RESEARCH ARTICLE

Uncovering *unc-5*: A Single-Nucleotide Polymorphism Affecting *C. elegans* Locomotion

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Abstract

This study investigates an unknown mutation (A) in *C. elegans*, focusing on its genetic location, phenotype, and physiological effects. *C. elegans* is a common model organism due to its sequenced genome, well-described development, and ease of lab maintenance. The research employs several techniques, including two-factor-, three-factor crosses, complementation tests, and microscopic visualization. Initially, locomotion and defecation assays revealed a significant difference in movement between the mutant A strain and the wild type, with the mutant showing a severe impairment in locomotion, but no change in defecation. Two-factor crossing determined that the mutation is located on linkage group IV (LGIV). Further analysis using three-factor crossing pinpointed the mutation's location to be approximately 1.82 cM away from *dpy-13* and 6.82 cM from *lin-54* on LGIV. Based on these locations, the candidate genes were narrowed down to *unc-5* or *unc-43*. Complementation testing with known mutant strains revealed that the unknown mutation is in fact *unc-5*. This was supported by neuronal visualization, which showed that the AVM neurons of the mutant strain grow ventrally, instead of toward the ALM neurons as in the wild type. Additionally, in the mutant strain, the dorsal nerve chord is missing, and no axons grow dorsally. These observations align with previous studies on *unc-5* mutants. In conclusion, the study successfully identified the unknown mutation as *unc-5* through a combination of genetic mapping techniques and phenotypic analysis. The findings highlight the role of *unc-5* in neuronal development, specifically in axon guidance and the formation of the dorsal nerve chord, which are necessary for normal locomotion.

Introduction

The nematode worm *Ceanorhabditis elegans* is one of the more common model organisms used for genetic studies due to its numerous benefits. It is about 1mm in length, has a life span of nearly 2.5 weeks and can reach the adult phase in about 3 days. It has 6 chromosomes which are called linkage groups (LG) where five are autosomal (I, II, III, IV, V) and one is a sex chromosome (X). Males have single X whereas hermaphrodites have double X. The latter can reproduce either by self-fertilization or by cross-fertilization with males. It is also important that its whole genome has been sequenced, and its developmental process has been described in detail by previous studies. Its growth and maintenance in the lab are easy, it feeds on *Escherichia* coli and can give many offsprings. The scientific community

around C. elegans is well connected, collaborative and online tools such as WormBase provide extensive information (Rose J. K. et al., 2019).

Some mutation phenotypes that provide key insight into biological processes are for example mutations in the *dpy* gene that result in worms with short and chunky body shape (Riddle et al., 1988). Mutations in the *bli* gene causes a distinct anomaly in the worm's cuticle, resulting in the formation of fluid filled blisters along the body (Rose A. M. et al., 1991) and lastly mutations in *unc* lead to worms with abnormal movement patterns or uncoordinated movement. For example, the mutation *unc-86,* associated with motor and sensory neurons, lead to reduced sinusoidal body waves and uncoordinated movement. Mutations in *unc-24*, which is part of the mechanosensory channel complex can enhance the Mec phenotype, which is a mechanosensory condition where the worm does not react to gentle touch.

In general, mutations in these genes lead to defects in the development of the nervous system such as cell migration and axon guidance. Some genes that control these mechanisms are *unc-5, unc-6* and *unc-40*. Specifically, *unc-5 and unc-40* encode receptors for the protein UNC-6 that acts as a guidance cue. Mutations in these genes result in disrupted neuronal circuits. For instance, mutations in *unc-5* can prevent axons from growing dorsally as they normally do or even lead to them growing ventrally. (Wadsworth W. G. et al., 2016)

These known mutations can give the ability to researchers to identify the location of other mutations in a chromosome. Such a technique is three-factor crossing that uses two linked morphological markers. By crossing a mutation to a strain containing these markers, isolating the progeny and observing their recombination patterns and frequency, it is possible to find a mutation's location relative to these markers. It is also possible to use green fluorescent protein (GFP) as a reporter gene, and since the animal is transparent, it can help cell visualization in live cells. Other dominant markers such as ol-6 (su6000) (pRF4) help to find transgenic animals by making them move in a corkscrew pattern and lastly, tissues specific promoters can be used to lead GFP into specific tissues we want to study. For instance, snb-1::gfp, which is a pan-neuronal gfp reporter, can be used to describe nervous system defects in uncoordinated animals (Fay D., 2006).

The aim of this study is to determine the genetic location of an unknown mutation, in which linkage group it is located and its specific location. Also, we aim to describe its phenotype and how it affects the worm's physiology.

Materials and Methods

Worm strains

Increased *X* chromosome loss (*him-5*), body shape effecting (*dpy-5* and *dpy-13*), uncoordinated movement (*unc-5* and *unc-43*), unknown (*A*), single-nucleotide polymorphism (SNP) mutation-carrying and Wild type (*N2*) strains of *C. elegans* nematodes were used during the experiment provided by the corresponding University of Gothenborg laboratory leaders (Delaney Kaper and Uros Radovic). The strains were grown on solid NGM media, fed with *E. coli* strain and presented in small petri dishes.

Maintenance and general steps

The day-to-day maintenance of the C. elegans strains have been done by picking 3 worms containing at least 1 hermaphrodite and placing them on a separate petri dish with enough

bacteria for 3-4 days on NGM solid media then closing the lid of the container, sealing it with parafilm and storing at 20° (Stiernagle T., 2006). The general steps of the experiment have been done similarly to the method described but with different amounts of worms picked.

Locomotion and Defecation assay

For the Locomotion and the Defecation assays 2 plates were used with A and N2 strains, 3 worms each plate and evaluated independently.

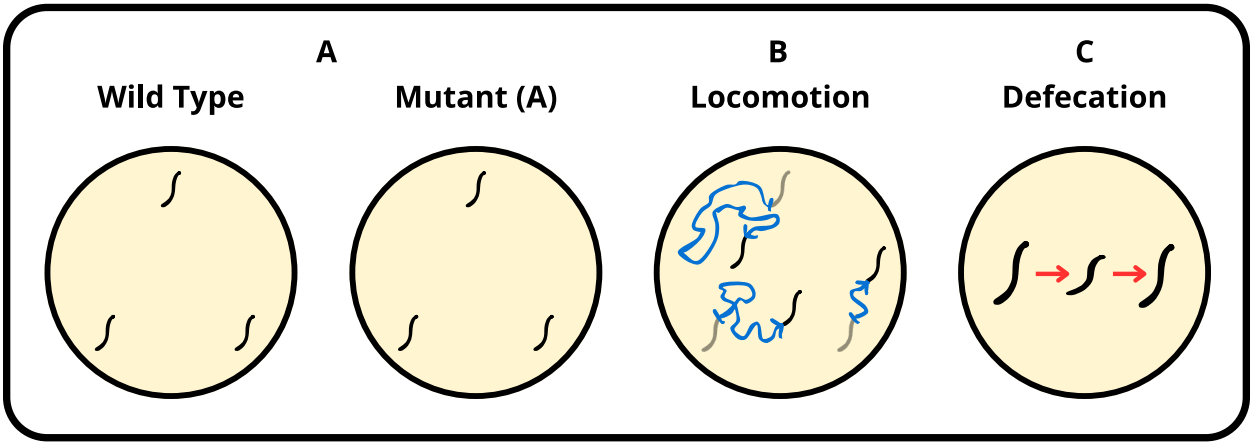

Fig. 1. Locomotion and Defecation assay in principle. (A) For the assays we used 2 plates that are assigned to either Mutant A or Wild type strains containing 3 worms each. **(B)** For the locomotion assay each worm's movement was marked every minute for 30 minutes on the bottom of the plate and connected to form a path that has been evaluated later as millimeters travelled. **(C)** Defecation in worms happens with a distinct movement phenomenon. They stop and contract for a short period of time and then continue their movement. The time between 2 defecations has been observed and noted for each worm 3 times.

Two-factor crossing

Accurately assessing if a mutation is present in specific chromosomes is measured with two-factor crossing.

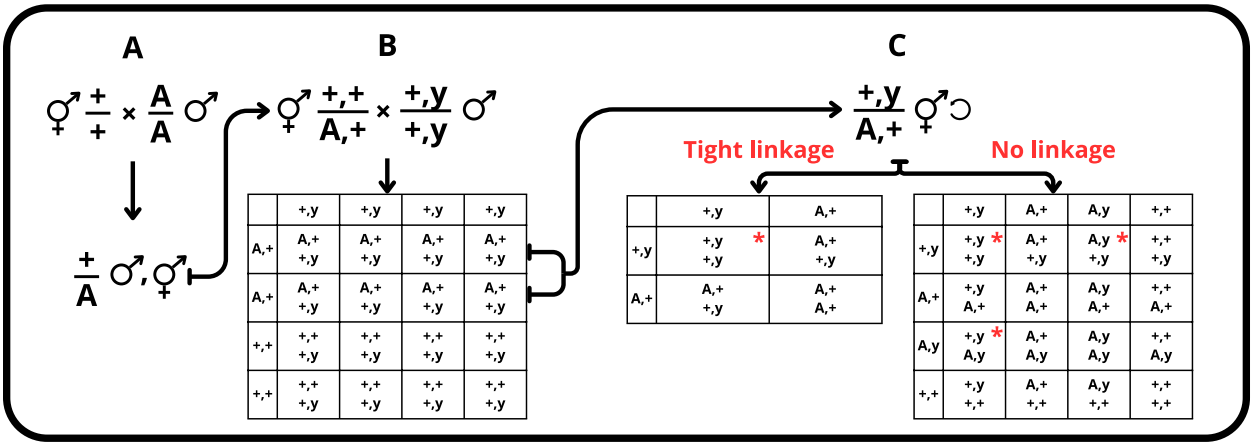

Fig. 2. Two-factor crossing in principle. (A) The experiment starts with creating a P heterozygous strain of our unknown mutation *A*. **(B)** Crossing heterozygous *A* with homozygous

markers (*y* which in our case is *dpy-5* and *dpy-13* separately) yields double heterozygous and marker heterozygous with wild type homozygous F1 progenies with a 50-50% chance of both offsprings. **(C)** Trying to self-fertilize the offsprings from (B) is going to yield results that either have no or some mutation carrying F2 progenies. We should continue the research with those that show mutation A phenotypes. If *A* is not located on the same linkage group (no linkage) as a marker then the genotypical distribution follows Mendel's unmodified dihybrid ratio. If both are on the same linkage group (tight linkage) the genotype would follow a non-Mendelian ratio shown in the figure. Picking only the marker phenotype showing F2 progenies and observing their F3 progenies will determine whether a marker and mutation A are on the same linkage group (no or only a few percentages of the F3 progenies should show *A* phenotype too), or if they are on different linkage groups (about 66% of the progenies should show the presence of mutation A).

Three-factor crossing

A statistically sufficient method for determining where a mutation lies between two markers (provided that the mutation and the markers are on the same linkage group) is three-factor crossing. With methods demonstrated in **fig. 2.** Preparation for the experiment requires 2 strains. A double homozygous for the 2 markers and a heterozygous mutant A strain.

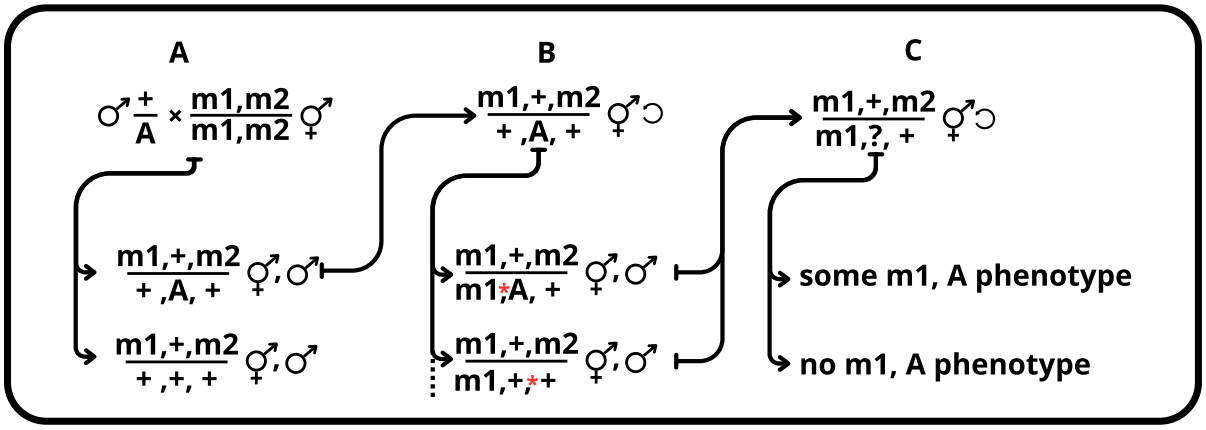

Fig. 3. Three-factor crossing in principle. (A) Heterozygous mutation A strain crossed with double homozygous for *m1* and *m2* (markers, in this case *lin-54* and *Dpy-13* both in the same experiment) strain creating triple heterozygous and double heterozygous for markers F1 progenies with 50-50% probability. **(B)** Allowing the F1 progenies to self-fertilize separately will show F2 progenies containing *A* and F2 progenies that do not. The experiment is continued with the batch showing any number of mutation A. From these F2 progenies one of the markers is chosen and only that marker phenotype is selected to create F3 progenies. This is important because single marker homozygous strains have undergone recombination between *m1* and *m2*. **(C)** In the F3 progenies we determine which side of mutation A could the recombination have happened to. Separate F3 progenies self-fertilize, and they are evaluated based on whether *m1* and *A* but non *m2* phenotype is present or not. If present, it means the recombination happened between *A* and *m2*. If absent, it means the recombination happened between *m1* and *A*. Measurement is made in dozens of plates and the place of mutation *A* is determined by the ratio of the recombination places. *M1* marker is closer to the mutation than the other marker if recombination between *m1* and *A* has happened fewer times than between *m2* and *A*.

Complementation

If multiple genes are found at the place of the genome where the three-factor crossing pointed it will be required to narrow down the possible gene affected to one. In the complementation test heterozygous mutant A is crossed with homozygous candidates separately and checked if their male progeny showed signs of the A phenotype or not. Phenotype showing means that the candidate and the mutation affect the same gene therefore there are no complementation. If phenotype is not or very rarely shown, then the two mutations complement each other, and they are affecting different genes.

Neuron visualization

To visualize mechanosensory and motor neural networks and determine any neuronal growth defects, 5 worms are picked on 2% agarose plates and paralyzed with 5µl of 100µM levamisole. To score their mechanosensory (ALML/R, PLML/R, AVM and PVM) and motor neurons (DD, VD) we used *Pmec-7::GFP* and *Punc-25::GFP*, respectively, with an epifluorescence microscope at 400x magnification.

Results

Locomotion and defecation assays

The assays were important in having a general understanding of mutation A and highlighted differences between it and the Wild type strain.

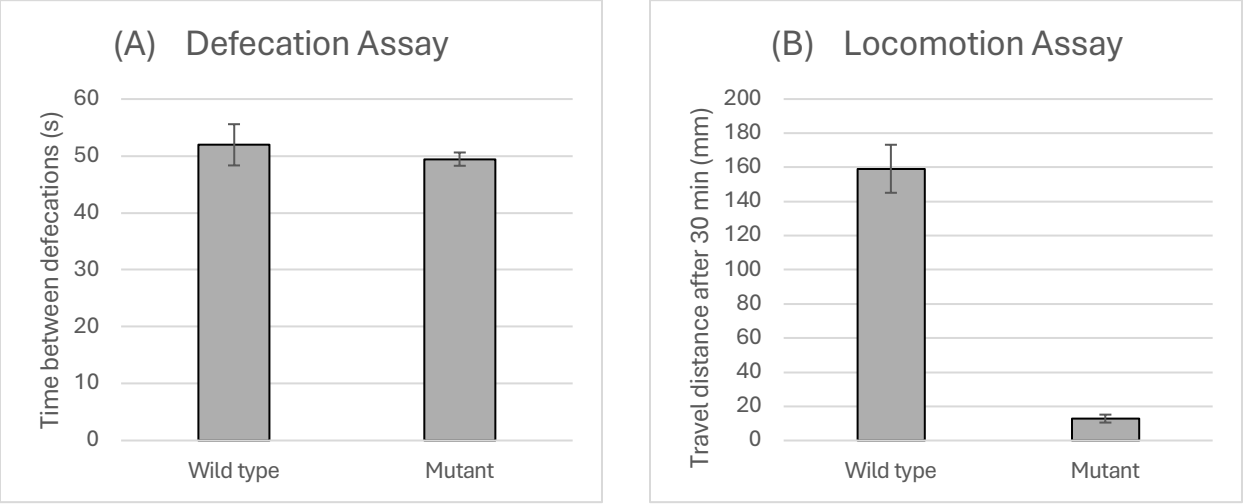

Fig. 4. Defecation and Locomotion assays. (A) The mutant A strain's defecation assay showed little to no difference in the time between defecations compared to Wild type which is indicative of no behavioral changes connected to defecation. **(B)** The locomotion assay on the other hand showed significant difference as the travel distance of mutant *A* strains have presented an order of magnitude difference which resulted in low p-value therefore high statistical significance indicative of massive defect in locomotion related growth.

Two-factor crossing

Markers used for the two-factor crossing were *dpy-13* (located on LGIV) and *dpy-5* (located on LGI). The results of the previously mentioned two-factor crossing showed that out of 20 assessable F3 plates only 4 (20%) showed *dpy-13* and mutation A phenotypes and 16 did not show *A*, only *dpy-13* phenotype. This indicates that mutation *A* and marker *dpy-13* are on the same linkage group based on **Fig. 2**. According to the previously known fact that *dpy-5* is on a different linkage group than *dpy-13*, this foreshadowed the result of the *dpy-5* experiment. Indeed, out of 18 viable samples, 13 (72%) showed *dpy-5* and mutant *A* phenotypes, further proving that mutant *A* is not on LGI but LGIV.

Three-factor crossing

Dpy-13 is considered by the scientific community to be the center of LGIV in genetic mapping. Out of 220 F3 progenies only 4 showed to be mutant *A*, *dpy-13* but not *lin-54* phenotypes and 15 showed mutant *A*, *lin-54* but not *dpy-13* phenotypes. The first calculations show that *lin-54* is placed about 8.64cM from *dpy-13* and that mutant *A* is about 1.82cM away from *dpy-13* and 6.82cM away from *lin-54*.

Phenotypic visualization using GFP markers

The mechanosensory neurons and motor neurons were visualized using Pmec7::gfp and Punc-25::gfp respectively. The results were captured using an epifluorescent microscope as shown below.

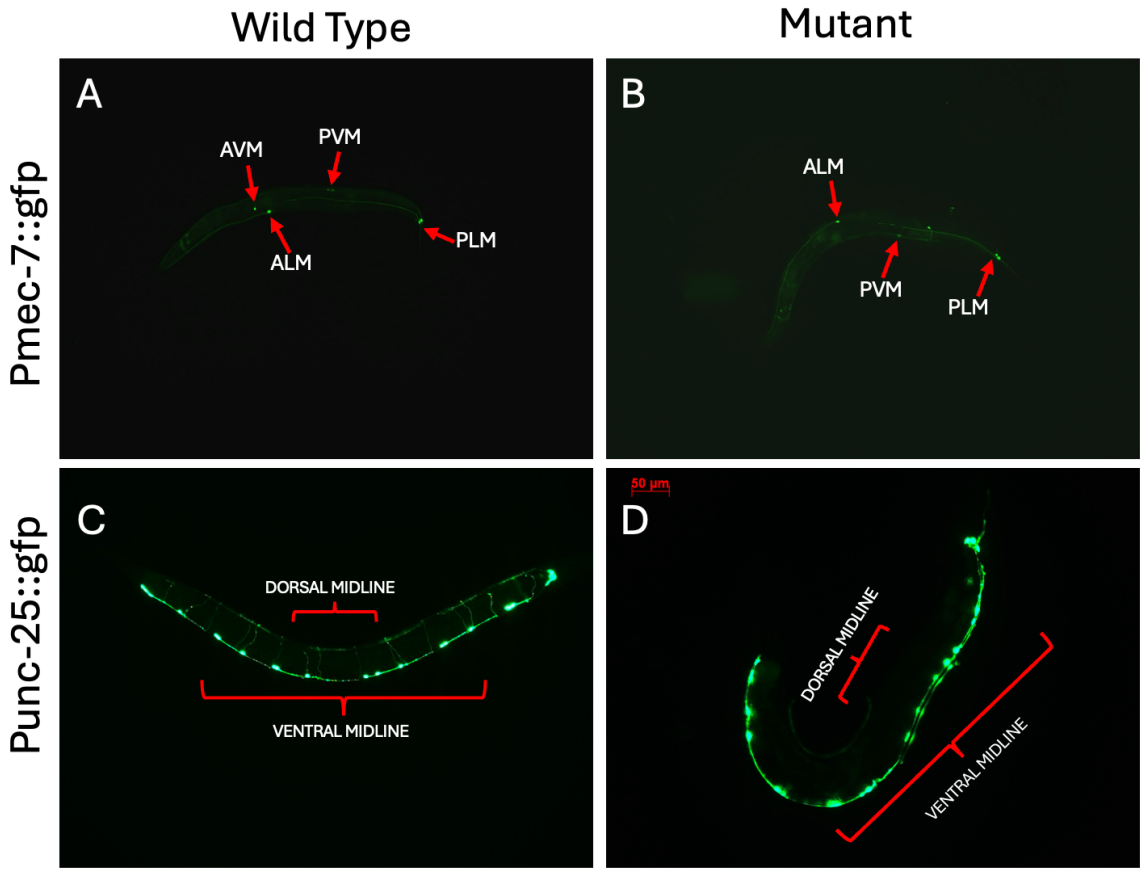

Fig. 5. Visual representation of mechanosensory and motor neurons in wild type and mutant worms. (A and B): Depiction of mechanosensory neurons in wild type N2 and mutant A worms respectively using Pmec-7::gfp. **(C and D)**: Depiction of motor neurons in wild type N2 and mutant A worms respectively using Punc-25::gfp. Images captured with epifluorescence microscope (400X).

Complementation

During the complementation test, the observed progeny in males for the *unc-45* x A cross was wild type, whereas the observed male progeny in the *unc-5* x A cross was mutated.

Genetic map

Based on the previous experiments we show an illustration of the mutant's genetic location.

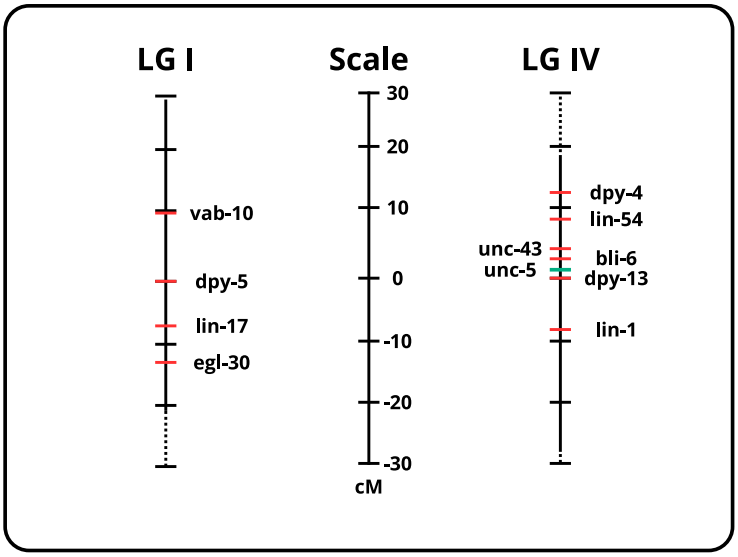

Fig. 6. Genetic mapping. The two possible linkage groups where the mutation A could be located shown with a scale using centimorgans. **Linkage group I** with *vab-10*, *dpy-5*, *lin-17*, *egl-30* markers. **Linkage group IV** with *dpy-4*, *lin-54*, *unc-43*, *dpy-13*, *lin-1* and our concluded mutation, *unc-5*.

Discussion

The experimental process we followed proved that mutant A has significant phenotypic differences with wild type worms. By using a locomotion and defecation test we proved that the mutant strain shows significant impairment in its movement compared to wild type. This leads to the initial thought that the mutation affects a gene that is important for motor functions of the animals (**Fig. 1B**). However, we could not show any differences in their defecation patterns or times between defecation (**Fig. 1A**).

After this we did two-factor crossing to identify in which linkage group the mutation is located. We suspected from previous knowledge that it is either on LGI or LGIV. The results showed that our mutation is on LGIV.

To find the specific mutation, we had to narrow down our candidates by finding the specific location of the mutant on LGIV. This was done by implementing three-factor crossing. The results showed that it is located 1.82cM away from *dpy-13* and 6.82cM away from *lin-54*. By looking on

WormBase to find out which genes are in that area, we conclude that it could be either *unc-5* or *unc-43*.

Results from the complementation test finally verified that the unknown mutant A is in fact *unc-5* since there is no complementation between the two strains used in the crossing.

During the microscopy visualization, Pmec-7::gfp worms showed significant differences in their neuronal phenotype compared to wild type. Specifically, while in wild type the AVM grows towards the ALM (**Fig. 4A**), that characteristic is absent in mutants and seems to only grow ventrally. Also, the latter seems to be absent in mutant worms (**Fig. 4B**). When using Punc-25::gfp we notice that the wild type strains have a normal development with neurons along the ventral midline and axons growing dorsally (**Fig. 4C**). However, in the mutant strain we notice that the neurons growing along the dorsal midline are missing completely and there are no axons growing dorsally (**Fig. 4D**). This specific characteristic was also observed by Brenner S. in 1973 where they did similar experiments and found that the dorsal nerve chord was absent in unc-5 mutants. Previous studies also show that in unc-5 mutant worms all touch cell axons extend dorsally and AVM and PVM adapt new trajectory moving dorsally instead of ventrally (Hemelin M. et al., 1993)

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