of the plate. The chemotaxis of nematodes was examined 60 min after treatment. B: (a) chemotaxis rate in response to DL-linalool treatment. The graph illustrates the mean ± standard error. N = 300 nematodes, 10%: $p = 0.053$. (b) Chemotaxis rate of nematodes treated with L-linalool. The vertical axis indicates the repelling index, and the horizontal axis the concentration of the reagent. The graph illustrates the mean ± standard error. N = 180 nematodes, $*p < 0.05$, $** p < 0.01$.

Figure 2. Fat accumulation and eating movement in nematodes treated with linalool

The synchronized nematodes were treated with linalool odor stimulation for 24 h. A: (a) Nematodes were stained with a Nile Red solution, fixed with 8% ethanol, and observed with a fluorescence microscope. CT: DMSO, DL: 1% DL-linalool, L: 1% L-linalool. N = 92, 91, 101, in order from the left, $**p < 0.01$, (b) Nematodes were observed by fluorescence microscopy; the scale bars indicate 100 μm. B: The pharyngeal pumping motion of worms was measured for 15 s. The vertical axis shows

the number of pumps per 15 s, and the horizontal axis shows the respective odor treatments. The graph shows the mean ± standard error. CT: DMSO, DL: 1% DL-linalool, L: 1% L-linalool. N = 10.

Figure 3. Nematode motility after thermal stress

Synchronized nematodes were given linalool odor stimulation for 24 h. (a) N2 wild type, (b) *daf-16* mutant, (c) *daf-2* mutant, and (d) *odr-3* mutant. The nematodes were cultured at 35°C for 4 h, then returned to 20°C before whole-body movement was measured at 12 and 24 h (or at 0, 3, 6 h). The number of whole-body movements per 15 s was measured. The vertical axis shows the ratio of the amount of exercise at 35°C to the number of movements at 20°C. The horizontal axis shows the elapsed time after thermal stress. The graphs represent the mean ± standard error. CT: DMSO, DL: 1% DL-linalool, L: 1% L-linalool, N = 10, $*p < 0.05$, $** p < 0.01$.

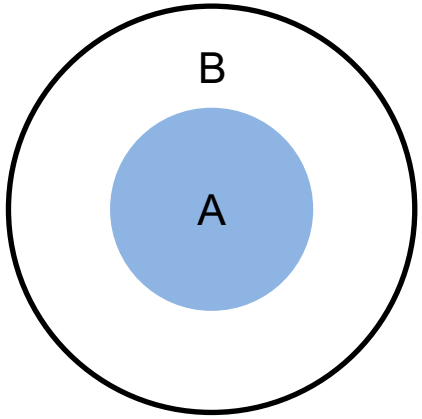
Figure 4. Nematode survival rate and gene expression during thermal stress

The synchronized nematodes were given odor stimulation for 24 h. A: The nematodes were cultured at 35°C and the survival rate was measured 10 h after heat treatment. The vertical axis indicates the percent survival rate, and the horizontal axis indicates

time. CT: DMSO, DL: 1% DL-linalool, L: 1% L-linalool, N = 40, *$p < 0.05$. B: Gene expression was examined using quantitative PCR. The vertical axis represents the mRNA expression level relative to the internal *actin* control, and the horizontal axis represents each gene analyzed. The graph represents the mean ± standard error. CT: DMSO, DL: 1% DL-linalool, L: 1% L-linalool, N = 3, *$p < 0.05$, **$p < 0.01$.

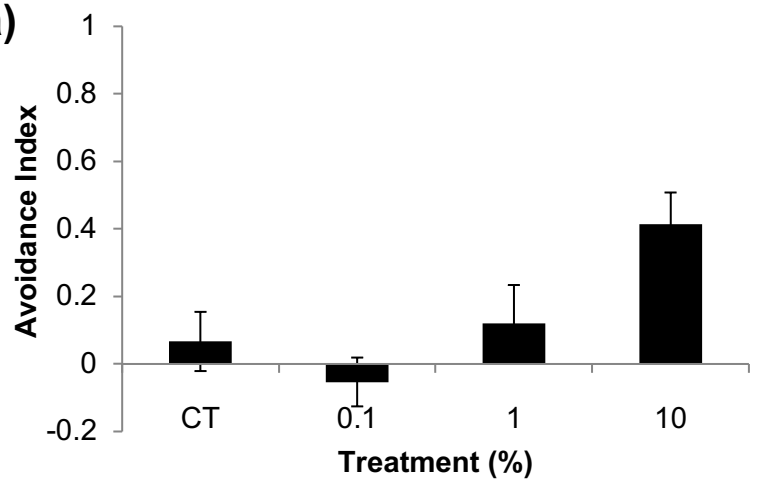
Fig1

A



Avoidance Index = (B-A)/(B+A)

B (a)



(b)

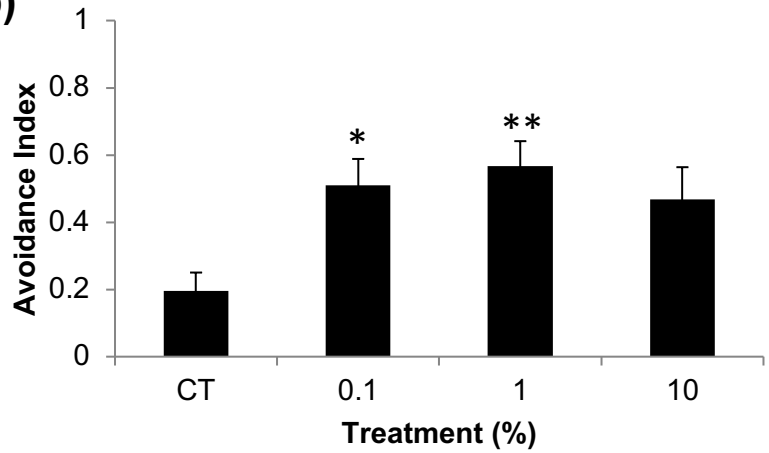


Fig2

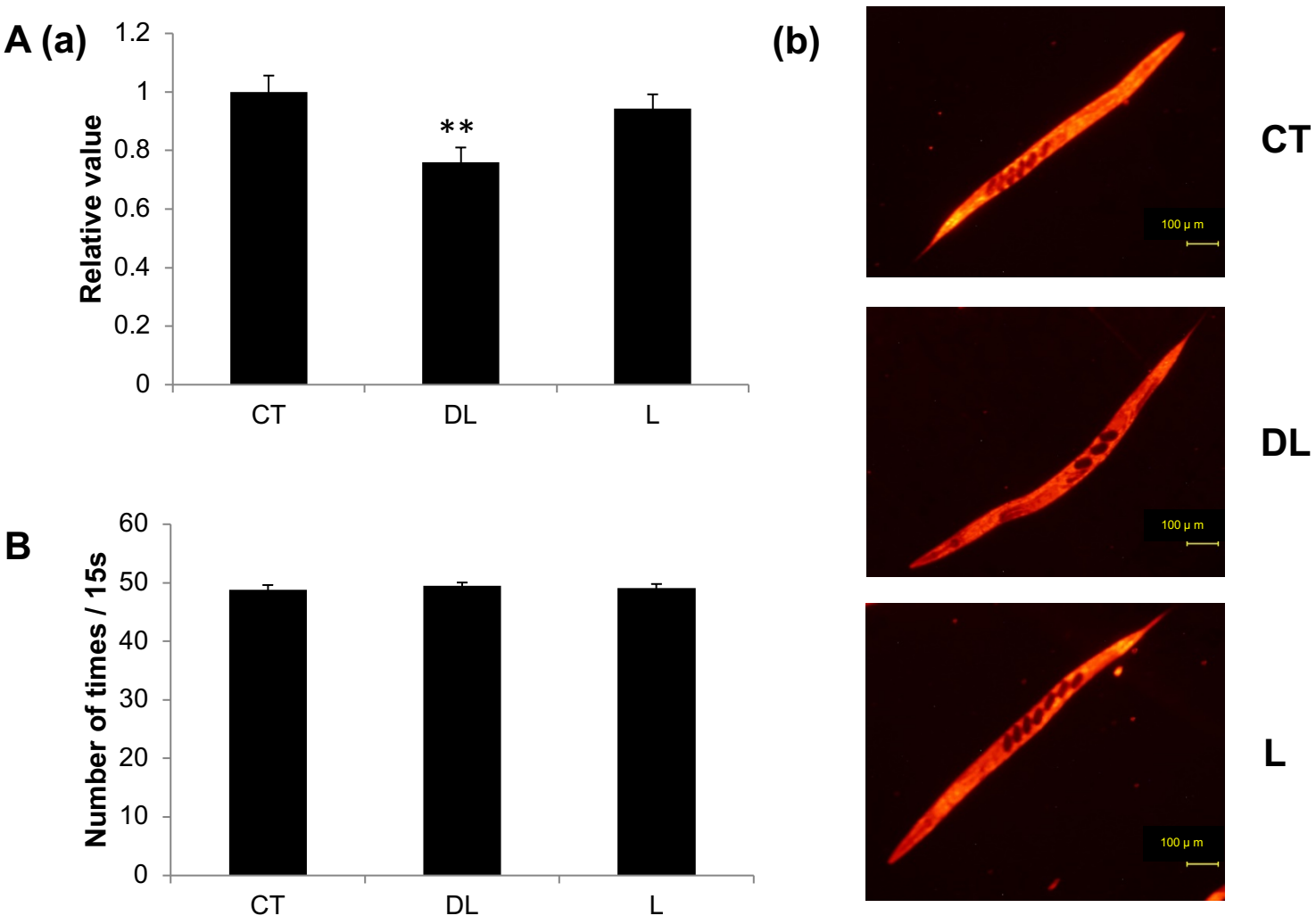

A (a)
Relative value
1.2
1
0.8
0.6
0.4
0.2
0
**
CT
DL
L
B
Number of times / 15s
60
50
40
30
20
10
0
CT
DL
L
(b)
100 μ m
100 μ m
100 μ m
CT
DL
L

Fig3

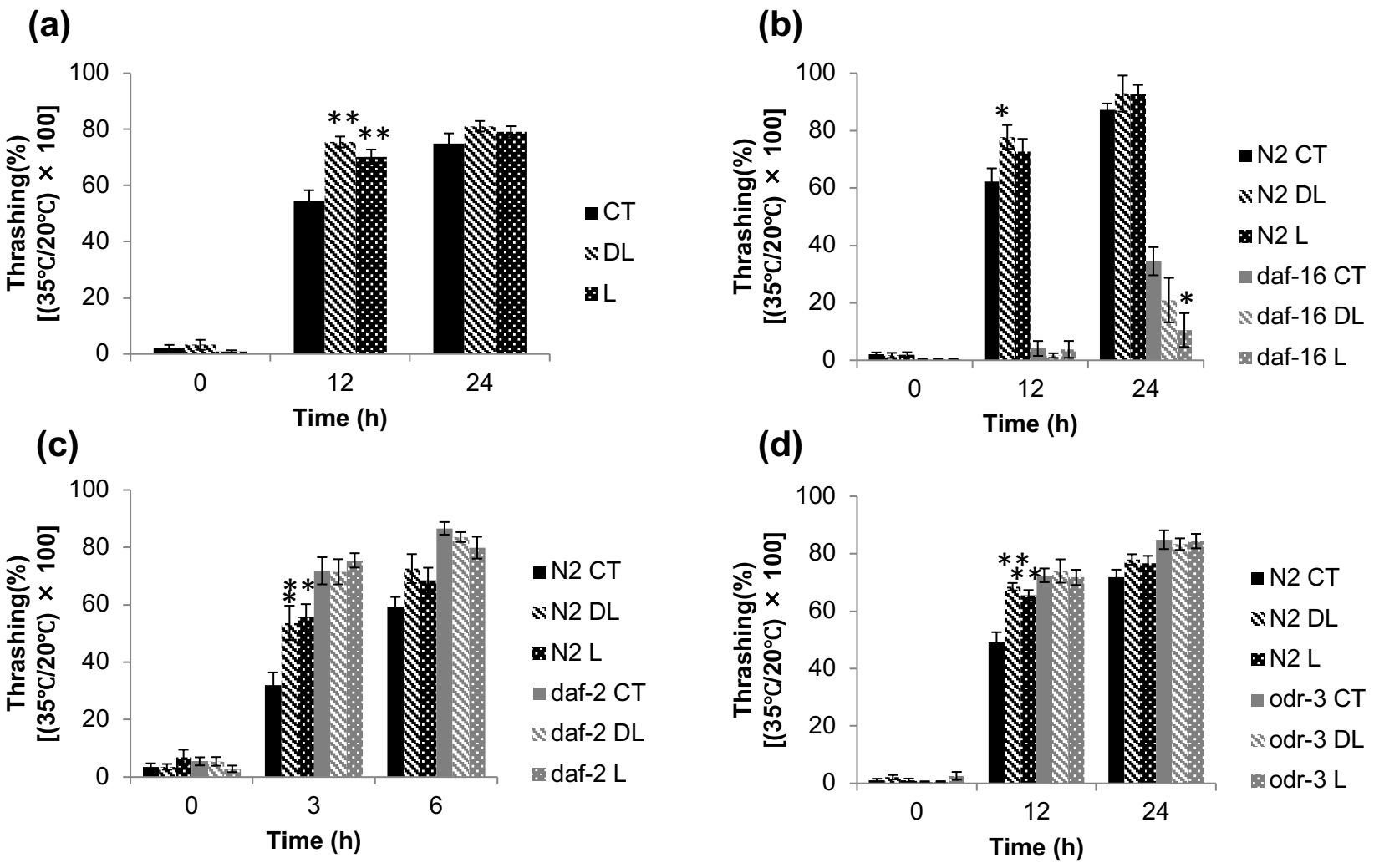
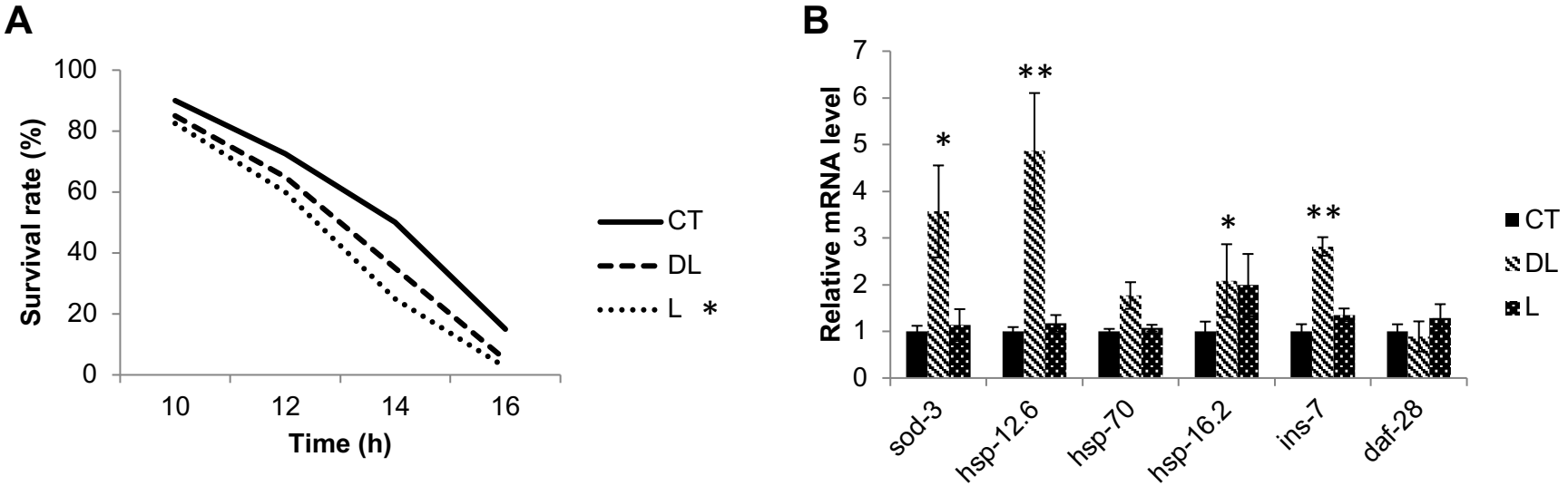
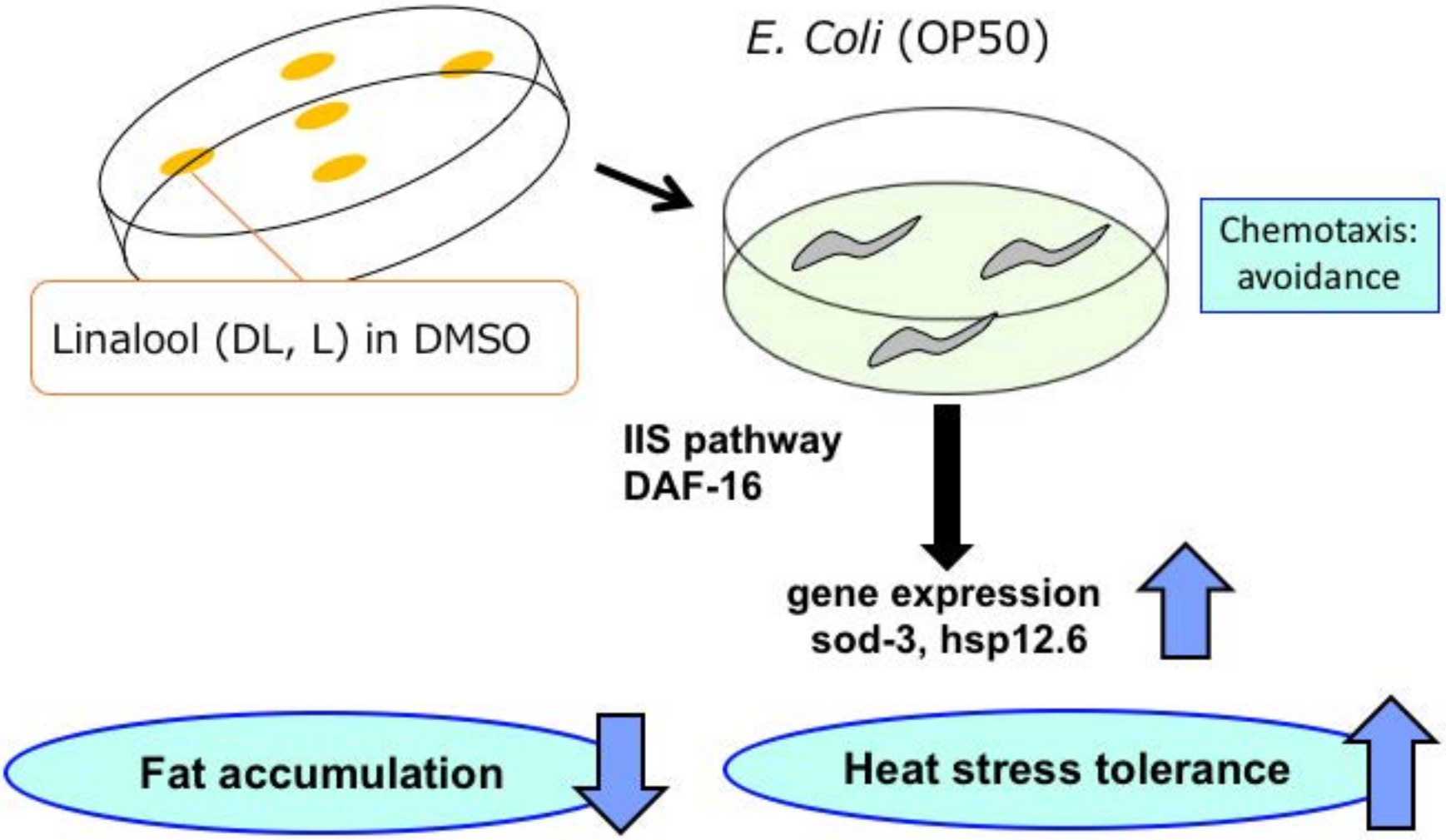

(a)
Thrashing(%) [(35℃/20℃) × 100]
100
80
60
40
20
0
0
12
24
Time (h)
CT
DL
L
(b)
Thrashing(%) [(35℃/20℃) × 100]
Time (h)
N2 CT
N2 DL
N2 L
daf-16 CT
daf-16 DL
daf-16 L
(c)
Thrashing(%) [(35℃/20℃) × 100]
0
3
6
Time (h)
N2 CT
N2 DL
N2 L
daf-2 CT
daf-2 DL
daf-2 L
(d)
Thrashing(%) [(35℃/20℃) × 100]
Time (h)
N2 CT
N2 DL
N2 L
odr-3 CT
odr-3 DL
odr-3 L

Fig4


A
Survival rate (%)
100
80
60
40
20
0
10
12
14
16
Time (h)
CT
DL
L *
B
Relative mRNA level
7
6
5
4
3
2
1
0
*
**
*
**
sod-3
hsp-12.6
hsp-70
hsp-16.2
ins-7
daf-28
CT
DL
L



Odor stimulation by Linalool (DL, L) reduced fat accumulation in nematode. Furthermore, Linalool odor increased the heat stress tolerance of nematode via insulin/IGF-1 (IIS) signal pathway.