Master's Thesis

석사 학위 논문

Identification of molecular mechanisms underlying developmental decision of *Caenorhabditis elegans*

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A thesis submitted to the faculty of DGIST in partial fulfillment of the requirements for the degree of Master of Science in the Department of Brain and Cognitive Sciences. The study was conducted in accordance with Code of Research Ethics[1)]

06. 01. 2016

Approved by

Professor (Advisor) Kyuhyung Kim (Signature)

Professor (Co-Advisor) Eunjoo Kim (Signature)

[1)] Declaration of Ethical Conduct in Research: I, as a graduate student of DGIST, hereby declare that I have not committed any acts that may damage the credibility of my research. These include, but are not limited to: falsification, thesis written by someone else, distortion of research findings or plagiarism. I affirm that my thesis contains honest conclusions based on my own careful research under the guidance of my thesis advisor.

Identification of molecular mechanisms underlying developmental decision of *Caenorhabditis elegans*

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Accepted in partial fulfillment of the requirements for the degree of Master of Science.

06. 01. 2016

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Abstract

Animals adapt to ever-changing environmental conditions to regulate their development. Our goal is to understand the mechanisms by which environmental signals are transduced and integrated with internal status to regulate development. The free-living nematode *Caenorhabditis elegans* undergoes reproductive development from egg to adult through four larval stages (L1-L4) in favorable environments. When L1 animals exposed to unfavorable environments, animals fail to initiate reproductive development and are arrested as stress-resistant and long-lived larva at dauer diapause, which is an alternative third developmental stage. However, how young animals make this developmental decision depending on environmental conditions at the time is not fully elucidated. To address this question, we first searched for genes of which expression pattern is altered at dauer diapause. Previous genetic studies have demonstrated that *flp-8* or *flp-4* FMRFamide-related peptide genes are differentially expressed in all touch receptor neurons of the dauer stage (Kim and Li, 2004). To identify the regulatory mechanism of *flp* gene expression, we first analyzed the *flp* gene promoter and found the putative *cis*-regulatory elements essential for expression in touch receptor neurons. We next found that expression of *flp*-8 in touch receptor neurons are regulated by MEC-3 and UNC-86, which are the transcription factors required for production and differentiation of the touch receptor neurons. To investigate whether this *flp* gene expression is regulated by the genes which are dauer-specific or involved in epigenetic mechanisms, we tested several candidate genes but none of animals carrying mutations in the genes displayed modified expression pattern both in non-dauer and dauer animals. Thus, our results suggest that unidentified genes or signaling pathways are likely to modify the expression of *flp* genes during dauer diapause, and that future genetic screen by mutagenesis may allow us to identify the nature of the genes or pathways.

Keywords : dauer, neuropeptide, touch receptor neuron

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I. Introduction

Animals adapt to ever-changing environmental conditions to regulate their development. In particular, they have developed various mechanisms in order to survive under harsh environmental conditions. Among them, the common strategy for their survival is entering the dormancy, which is a resting state and a period when the reduction of metabolic activity and temporal developmental suspension occur, and it has been adopted by many animals and also even by viruses and plants. Several types of dormancy (e.g., hibernation, estivation, brumation and diapause) have been reported from various organisms, but the detailed mechanisms of dormancy and how animals regulate their development and behavior in response to environmental cues are not well known.

To address this question, the free-living nematode *Caenorhabditis elegans* is a great model since it also has a form of dormancy described as dauer (a German word meaning for “endurance” or “permanent”). Under favorable conditions, *C. elegans* develops from egg to adult through four larval stages (L1-L4). However, when the environmental conditions are unfavorable, their development is arrested at L3 stage as stress-resistant dauer larva (**Cassada and Russell, 1975**). Once they become dauer larvae, they are under non-feeding, stationary and long-lived state; despite the absence of food intake, they can survive for up to few months, which is much longer than average lifespan of about 2-3 weeks under normal reproductive development (**Klass and Hirsh, 1976)**. This dauer arrest is influenced by three environmental conditions: food supply, population density (pheromone), and ambient temperature. Once environmental conditions improve, dauer animals emerge from diapause and re-enter the reproductive development as L4 stage animals. Actually this developmental decision is made at L1 stage when animals become either a reproductively growing L2 or a dauer growing pre-dauer L2d depending on their environmental conditions, but the molecular mechanisms of this developmental decision-making are not fully understood.

Previous genetic studies have focused on gene regulation during dauer formation and identified a number of genes in cellular and molecular pathways involved in this developmental plasticity. Loss of genes involved in insulin/IGF signaling (IIS) pathways including *daf-2* (insulin/IGF receptor homolog), *age-1* (PI-3 kinase), and *daf-16* (FOXO transcription factor) results in constitutive dauer formation (Daf-c; mutants always enter dauer diapause), while loss of genes in transforming

growth factor β (TGFβ) pathways including *daf-3* and *daf-5*, results in defective dauer formation (Daf-d; mutants avoid dauer diapause) (**Riddle and Albert, 1997; Liu et al. 2004**). In addition to these signaling pathways, genes encoding neuropeptides have been implicated to be likely to participate in dauer formation. In *C. elegans*, 113 neuropeptide genes have been identified; 31 genes encode FMRFamide-like peptides ("*flp*" family) (**Li et al., 1999a; Li and Kim, 2008**), 40 genes encode insulin-like peptides ("*ins*" family) and the rest 42 genes encode neuropeptide-like peptides ("*nlp*" family) (**Li et al., 1999b**). Among them, previous studies have identified that genes encoding insulin-like peptide *ins-1*, *ins-9*, and *daf-28* have been known for their critical roles in dauer formation (**Bargmann and Horvitz, 1991; Li et al., 2003**), but the functions of genes in other family in dauer formation are not well elucidated.

The previous study demonstrated that expression of the FMRFamide-related peptides *flp-8* and *flp-4* genes are differentially regulated between non-dauers and dauers (**Kim and Li, 2004**). In non-dauers, *flp-8* is expressed in the head region (e.g. URX and AUA) and PVM neurons, which is one of touch receptor (also called as mechanosensory) neurons (TRNs) in *C. elegans*, while dauers also expresses in other TRNs (AVM, ALM, and PLM). Likewise, non-dauers express *flp-4* in the several head, body neurons and AVM touch receptor neurons, while dauers also express *flp-4* in TRNs except for PVM. However, the molecular mechanisms by which their expression in TRNs is regulated, and their functions in dauer formation have not been studied.

To study how *flp* gene expression in TRNs is modified in dauers, we first examined promoter of *flp* genes and identified the putative *cis*-regulatory elements required for additional expression in TRNs in dauers. We also found that two transcription factors MEC-3 and UNC-86, which are essential for production and differentiation of TRNs, are required for *flp* gene expression in TRNs in dauers. To identify the putative repressors or activators acting on *flp* gene promoter, we next searched and examined candidate genes which are known for involved in dauer formation and chromatin remodeling, and found that these genes are not required for alteration of *flp* gene expression. We are currently working on finding mutants that show abnormal *flp* gene expression in both non-dauers and dauers via mutagenesis. We expect that this study will help to understand the mechanisms by which environmental signals are transduced and integrated with internal status to regulate gene expression and development.

II. Materials and Methods

2.1. Strains and transgenic animals

All strains used in this study were maintained as described previously (**Brenner, 1974**). For wild-type strain, N2 Bristol was used. Strains were cultivated at 20°C on 60mm NGM(nematode growth medium) plates seeded with the *E. coli* strain OP50 as a food source. Following strains were obtained from Caenorhabditis Genetic Center (CGC): *daf-2(e1370)* III, *mec-3(gk3299)* IV, *unc-86(n846)* III, CX5974(*unc-86*p::*myrGFP*), *hpl-1(tm1624)* X, *hpl-2(tm1489)* III, *set-2(ok952)* III, *hda-2(ok1479)* II, *zfp-1(ok554)* III, *gfl-1(gk321)* IV, *hil-1(gk229)* V, *isw-1(n3294)* III, and *lin-35(n745)* I. *daf-2(e1370)*;*flp-8*::*gfp* and *daf-11(sa195)* V was obtained from Dr. Piali Sengupta. *daf-7(e1372)* III was obtained from Dr. Jeong-Hoon Hahm. *Ex*[*flp-4*p::*gfp*] transgenic animal was generated by microinjection of plasmid (50ng/μl) into wild-type N2 with *unc-122*p::*DsRed* (50ng/μl) as co-injection marker. *flp-8*p::*gfp* extrachromosomal arrays were integrated into the genome using UV irradiation.

2.2. Generation of *flp-8*p::*gfp*-expressing strains

Following strains were produced by crossing each single mutant hermaphrodites with *flp-8*p::*gfp* males: To identify *hpl-1(tm1624)*;*flp-8*p::*gfp*, *hpl-2(tm1489)*;*flp-8*p::*gfp, set-2(ok952)*;*flp-8*p::*gfp, hda-2(ok1479)*;*flp-8*p::*gfp, zfp-1(ok554)*;*flp-8*p::*gfp, gfl-1(gk321)*;*flp-8*p::*gfp, hil-1(gk229)*;*flp-8*p::*gfp,* genotypes of F2 animals were confirmed by polymerase chain reaction (PCR) and sequencing. *mec-3(gk3299)*;*flp-8*p::*gfp*, *unc-86(n846)*;*flp-8*p::*gfp*, *daf-7(e1372);flp-8*p::*gfp,* and *daf-11(sa195);flp-8*p::*gfp* were identified by examining F2 animals showing mutant phenotypes.

2.3. Dauer formation assay

Dauer formation assay was performed as described previously (**Neal et al., 2013**). 35mm plates for assay were prepared with noble agar (Difco) without peptone. Each plate contains 100μl of crude pheromone extracted from wild-type N2 animals and seeded with 20μl of heat-killed *E. coli* strain OP50. Five healthy well-fed young adult animals were transferred to each plate and allowed to lay eggs at 25°C for 4-5 hours. Adult animals were removed when the number of eggs is about 65-85

in the plate. Plates with eggs were incubated at 25°C for 72~84 hours. Dauer animals were isolated by visual inspection in consideration of size, movement, and body posture.

2.4. Plasmid construction and quantification of GFP expression

To construct *mec-3*p::*gfp* plasmid, about 2.0kb promoter region of *mec-3* was extracted from wild-type N2 genomic DNA by PCR and subcloned into the GFP(green fluorescent protein)-containing vector pPD95.77. For promoter analyses, several promoter regions of *flp-8* and *flp-4* were digested by appropriated restriction enzymes and subcloned into pPD95.77. All plasmids (50ng/μl) were injected into wild-type N2 with *unc-122*p::*DsRed* (50ng/μl) as co-injection marker. The plasmid for point mutation (site-directed mutagenesis) is generated by PCR with mutagenic designed primers.

GFP expression patterns were examined in extrachromosomal transgenic animal lines. The list of restriction enzymes and primer sequences used were described in **Table 1**. We set the following standards to score arbitrary GFP intensity: + (faint cell body), ++ (cell body and partial process), +++ (overall faint cell body and process), ++++ (vivid cell body and process).

2.5. Mutagenesis screen

The mutagenesis protocol is modified from **Brenner (1974)**. Wild-type N2 worms were grown to the L4 stage and washed off plates with M9 buffer solution into 15ml conical tube. After worms were sunk, the supernatant was removed. Worms were washed and resuspended once or twice with 2ml M9 buffer and 2ml 0.1M EMS(ethyl methanesulfonate) was administrated to the 2ml worm suspension. The tube was sealed with paraffin film and placed on shaker for 4 hours at room temperature. Then worms were washed twice with M9 buffer and transferred to plates seeded with *E. coli* strain OP50 using sterile glass pipette. After 2 hours later, healthy looking late L4 animals were picked to new plates as P0 generation and incubated at 15℃. After 3 days, three of F1 animals were picked to new individual plates and incubated at 15℃.

III. Results

3.1. *flp* gene expression in TRNs is dependent on two transcription factors, MEC-3 and UNC-86

To investigate the molecular mechanisms underlying dauer entry of *C. elegans*, we focused on two neuropeptide genes, *flp-8* or *flp-4,* of which expression is differentially regulated between non-dauers and dauers (**Kim and Li, 2004**). In addition to other neurons, non-dauers express *flp-8* in PVM and *flp-4* in AVM, while dauers additionally express *flp* genes in all TRNs, except for PLM in *flp-4* expression (**Figure 1**). However, the mechanisms of the regulation of *flp* genes in TRNs depending on developmental stage and its functional significance are still unclear.

C. elegans has six TRNs (AVM, ALML/R, PVM, and PLML/R) and it has been known that they express neuropeptides *flp-4* or *flp-8.* (**Kim and Li, 2004**). Previous genetic studies have identified two genes, *mec-3* and *unc-86*, which are necessary for the production and differentiation of these TRNs. These genes are known as transcription factors and also terminal selectors of TRNs (**Way and Chalfie, 1988; Zhang et al., 2014**). *mec-3* encodes a LIM-type homeodomain protein needed for the differentiation of the TRNs, while *unc-86* encodes a POU-type homeodomain protein needed for the production (**Finney et al., 1988; Way and Chalfie, 1988)**. They can interact on each other to form a heterodimeric complex and bind on the promoters of downstream target genes to regulate their transcription (**Xue et al., 1993**). Mutations in *mec-3* result in failure to express specific features of TRNs and mutations in *unc-86* cause cell lineage defects such that no TRNs are produced (**Chalfie et al., 1981; Way and Chalfie, 1988**). Therefore, we wondered whether *flp* genes are also regulated by *mec-3* and *unc-86.*

To investigate whether *flp* gene expression is dependent on *mec-3* and *unc-86*, we expressed GFP-expressing vector under the control of *flp-8* promoter in these mutant backgrounds and examined whether *flp-8* gene expression is lost in TRNs. Mutation of *mec-3* and *unc-86* results in the complete loss of *flp-8* expression in TRNs both in non-dauer and dauer animals (**Figure 2**). These results suggest that *mec-3* and *unc-86* are required for *flp-8* expression in TRNs. We also tested whether *mec-3* and *unc-86* expression is also modified in dauers by observing GFP-expressing vector under their own promoters, but we didn't observe any differences in *mec-3* and *unc-86* expression in dauers compared to non-dauers, suggesting that *mec-3* and *unc-86* expression is not altered in dauer

diapause and not key regulators for dauer-specific *flp-8* or *flp-4* expression in TRNs (**Figure 3**).

3.2 Potential c*is*-regulatory elements for *flp* gene expression in TRNs are identified.

To identify the *cis*-regulatory elements on *flp* gene promoter, we performed promoter analyses of *flp* genes. We constructed several reporter plasmids containing GFP under *flp* gene promoter by subcloning and examined expression patterns in TRNs (**Figure 4 and 5**). We found that deletion of a half of the first part of *flp-8* promoter (deleted sequence (▲) 1) results in the loss of expression in TRNs except for PVM. Hence, we investigated this deletion site in more detail and found that deletion of 331bp between promoter sequence ▲4 and ▲6 results in identical consequence as shown in ▲1 transgenic animals; both ▲1 and ▲5 transgenic dauer animals lost additional *flp-8* expression in TRNs. However, ▲6 transgenic dauer animals do not lose *flp-8* expression in all TRNs, indicating that the *cis*-regulatory element for *flp-8* expression in additional TRNs is located between ▲5 and ▲6.

Since *flp-8* expression in TRNs is dependent on MEC-3 and UNC-86, we anticipated that this *cis*-regulatory element may contain potential binding site for MEC-3::UNC-86 heterodimeric complex. Therefore, we examined and found the well-conserved putative MEC-3::UNC-86 binding site (Figure 6). To test whether this sequence is indeed the binding site for MEC-3::UNC-86, we performed site-directed mutagenesis to induce point mutation in the middle of this sequence and investigated *flp-8* expression pattern. As a result, dauer animals expressing GFP under the control of mutated promoter do not express *flp-8* in additional TRNs (**Figure 6**), indicating that *flp-8* expression in TRNs is directly regulated by MEC-3 and UNC-86.

In addition to *flp-8*, we also found that the deletion of 350bp in the anterior *flp-4* promoter sequence results in the loss of expression in all TRNs in dauer animals, indicating that this region contains the putative *cis*-regulatory elements for *flp-4* expression in TRNs.

From these results, we generated a possible model (**Figure 7**) and hypothesized that an unknown repressor exists and it blocks *flp* gene expression in most TRNs in non-dauer animals. However, this repressor might be repressed itself or by an unknown co-factor during dauer diapause to allow expression of *flp* gene in additional TRNs. To test our hypotheses, we decided to identify an unknown repressor with its binding sites for *flp* gene expression in TRNs.

3.3 *flp* gene expression in dauer animals is not affected by the genes involved in major dauer formation pathways.

Next, we tested that expression of *flp* genes are also regulated by the genes involved in the pathways critical for dauer formation; *daf-2* in insulin/IGF signaling encodes insulin/IGF receptor and *daf-7* in TGFβ signaling encodes a member of the TGFβ superfamily. In addition, *daf-11* in cGMP signaling encodes a transmembrane guanylate cyclase (GCY). Animals carrying mutation in these genes result in constitutive dauer formation at 25°C. Because of their important roles in dauer formation, we expected that these genes might act in regulating *flp* genes in dauer diapause. Hence, we expressed GFP-expressing vector under the control of *flp-8* promoter in these mutant backgrounds and examined the expression pattern between non-dauers and dauers. However, contrary to our expectations, we did not observe the alteration of expression in both non-dauer and dauer mutant animals (**Figure 8**), suggesting that the functions of these genes are not involved in *flp-8* expression in dauer diapause.

3.4 Alteration of *flp* gene expression in dauer animals is not affected by the genes associated with chromatin remodeling.

Regulation of gene expression by epigenetic mechanisms, including DNA methylation, RNA interference (RNAi) and histone modifications, enables the animals to integrate internal and environmental (external) cues in the genome and therefore modulations in gene expression can promote the adaptation of animals in response to ever-changing environments (**Jaenisch and Bird, 2003**). In the case of *C. elegans*, previous studies demonstrated that the expression of chromatin-associated genes was altered during dauer diapause by using serial analysis of gene expression (SAGE) (**Jones et al., 2001**), and that genome-wide modulations in active chromatin marks, including H3K4 methylation and H4 acetylation, is associated with the alteration of expression profiles between dauer and post-dauer animals by using chromatin immunoprecipitation (ChIP) (**Hall et al., 2010**). Thus, we hypothesized that *flp* gene expression in dauer TRNs may be regulated by the chromatin-associated genes, and searched candidate genes that encode proteins acting in histone modifications.

We tested the following genes implicated in chromatin remodeling in *C. elegans*: *hpl-1* and *hpl-2*

encodes *C. elegans* heterochromatin protein homologs (**Couteau et al., 2002**) and *hil-1* encodes histone H1-like protein (**Jedrusik et al., 2002**). *isw-1* encodes a chromatin remodeling protein acting as the ATPase which is component of nucleosome remodeling factor (NURF)-like complex (**Andersen et al., 2006**). *set-2* encodes a histone H3K4 methyltransferase and plays a role in germline development, postembryonic development, and RNAi (**Xu and Strome, 2001**). *gfl-1,* which is predicted to associate with chromatin, encodes an ortholog of human glioma-amplified sequence-41 (GAS41). *hda-2* encodes a class I histone deacetylase, which is predicted to function in deacetylation of histone residues and transcriptional regulation (**Shi and Mello, 1998**). *lin-35* encoding retinoblastoma suppressor protein ortholog, and *zfp-1* encoding a chromatin-associated protein are known to be associated with RNAi (**Dudley et al., 2002; Lehner et al., 2006**). We expressed *flp-8* in mutant animals defective in these genes, except for *lin-35* and *isw-1*, and examined whether the expression pattern is altered compared to wild-type. Similar to the genes involved in dauer formation, we did not observe any changes in the expression patterns between non-dauer and dauer mutant animals (**Figure 9**), suggesting that mutations in chromatin-associated genes do not affect in *flp-8* expression in TRNs.

IV. Discussion

Despite the expression patterns of the FMRFamide-related neuropeptides *flp-8* and *flp-4* were identified more than a decade ago (**Kim and Li, 2004**), what functions of these genes are in dauer animals and the mechanisms how additional expression in dauer TRNs is regulated have not been understood. Indeed, it has not been reported so far that loss of *flp-8* results in defects in dauer formation. In addition, although *flp-8* and *flp-4* are expressed in TRNs, definite functions of *flp* genes in touch response still have not been known. Since it is reported that touch sensitivity can be modulated by dauer formation via insulin/IGF signaling (**Chen and Chalfie, 2014**), it is likely that the additional expression of *flp* genes in dauer TRNs may be involved in the modulation of touch response in dauer animals.

We have shown that modification of the expression of *flp-4* and *flp-8* in TRNs is controlled by two transcription factors MEC-3 and UNC-86, which play important roles in TRNs. **Zhang et al. (2002)** previously identified the consensus binding site for MEC-3::UNC-86 heterodimer and about 70 *mec-3*-dependent genes from DNA microarrays, but *flp-8* and *flp-4* were not included in the results. From our promoter analyses and site-directed mutagenesis, we found the potential MEC-3::UNC-86 binding site in *flp-8* promoter and point mutation in this site results in the loss of additional expression of *flp-8* in TRNs during dauer diapause, indicating that the putative site is indeed the *cis*-regulatory binding motif for MEC-3::UNC-86 and that *flp-8* is the novel transcriptional target of MEC-3 and UNC-86. However, *flp-8* expression in PVM do not disappear even in non-dauer and dauer animals, suggesting the possible presence of another MEC-3::UNC-86 binding site for regulation of *flp-8* expression in PVM.

In this study, we proposed a model suggesting the presence of a potential repressor that may regulate *flp* gene expression in TRNs during dauer diapause (**Figure 7**). We first assumed that potential binding site for a repressor that blocks *flp-8* expression in additional non-dauer TRNs may exist in the dissected regions in subcloned *flp-8* promoters and if so, transgenic animals expressing *flp-8* under a certain modified promoter without putative repressor-binding site will show ectopic expression even in all non-dauer TRNs. However, we observed that none of transgenic non-dauer animals (▲1 to ▲6) ectopically express *flp-8* in the additional TRNs, suggesting that dissected sequences in used promoters may not contain the sites for a putative repressor. Thus, we anticipate

that the posterior region of *flp-8* promoter may contain the potential binding site for a repressor, and performed additional promoter analyses; we gradually dissected from posterior part of promoter, keeping the presence of *cis*-regulatory element for *flp-8* expression in TRNs. (**Figure 10**). However, we found that all of transgenic animals also do not ectopically express *flp-8* in TRNs, suggesting that further analyses will be required for identification of putative repressor-binding site.

The treatment of chemical mutagen EMS (ethyl methanesulfonate) to *C. elegans* is the most common strategy for genome-wide mutagenesis to induce genetic mutation and identify gene function (**Brenner, 1974**). Thus, we have performed genetic screen to isolate mutant animals that show adverse *flp-8* expression patterns in non-dauer animals to find the genes controlling *flp* gene expression. We have screened 3,000 haploid genomes of *C. elegans*, but did not find any candidate mutants yet (**Figure 11**). Further genetic screen may allow us to find the unknown repressor or *trans*-acting factor regulating *flp* gene expression in TRNs.

V. Figures and Tables

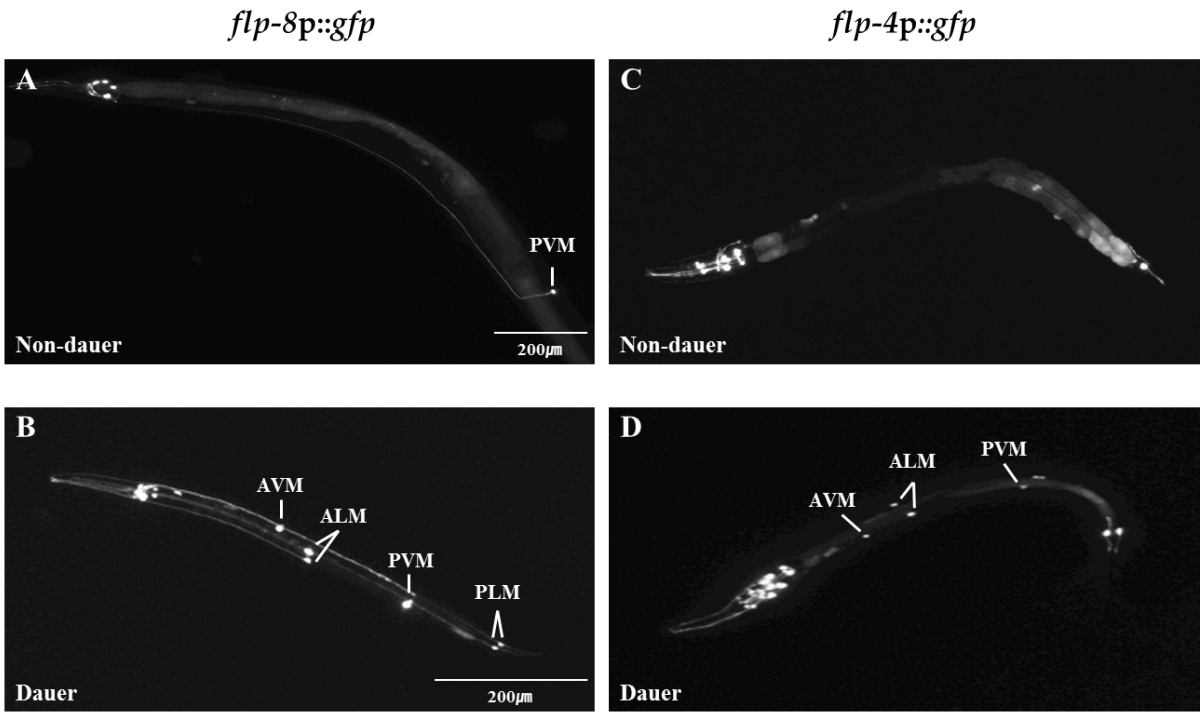

Figure 1. Expression of few *flp* genes is altered in dauer diapause.

(A) Wild-type non-dauer animals express *flp-8* in PVM, (B) while dauer animals express *flp-8* in all TRNs (AVM, ALML/R, PVM, and PLML/R). Likewise, *flp-4* expression is altered during dauer diapause; **(C)** non-dauer animals express *flp-4* in AVM (which is too faint to detect), (D) while dauer animals express *flp-4* in four TRNs (AVM, ALML/R and PVM).

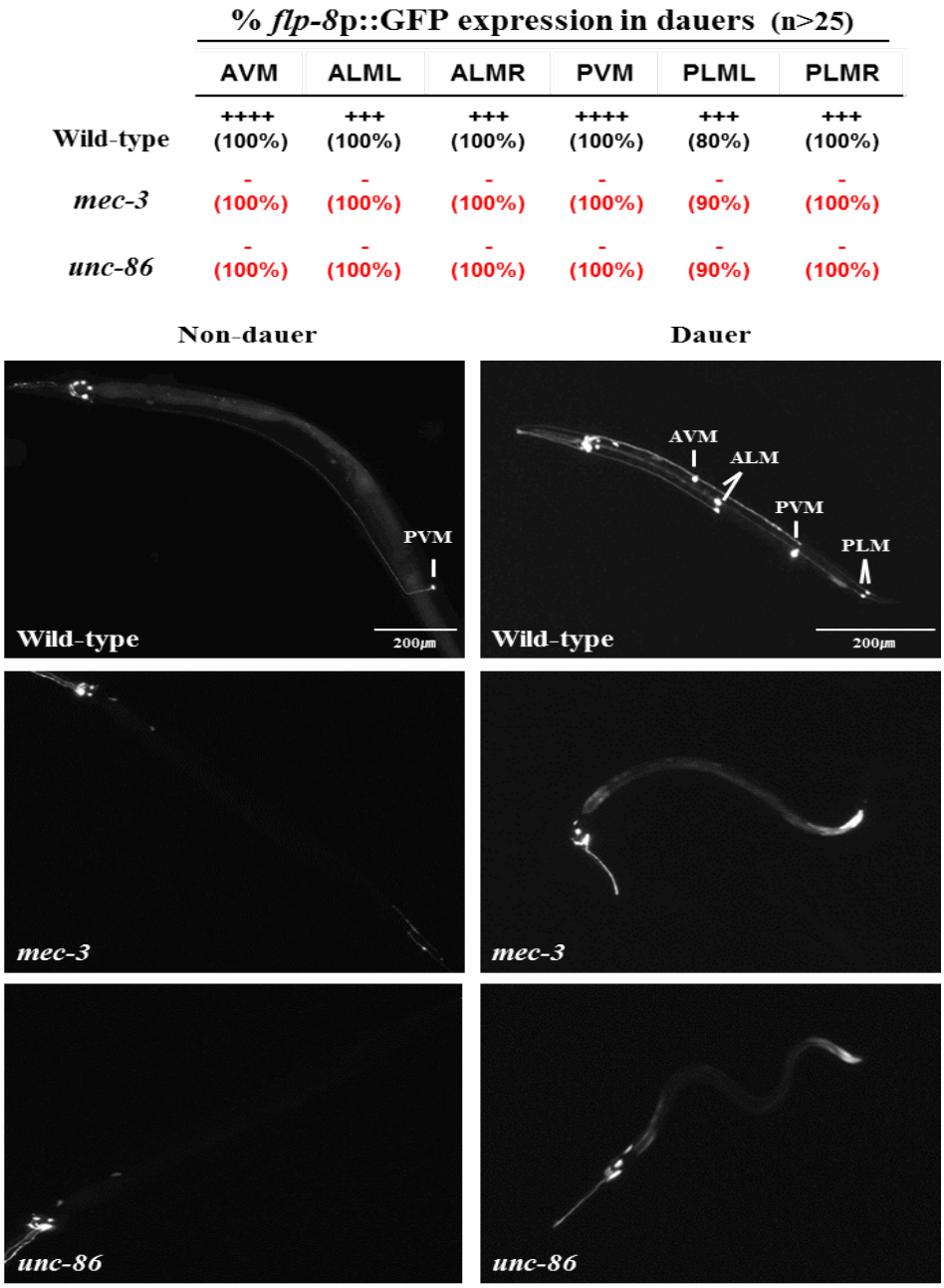

Figure 2. Expression of *flp* genes in TRNs is dependent on MEC-3 and UNC-86.

Wild-type animals normally express *flp-8* in TRNs, while mutants defective in the genes encoding transcription factors MEC-3 and UNC-86 do not express *flp-8* in TRNs both in non-dauer and dauer animals. The number of percentage means the portion of animals that express that level of GFP intensity.

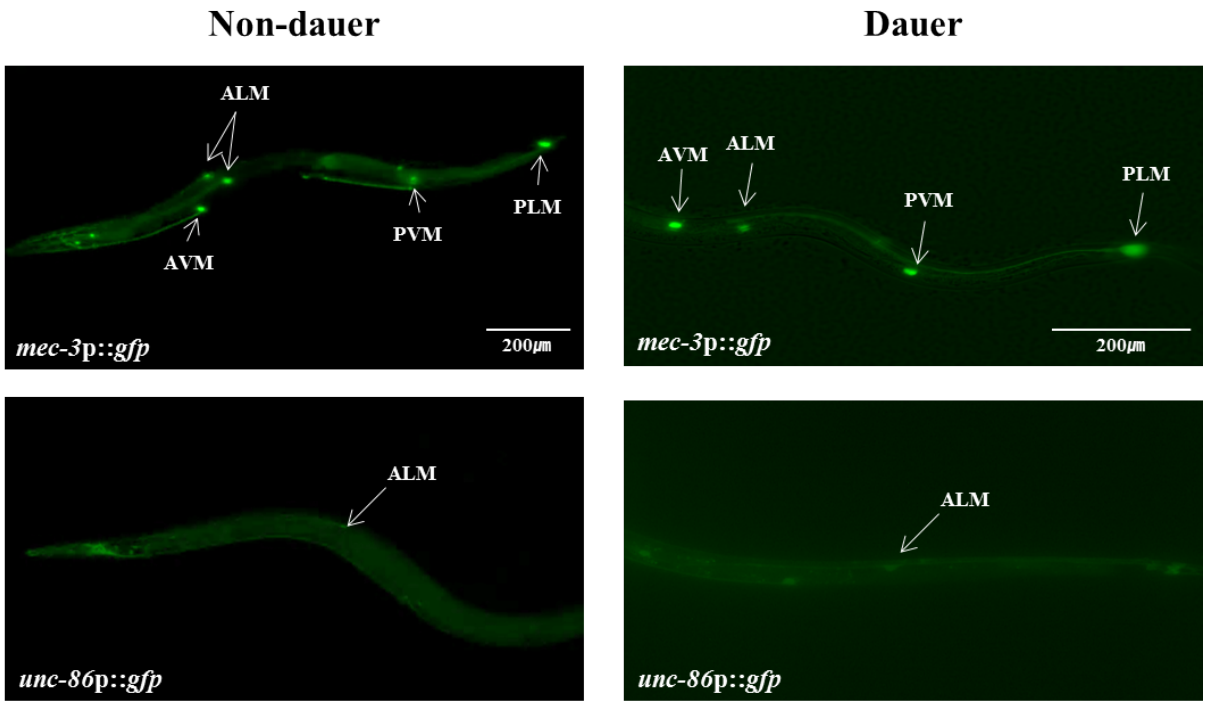

Figure 3. Expression of *mec-3* and *unc-86* is not altered in dauer diapause.

Wild-type non-dauer animals express *mec-3* in all TRNs, and this expression pattern is not altered in dauer diapause; dauer animals also express *mec-3* in all TRNs. Likewise, the expression pattern of *unc-86* is not modified in dauer diapause; both non-dauers and dauers express *unc-86* in ALM; other TRN expression is not shown in this picture.

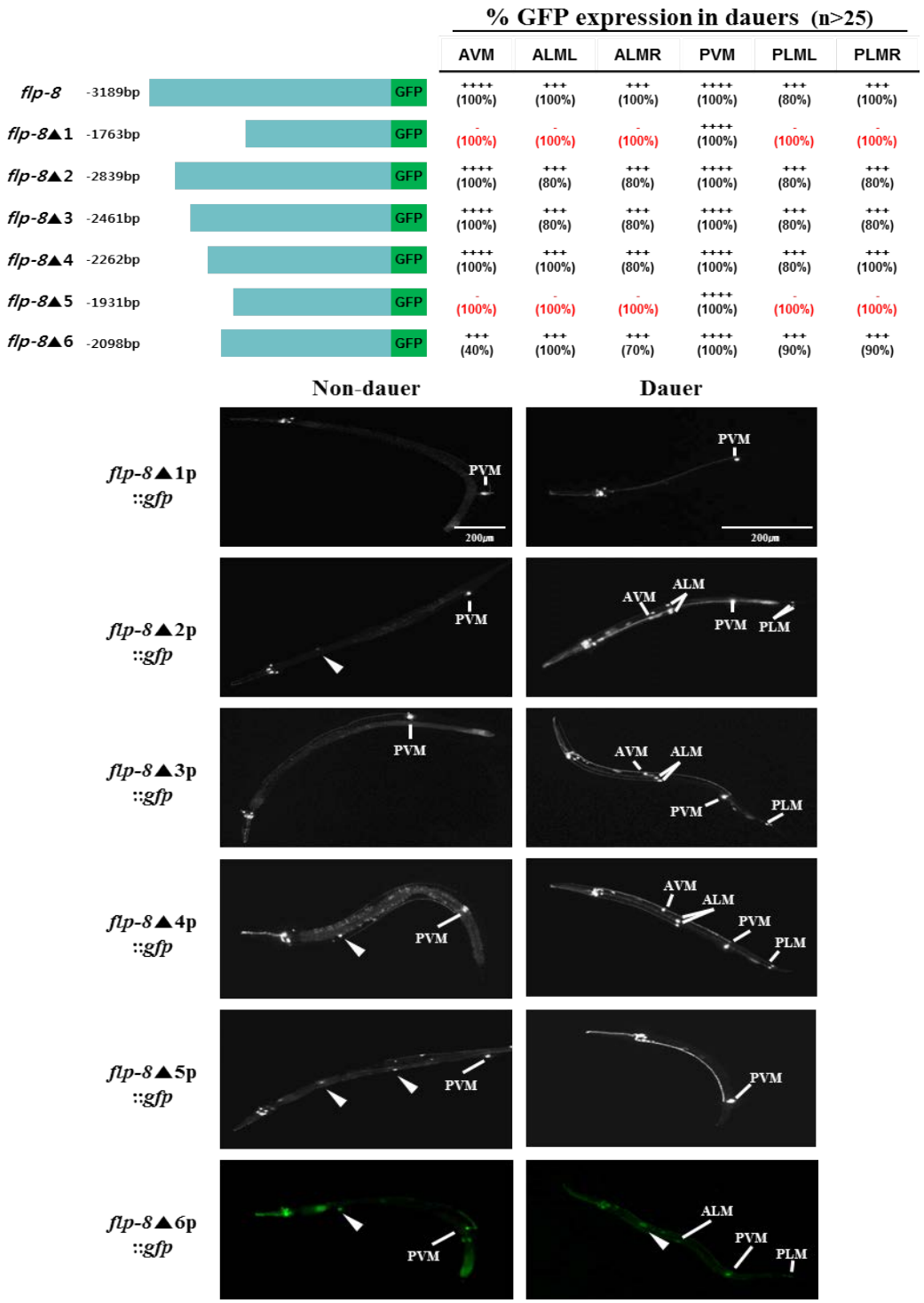

Figure 4. *flp-8* promoter analyses to identify *cis*-regulatory elements for expression in TRNs.

Expression patterns of *flp-8*▲5p::*gfp* and *flp-8*▲6p::*gfp* transgenic dauer animals indicates that *cis*-regulatory elements for expression in TRNs may exist between ▲5 and ▲6 sequences. The *cis*-regulatory element for PVM is assumed to be in the ▲1 sequence. Arrowheads indicate the sites of coelomocytes for the injection marker expression.

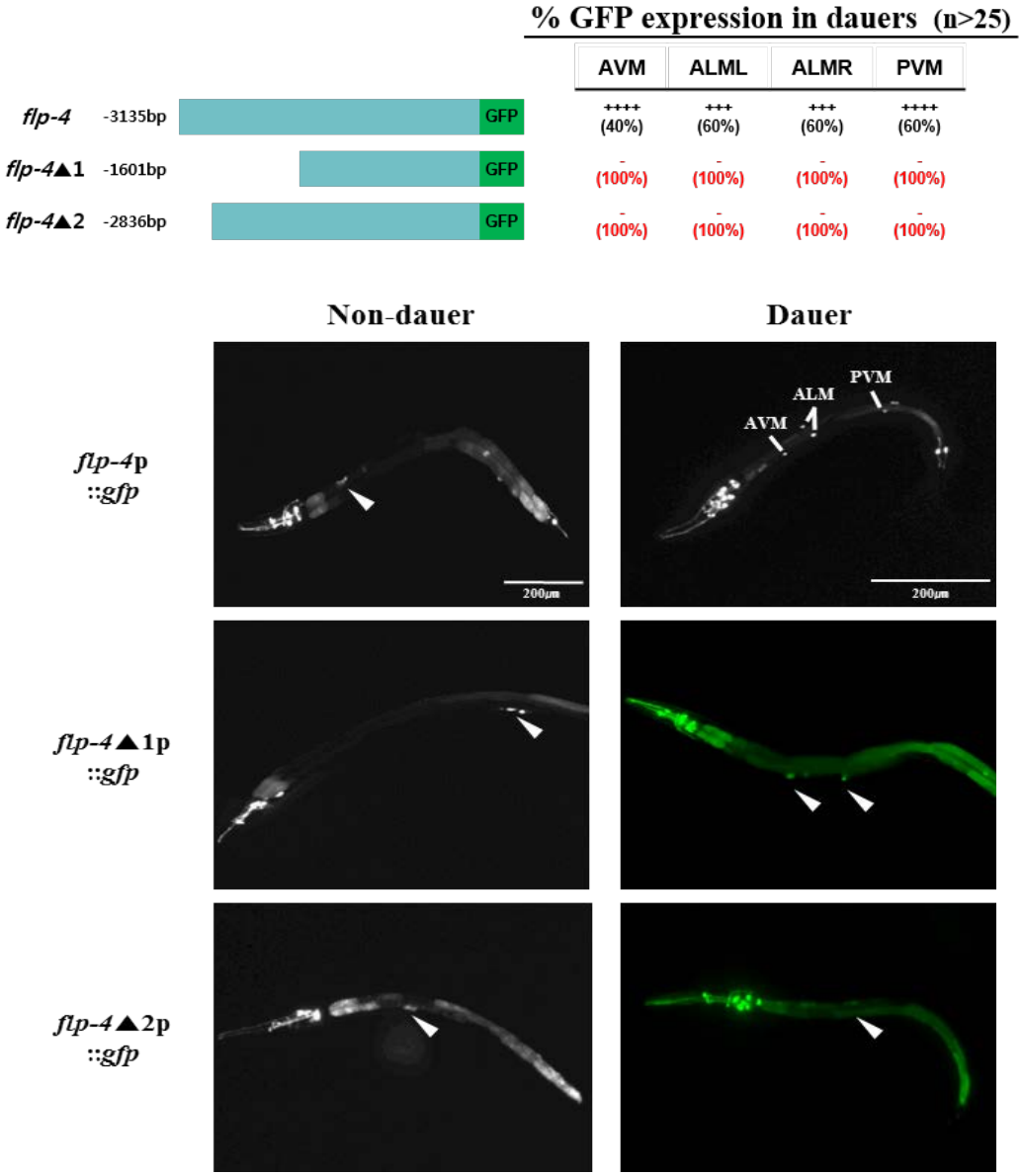

Figure 5. *flp-4* promoter analyses to identify *cis*-regulatory elements for expression in TRNs.

Expression patterns of *flp-4*▲1p::*gfp* and *flp-8*▲2p::*gfp* transgenic dauer animals completely lost expression in TRNs, indicating that *cis*-regulatory elements for expression in TRNs may exist anterior sequences of *flp-4* promoter. Arrowheads indicate the sites of coelomocytes.

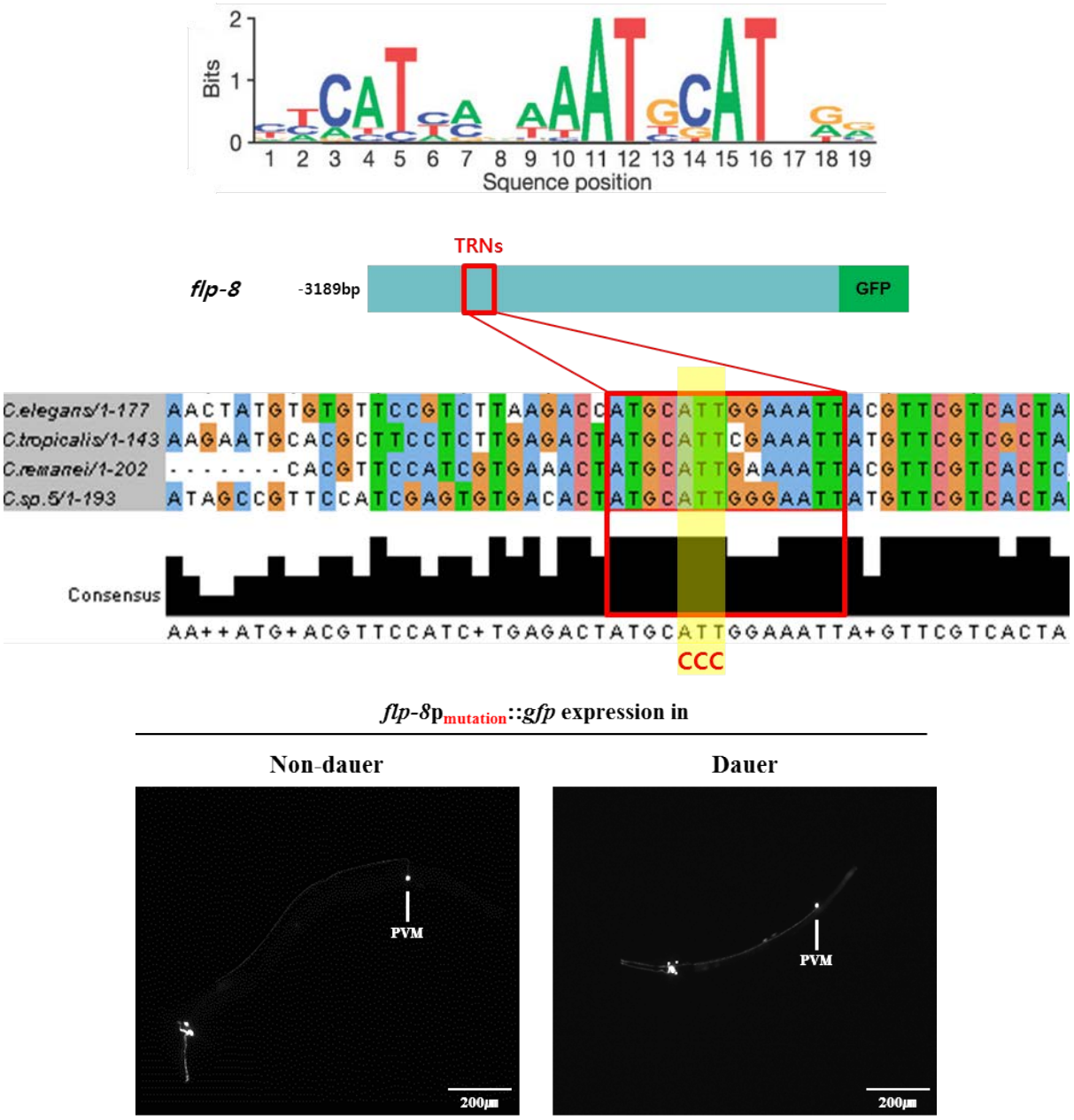

Figure 6. Identification of MEC-3::UNC-86 binding site in *flp-8* promoter.

(Upper panel) Consensus MEC-3::UNC-86 binding site. The image is adapted from **Zhang et al. (2002)**. (Middle panel) Point mutation in the potential binding sequence for MEC-3::UNC-86. (Lower panel) Dauer animals expressing GFP under the control of the mutated promoter completely lost the expression in additional TRNs.

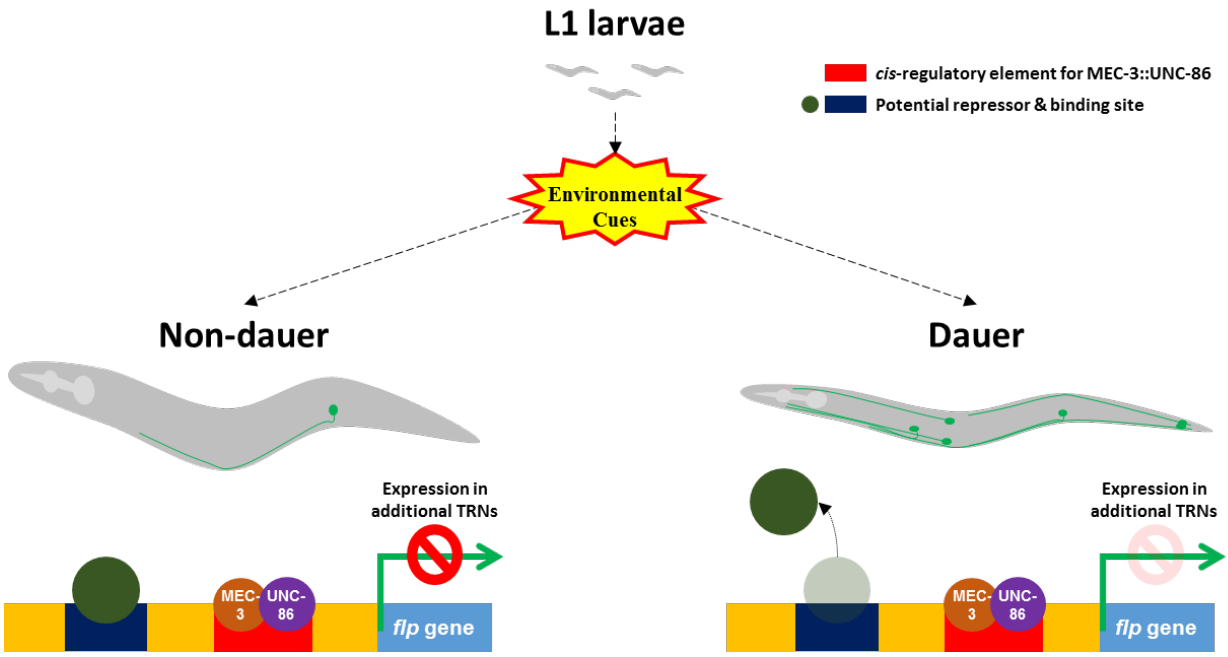

Figure 7. A model for regulation of *flp* gene expression in TRNs between non-dauers and dauers.

This model suggests the presence of potential binding sites for MEC-3::UNC-86 heterodimer and a repressor which may block *flp* gene expression in most TRNs. In our model, *flp* genes can be expressed in additional TRNs in dauer animals by the mechanisms that 1) MEC-3::UNC-86 complex binds on the putative *cis*-regulatory element and activate *flp* gene expression. 2) As dauer formation progressed, a repressor may lose its function itself or by an unknown factor.

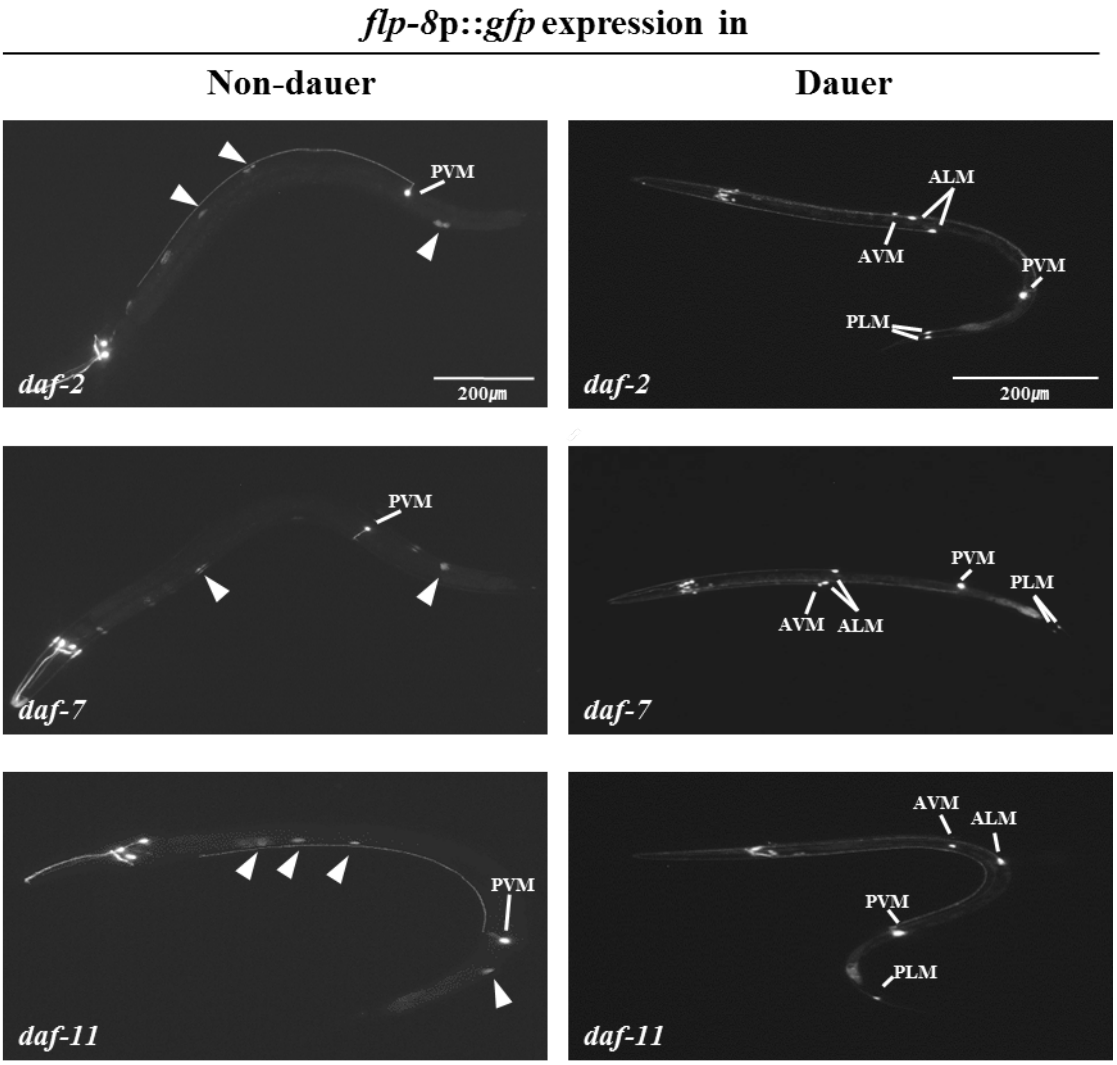

Figure 8. Expression patterns of *flp-8* in TRNs in mutants defective in dauer formation.

daf-2 insulin receptor, *daf-7* TGFβ superfamily, and *daf-11* guanylate cyclase (GCY) have been implicated for the major components acting in dauer formation pathways. Mutations in these genes did not alter the expression patterns of *flp-8* during dauer diapause, as shown in wild-type animals (**Figure 1**). Arrowheads indicate the sites of coelomocytes.

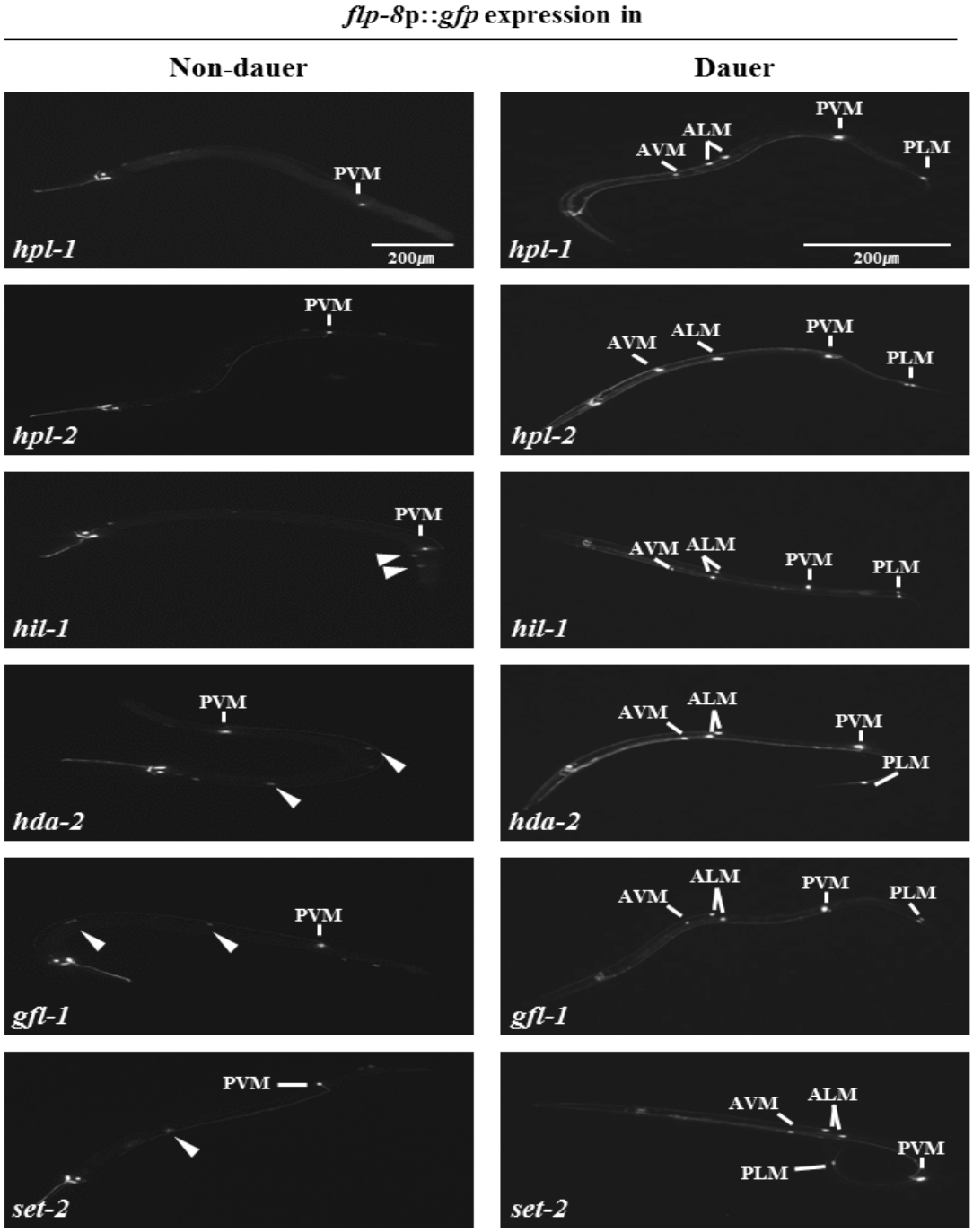

Figure 9. Expression patterns of *flp-8* in TRNs in animals carrying mutations in histone modification genes.

Both non-dauer and dauer mutant animals expressing *flp-8* show the same expression pattern of wild-type, indicating that chromatin-associated genes we tested may not regulate *flp-8* expression in TRNs. Arrowheads indicate the sites of coelomocytes.

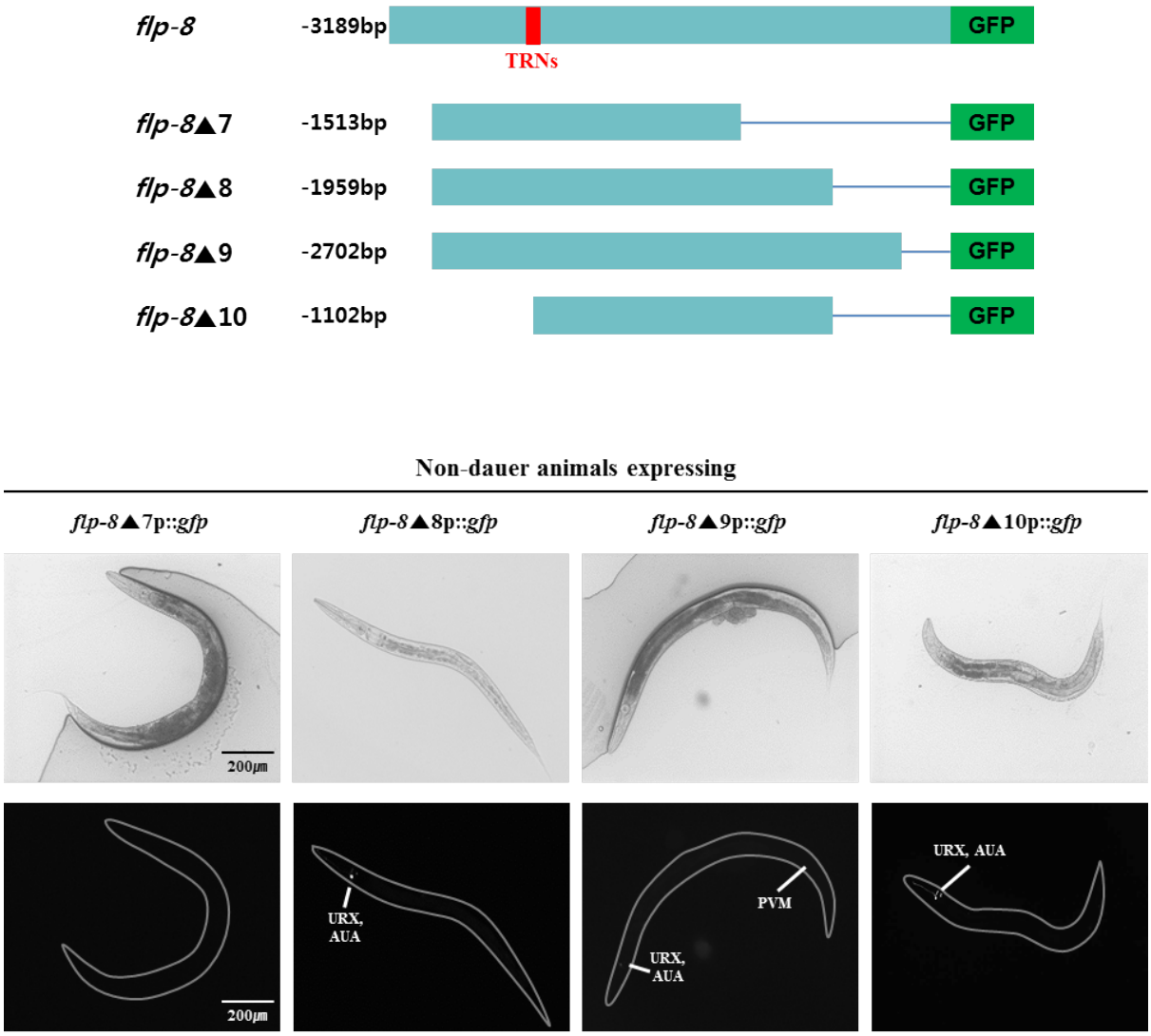

Figure 10. Search for the potential repressor-binding site in *flp-8* promoter.

None of transgenic non-dauer animals expressing GFP under the control of modified promoter ectopically express *flp-8* in additional TRNs, indicating that the deleted sequences do not contain the potential repressor binding site. These promoter analyses show the presence of distinct *cis*-regulatory elements for *flp-8* expression in: head neurons (URX and AUA, between ▲7 and ▲8), PVM (between ▲8 and ▲9)

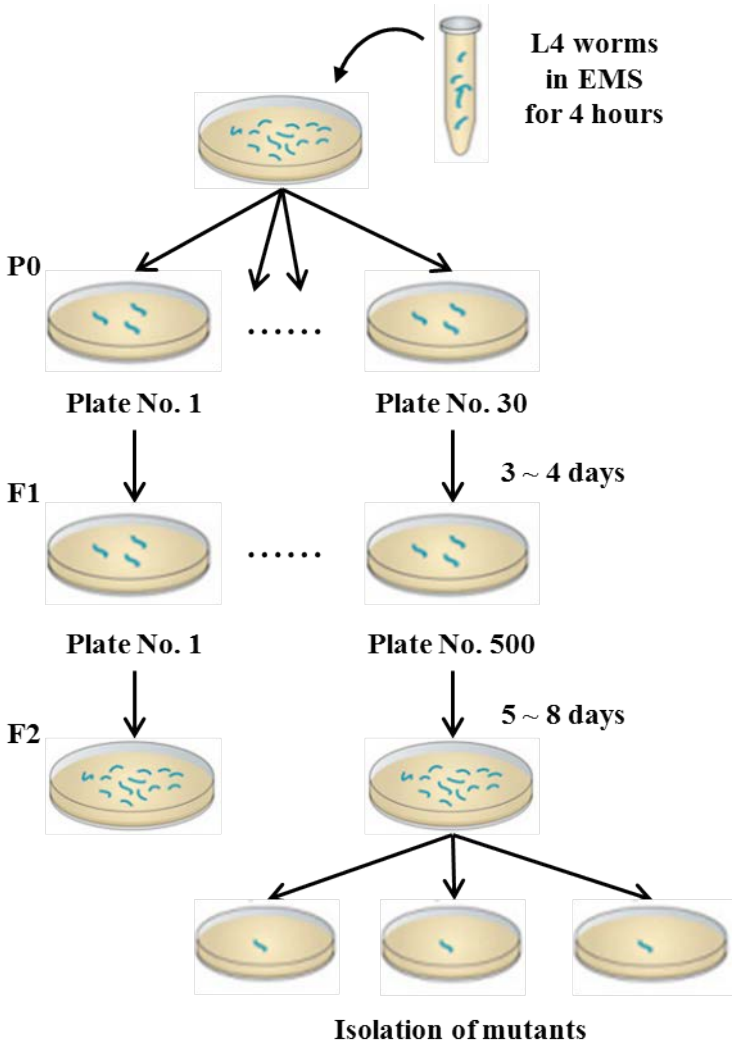

Figure 11. Forward genetic screen via EMS mutagenesis.

We performed EMS mutagenesis for genetic screen to find mutants that have defects in *flp*-8p::*gfp* expression in TRNs. The detailed protocol is described in **Materials and Methods**. We picked three of EMS-treated L4 animals as P0 in each plate and also transferred three of F1 animals per plate in total 500 plates (3,000 haploid genomes). *flp-8*p::*gfp* expression was examined in F2 animals and candidate mutants exhibiting unusual expression pattern were transferred to new plate.

Table 1. Construction of GFP plasmids.

Plasmid	Primer sequence (5'→3')	Subcloning site into pPD95.77
flp-8▲1	-	HindIII/HindIII
flp-8▲2	-	PstI/BamHI into PstI/BamHI
flp-8▲3	-	SnaBI/BamHI into HincII/BamHI
flp-8▲4	AAAAAGTCGACTCGTCAGTCTTCATCGGTCTTCAC	SalI/BamHI into SalI/BamHI
flp-8▲5	AAAAAGTCGACCTCTTCTCACATTTGCGGTC	SalI/BamHI into SalI/BamHI
flp-8▲6	AAAAAGTCGACCCGGAAACCACTCCGAAACAATTC	SalI/BamHI into SalI/BamHI
flp-8▲7		PstI/PsiI into PstI/MscI
flp-8▲8		PstI/HincII into PstI/MscI
flp-8▲9		PstI/XbaI into PstI/XbaI
flp-8▲10		NsiI/HincII into PstI/MscI
flp-4▲1	-	HincII/HincII
flp-4▲2	-	NsiI/KpnI into PstI/KpnI
*mec-3*p::*gfp*	Forward (AAAAAGTCGACTCGATTGTGCTATTTTCGGA) Reverse (TTTTTGGATCCAGTTCAAATGAAATAAATCAG)	SalI/BamHI into SalI/BamHI
*flp-8*$p_{mutation}$::*gfp*	Forward (GACCATGCCCCGGAAATTAC) Reverse (GTAATTTCCGGGGCATGGTC)	

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요 약 문

예쁜꼬마선충의 주변 환경 조건에 따른 장수유충 발달 유도를 조절하는 근본적인 분자적 기작 연구

동·식물 및 바이러스를 포함한 여러 생명체들은 혹독한 환경 조건에서도 장기간 동안 살아남기 위한 방법으로 비활동 상태인 휴면기에 돌입한다. 이는 예쁜꼬마선충(*C. elegans*)에서도 장수유충(dauer)이라는 형태로 존재하는데, 어떻게 어린 유충이 악조건에서 살아남기 위해 정상적인 생식 발달 과정을 거치는 대신 장수유충으로 발달하게끔 유도되는지에 대한 분자적 기작은 정확히 알려져 있지 않다. 그래서 우리는 장수유충으로 발달하는 과정에서 나타나는 유전자 발현 변화에 초점을 두고, 이러한 발현 패턴이 달라지는 유전자들 중 신경펩타이드를 암호화하는 유전자인 *flp-8*과 *flp-4*를 이용해 이 근본적인 문제 해결에 접근하고자 했다. *flp-8*과 *flp-4*는 비(非) 장수유충에서는 각각 하나의 기계적 자극 감지 세포에서 발현되지만, 장수유충일 때는 더 많은 기계적 자극 감지 세포에서 추가적으로 발현되는 것으로 알려졌다. 하지만 두 신경펩타이드 유전자가 장수유충의 형성에 어떻게 관여하는지, 그리고 어떻게 이 두 유전자의 발현 패턴이 장수유충 상태에서 달라지는지에 대한 분자적 기작 또한 알려지지 않았다. 그래서 우리는 먼저 두 유전자의 발현 패턴 변화에 관심을 가졌고, 기계적 자극 감지 세포의 생성 및 분화에 중요하다고 알려진 두 전사인자인 MEC-3와 UNC-86이 장수유충 상태에서 두 유전자의 추가적인 발현에 관여한다는 사실을 발견했다. 또한 두 유전자의 프로모터 서열에서 장수 유충의 기계적 자극 감지 세포에서의 유전자 발현에 관여하는 *cis*조절서열을 찾기 위해 프로모터 분석을 수행했고, 이에 해당하는 잠재적인 서열을 발견했다. 다음으로 우리는 유전자 발현 패턴 변화를 조절하는 후보 유전자들을 찾아보았고, 이 중 이전 연구 결과들을 바탕으로 장수유충 형성에 중요하게 관여한다고 알려진 유전자들과 후생유전적 조절에 관여하는 유전자들에 의해 두 유전자가 조절되는 지를 시험해보기로 했다. 이들 유전자 돌연변이체들에 형광단백질을 발현시켜서 발현 패턴의 변화 유무를 조사해보

았으나 발현 패턴 변화를 관찰하지 못했다. 그래서 우리는 정상 유충에 돌연변이 유발 물질을 처리하여 두 유전자 발현 패턴의 변화에 영향을 미치는 유전적 돌연변이를 새롭게 찾고자 했으나, 아직까지 그런 가능성이 보이는 돌연변이체는 찾지 못했다. 본 연구를 통해 장수유충에서 나타나는 신경펩타이드를 암호화하는 유전자 발현 패턴 변화가 두 전사인자인 MEC-3와 UNC-86에 의해 조절됨을 밝혔고, 앞으로의 연구를 통해 두 유전자의 프로모터에 작용하는 잠재적인 조절 인자 및 발현 패턴 조절에 관여하는 새로운 유전자를 찾을 것으로 기대한다.

핵심어 : 장수 유충, 신경펩타이드, 기계적 자극 감지 세포