Caenorhabditis elegans: Determining the relationship between *srf-6* and *nsy-1* in *C. elegans*

A Major Qualifying Project Report
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Abstract

A major component contributing to the ability of nematodes to survive in a diversity of environments may be their ability to alter their surface antigen composition. In doing this, free-living nematodes may be able to avoid pathogenic microbial infections and parasitic nematodes may be able to avoid host's immune response, both of which rely on recognizable antigens and epitopes located in glycoproteins on the surface of the worm. Two genes, *srf-6* and *nsy-1*, have been shown to affect surface antigen presentation and prior evidence suggests that they are the same gene. This MQP aims to employ complementation tests to investigate whether *srf-6* and *nsy-1* mutations affect the same genetic function.

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Introduction and Background

A major component contributing to the ability of nematodes to survive in a diversity of environments is their ability to alter their surface antigen composition. In doing this, free-living nematodes may be able to avoid pathogenic microbial infections, and parasitic nematodes may be able to avoid a host's immune response, both of which rely on recognizable antigens and epitopes from glycoproteins on the surface of the worm (Politz & Philipp, 1992; Blaxter *et al.* 1992).

One way in which nematodes are able to switch their surface composition is through larval stage molting, during which the worms make a new external cuticle that replaces the old one, which is shed (Singh & Sulston, 1978; Politz & Philipp, 1992).

Parasite lifestyles can be complex, involving both free-living and parasitic stages, or movement between different host species or host tissues. However, all nematodes follow the same series of developmental stages. During the postembryonic life cycle, during which the nematode matures and grows, nematodes molt 4 times. The molts mark the transitions from L1-L4 and to adult (Singh & Sulston, 1978).

During post-embryonic development, a primarily collagenous extracellular matrix, termed the cuticle, forms an external layer that protects nematodes (Kramer, 1994). The cuticle can be subdivided into multiple layers. The outermost layer is the epicutule, which is composed mainly of lipids (Blaxter, 1993a). The surface of the epicutule is exposed to the environment, and is covered by a 5-20nm coat of glycoproteins known as the surface coat (Blaxter *et al.,* 1992). Underneath the surface coat and the epicuticle are the cortical, medial, and basal cuticle layers, which are primarily collagenous. Beneath the cuticle is the first cellular layer of the body wall, the hypodermis, which synthesizes the collagens of the cuticle layers, and in parasitic nematodes, may secrete surface coat proteins (Blaxter *et al.,* 1992; Page *et al.,* 1992).

In order to execute the developmental stage transitions, the underlying hypodermal layer forms a new cuticle underneath the old cuticle, which is shed at each larval stage transition. This process is called molting, and is accompanied by a period of several hours during which nematodes do not feed or move, termed lethargus. The new cuticle then replaces the function of the old cuticle from the previous larval stage (Singh & Sulston, 1978). After the shedding of the old cuticle, stage-specific surface molecules appear.

Nematode surface composition has also been observed to change within a larval stage. When infecting a new host, the surface antigen of observed parasitic nematodes changes too quickly to have been accounted for solely by larval stage molting. For example, *Trichinella spiralis* has been observed to alter its surface antigen presentation after the binding of effector cells from the host's immune system (Anderson, Morran, & Phillips, 2010).

This stage-specific presentation and dynamically changing surface composition may serve as a defense against host immune responses, in the case of parasitic nematodes, and pathogenic bacteria and fungi, in the case of non-parasitic nematodes (Politz & Philipp, 1992; Blaxter *et al. 1992*). As the mechanisms controlling changes in surface composition are not well understood, it is important to study them.

It is significantly easier to culture the free-living nematode *Caenorhabditis elegans* than it is to culture the parasitic nematodes, which often have a vector and a different definitive host. The entire lifecycle of *C. elegans* is observable on a plate, whereas parasitic nematodes require co-culturing with their natural host organism or organisms to view their entire life cycle (Gagliardo, 2002).

C. elegans has been shown to execute surface antigen switching. In particular, it has been found that multiple genes control a larval-stage specific surface antigen switch that is inducible by chemical signals from an extract of spent nematode liquid culture medium (Grenache *et al.* 1996). Normally, wild type L1s only display a certain surface epitope at the L1 stage, as evidenced by

immunofluorescence using a surface specific monoclonal antibody mAb37. When exposed to the extract of spent culture medium, the epitope continues to be displayed at later larval stages. Thus this change represents an inducible larval display (ILD).

Using the L1-specific monoclonal antibody mAb37 in mutant screens, mutant *C. elegans* were isolated that also show expression of the L1-specific epitope constitutively at all larval stages, even in the absence of the culture medium extract (Hemmer *et al*., 1991; Grenache *et al*., 1996). We refer to this mutant phenotype as constitutive larval display (CLD).

Chemosensation is required for many *C. elegans* behaviors, including chemotaxis, thermal detection, dauer formation, and mate finding (Bargmann, 2006). These behaviors depend on a chemosensory system that can detect volatile (olfactory) and water-soluble (gustatory) signals (Bargmann, 2006). This system uses chemosensory neurons, which extend dendritic sensory processes in contact with the aqueous environment (Bargmann, 2006). Of these neurons, gustatory neurons sense vital water-soluble attractants. In contrast, olfactory neurons detect volatile odors, such as alcohols or esters, which are natural products of bacterial metabolism. Olfactory neurons are thus more likely to contribute to long-range chemotaxis to a food source. The *C. elegans* genome contains multiple G-protein-coupled receptors, which in a few cases have been shown to be expressed in the olfactory neurons, and function as receptors for diacetyl and other molecules (Lans *et al*., 2004; Bargmann *et al.,* 1993).

Each chemosensory neuron species is bilaterally symmetrical, and the left-right paired neurons are structurally similar. In particular, in the amphid wing "C" (AWC) neurons, in wild type, either the left AWC (AWCL) or the right AWC (AWCR) stochastically expresses the G protein-coupled receptor STR-2 during development through a process of cell fate determination (Wes & Bargmann, 2001; Sagasti *et al.*, 2001). We will refer to the STR-2-expressing state of an AWC neuron as AWC+. This asymmetric cell fate is instrumental in olfactory chemosensation, in that it

enables *C. elegans* to both detect more odors and with higher resolution (Wes & Bargmann, 2001).

The *nsy-1* gene has a prominent role in AWC cell fate. Mutations in *nsy-1* result in a mutant AWC phenotype; for example, *nsy-1 (ky397),* which carries a nonsense mutation in the C terminal domain, expresses both AWCL+ and AWCR+, which we will refer to as the 2AWC+ phenotype (Sagasti *et al.,* 2001). *Nsy-1* mutants have weakened olfaction due to their mutant 2AWC+ phenotype (Sagasti *et al.,* 2001;Wes & Bargmann, 2001).

In previous experiments, it has been shown that ILD requires the activity of three genes, *che-3*, *osm-3*, and *tax-4* (Olsen *et al*., 2007). These genes are also essential for the function of *C. elegans* chemosensory neurons (Wicks *et al.,* 2000; Bargmann 2006). In chemotactically defective *che-3*, *osm-3*, and *tax-4* mutants, ILD was defective (Olsen et al., 2007). In addition, *srf-6* mutants are also chemotactically defective and display CLD (Olsen et al., 2007). Double mutants of *che-3, osm-3,* or *tax-4* with *srf-6* blocked the CLD of *srf-6* (Olsen et al., 2007). Double mutants of *srf-6* and other dauer-constitutive mutations enhanced constitutive dauer formation, suggesting that *srf-6* is required in parallel with the dauer-constitutive genes to prevent dauer formation under inappropriate conditions—a process which requires chemosensation (Olsen et al., 2007).

In addition to the evidence that ILD relies on proper chemosensory neuronal function, whole genome sequences of three different *srf-6* mutants were determined, and each one had a different mutation in the *nsy-1* gene (Politz *et al.,* unpublished). In addition to the genome sequences, one of the sequenced mutants, *srf-6(yj13)* expresses the 2AWC+ phenotype characteristic of *nsy-1* mutants. Additionally, *nsy-1 (ok593)* and *nsy-1(ag3)* mutants show the CLD phenotype characteristic of *srf-6* mutants. Exact locations of mutations can be seen in figure 1 below. These results suggest that *srf-6* and *nsy-1* are the same gene.

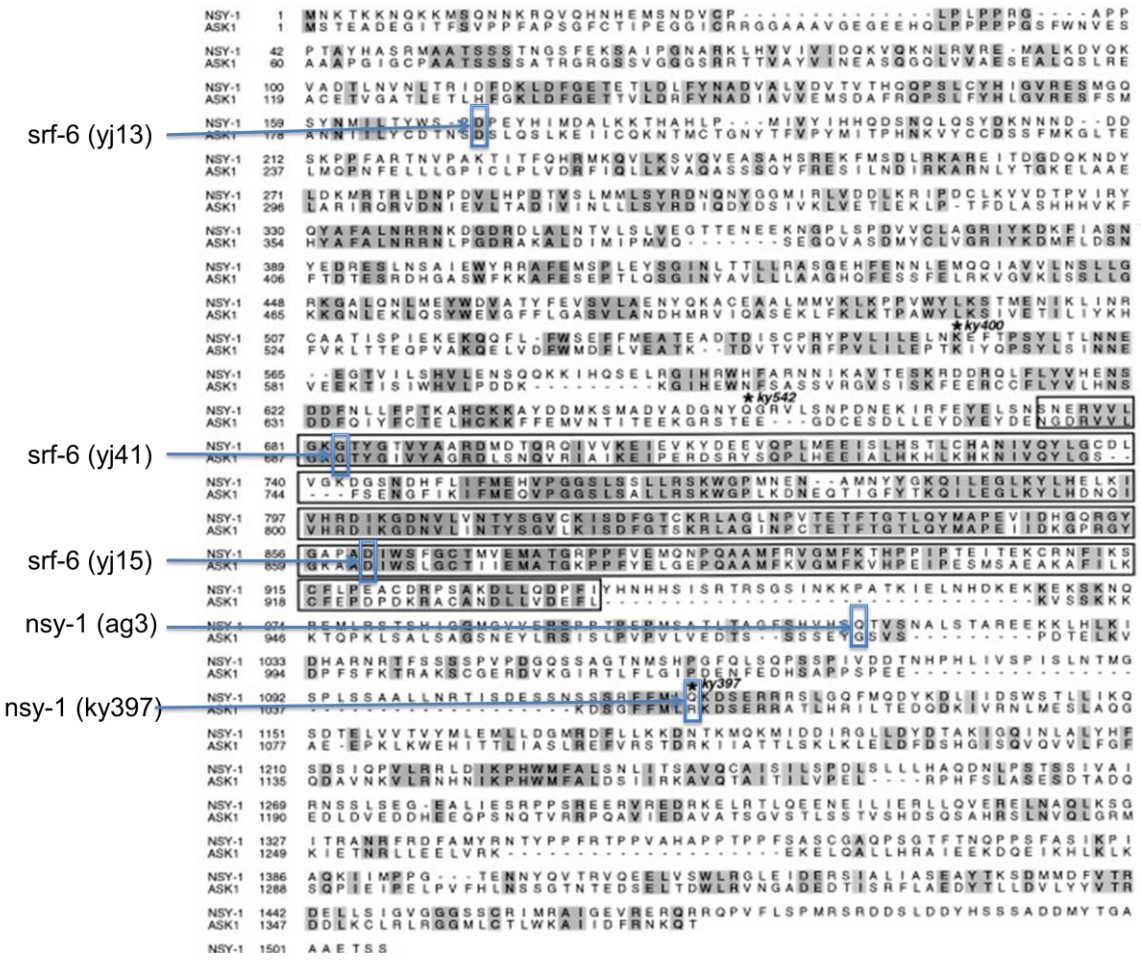

Figure 1: Genomic Sequence of *nsy-1* (Sagasti *et al*., 2001). The figure shows the entire genomic sequence of the *nsy-1* gene in comparison with *ASK1* (the human homolog of *nsy-1*), along with pertinent mutations. The exact location of the *nsy-1* mutations *ky397* and *ag3* are shown, as well as the *srf-6* mutations *yj13, yj41,* and *yj15. Yj13* is a missense mutation from G to A (D170N). *Yj41* is a missense mutation from G to A (G683E). *Yj15* is a missense mutation from G to A (G860E). *Ag3* is a nonsense mutation from C to T (Q1013X). *Ky397* is a nonsense mutation from C to T (Q1120X).

Nsy-1 encodes a MAP Kinase Kinase Kinase (MAPKKK) in the p38 pathway in *C. elegans* (Sagasti *et al., 2001*; Tanaka-Hino *et al.,* 2002*)*. The serine/threonine protein kinase encoded by *nsy-1* phosphorylates and activates downstream *sek-1*, which phosphorylates and activates the *pmk-1*-encoded p38 MAPK (Tanaka-Hino *et al*., 2002). The activation of the p38 pathway results in the stochastic repression of STR-2 expression in one of the 2 AWC neurons, among other effects such as egg

laying (Tanaka-Hino *et al*., 2002). The pathway downstream of *nsy-1* itself can be seen in figure 2 below.

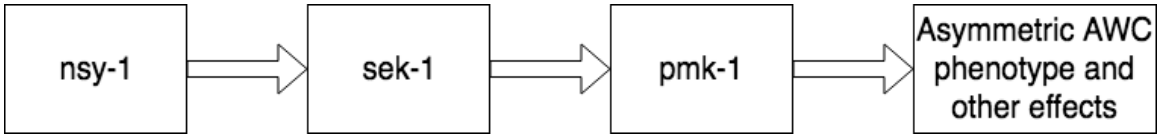

Figure 2: *nsy-1's* Role in the p38 Pathway. *Nsy-1* encodes for a serine/threonine MAPKKK that activates the MAPKK encoded by *sek-1*, which phosphorylates and activates the MAPK encoded by *pmk-1*. The transcription factor itself is unknown.

ASK1 is the mammalian gene most similar in coding sequence to *nsy-1*, and it encodes a MAPKKK that activates the p38 pathway and induces apoptosis in response to TNFα (tumor necrosis factor α) (Tobiume *et al.,* 2001; Sagasti *et al.,* 2001). Important to note is that *ASK1* homodimerizes, and that only the dimeric form of *ASK1* is active (Bunkoczi *et al.,* 2007). The ramifications of this dimerization will be described in more detail in the discussion section.

Project Purpose

The subject of this MQP project will be to test whether *srf-6* and *nsy-1* mutations affect the same genetic function, in order to obtain further evidence that they are the same gene. This will be accomplished by performing genetic crosses of *C. elegans* that will be tested for the 2AWC+ phenotype.

In a complementation test, *trans* double heterozygotes for the two mutations are tested for phenotype. There are two common results to this test. If the double heterozygote expresses a WT phenotype, then the genes complement each other; each mutant genome provides the WT version of the opposing mutation, indicating that the mutations affect different genetic functions. If the double heterozygote expresses a mutant phenotype, then the genes fail to complement and the mutations affect the same genetic function and likely occur in the same gene. An example of complementation can be seen in figure 3.

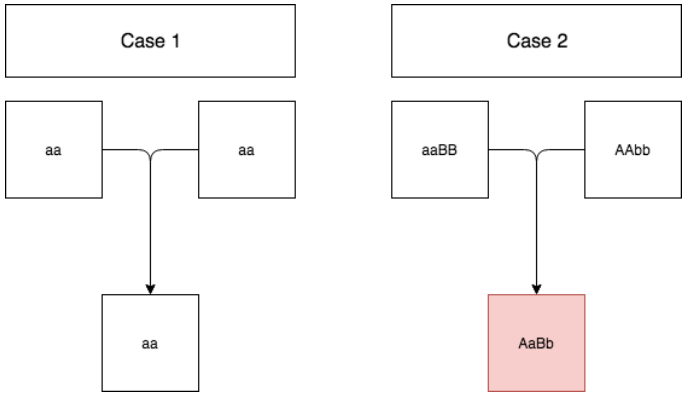

Figure 3: Complementation Example. In case 1, the mutations do not complement. In case 2, the mutations do complement. In case two, the wild-type F1 phenotype is indicated in color.

In case 1, the mutant phenotype (white box) is due to a mutation in the same gene from both parents. In case 2, the mutant phenotype is due to mutations in different genes from each parent. In case 1, the F1 progeny would be homozygous for the mutation, and thus would all display the mutant phenotype. In case 2, the F1 progeny would have a heterozygous mutation at either locus, and thus the progeny would all display the wild type phenotype (red box).

Methodology

Stock Maintenance

All *C. elegans* stocks were cultured at 16°C on 60 mm petri dishes containing 10 mL of NGM agar unless otherwise noted (Stiernagle, 2006). Plates were supplied with a central lawn of *E. coli* strain OP50.

List of Stocks Used

CX4998 *kyls140 (I) str-2*::GFP; *nsy-1(ky397)*

AT27 *kyls140 (I) str-2*::GFP*; srf-6(yj13)*

VC390 is a strain of *nsy-1 (ok593)*

AT18 is a strain of *srf-6 (yj13)*

AT28 *kyls-140 (I) str-2::GFP; unc-4 srf-6*

Producing *C. elegans* Male Stocks

C. elegans has two natural sexes: XX hermaphrodites and XO males. Hermaphrodites produce a limited amount of sperm for self-fertilization to produce offspring, (Brenner, 1974). When a male mates with a hermaphrodite, the hermaphrodite will use the male's sperm preferentially to its own. Consequentially, after mating, the hermaphrodite will only produce cross-progeny, as seen in figure 4, which will either be XX hermaphrodite or XO male in a 1:1 ratio. In this way, male mating is essential in facilitating the movement of mutations between strains.

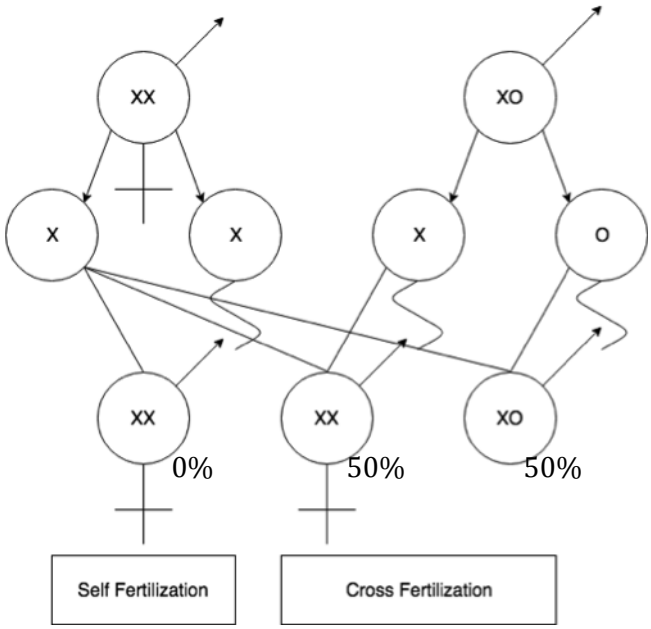

Figure 4: Male Sperm Preference. As seen in the figure, male sperm will outcompete the hermaphroditic sperm when creating progeny.

Males are produced through rare, spontaneous X-chromosome non-disjunction during meiosis I, in which XX eggs and null-X eggs are produced. The eggs then mate with the hermaphroditic sperm. A punnett square of the model can be seen in figure 5.

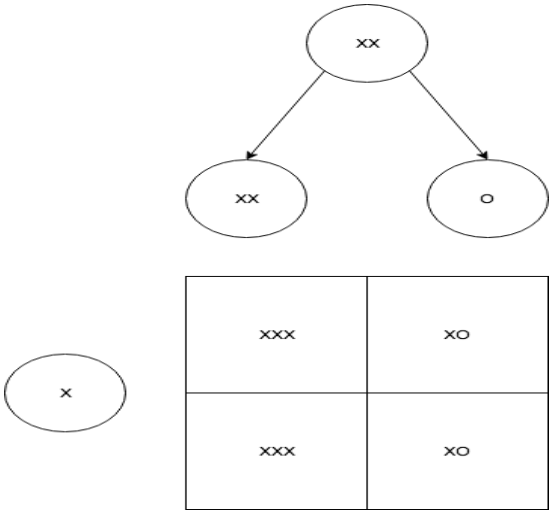

Figure 5: Punnett Square of X-Chromosome Non-Disjunction. During Meiosis I, x-chromosome non-disjunction occurs and both XX and null-O eggs are produced. The X sperm is then combined, which produces the viable XXX hermaphrodite and XO male in a 1:1 ratio.

In a homozygous hermaphroditic population, natural X-chromosome non-disjunction occurs at the relatively rare rate of 1:1000 (Anderson *et al.*, 2010). However, it has been shown that incubating L4 worms that are synthesizing their eggs in increased temperatures promotes X-disjunction (Sulston & Hodgkin, 1988). In order to create the males required for the *nsy-1* and *srf-6* complementation test, L4 worms were incubated at 27°C overnight, after which plates were transferred to 16°C and inspected for presence of males after one generation (approximately a week).

Upkeep of Mutant Strains

Due to the genomic nature of the mutations being worked with, strains had to be vigorously checked for reversion to wild type. For example, strains containing *nsy-1* mutations are kept as a marked strain containing a gene fusion between *str-2* and *GFP*, under control of an AWC-specific promoter (Sagasti *et al.,* 2001). The presence of the *nsy-1* phenotype is evaluated under the fluorescent microscope as a 2AWC+ phenotype, whereas reverted individuals in such strains would express a 1AWC+ phenotype. If a mutation were to revert, over several generations the resulting wild type strain would outgrow the mutant strain due to the wild type's increased viability. In particular, the *nsy-1 (ky397)* mutation, as seen in figure 1, is a C to T transition, Glutamine to stop, C-terminal nonsense mutation that can revert back to an amino acid codon. Another example is *nsy-1* (ag3), as seen in figure 1, which is another C to T, Glutamine to stop, C-terminal nonsense mutation.

Some mutations were very unlikely to revert to wild type. An example of this is *nsy-1 VC390 (ok593)*, which is a complex deletion of the entire *nsy-1* coding sequence accompanied by a small insertion of unrelated DNA (http://www.wormbase.org/db/get?name=WBGene00003822;class=gene).

Strategy for Producing a *nsy-1/srf-6* Genotype For Complementation Testing

Multiple factors had to be considered to create a *nsy-1/srf-6* heterozygote. Because *C. elegans* is hermaphroditic in nature, there exists the need to distinguish self progeny from cross progeny. Self progeny are always produced by self-

fertilization prior to mating. One way to do this is to incorporate a *str-2::GFP* AWC dominant expression marker in the parental males, so that the *str-2::GFP* AWC marker would only fluoresce in cross-progeny. Important to note is that the *str-2::GFP* gene is not located on chromosome II—the location of the *nsy-1* and *srf-6* mutations— it was integrated onto chromosome I. Thus, *str-2::GFP* will be inherited independently from both *nsy-1* and *srf-6*.

Another design factor is the mutant male's ability to mate. As seen in figure 6, sexual reproduction requires effort and interest primarily from the male.

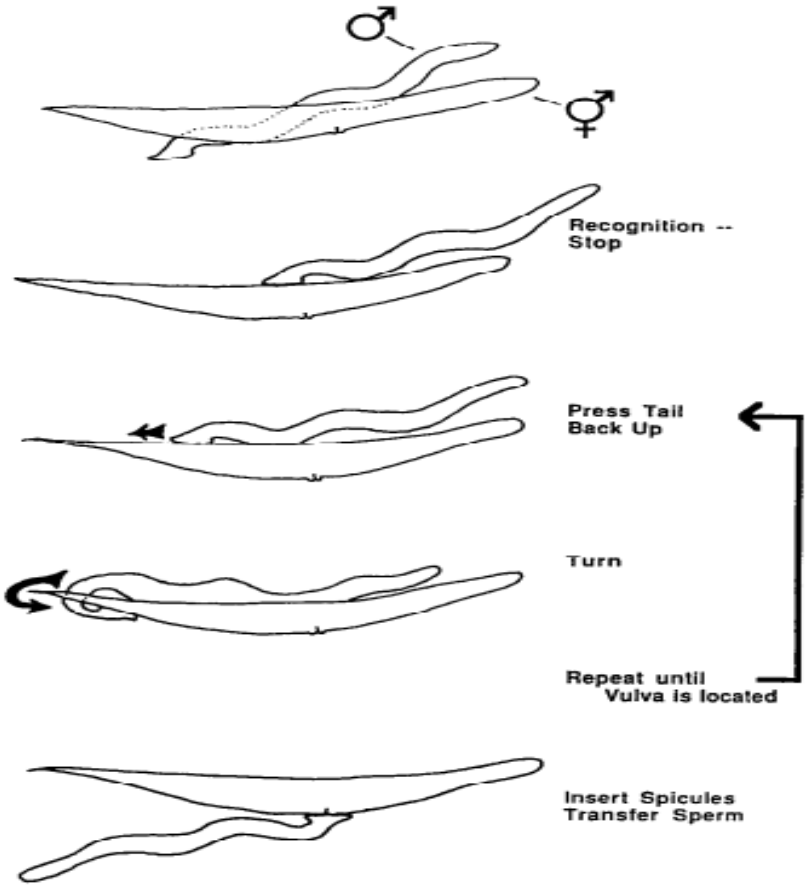

Figure 6: Male Mating Behavior (Loer & Kenyon, 1993). The steps in which males must take to mate with a hermaphrodite requires adequate physical ability and interest, as seen in the figure.

Initially, the ventral surface of male's tail recognizes the hermaphrodite's body. The male then backs up and turns around the hermaphrodite until the tail locates the vulva, into which the male ejaculates its sperm. Due to the immense

amount of physical ability required, the male may be unable to mate effectively if it is physically hindered. The male must also utilize chemotaxis to find the hermaphrodite. As such, mutations in males that affect these functions may hinder their mating abilities.

As an early attempt to create a *nsy-1/srf-6* heterozygote, *nsy-1* (*ky397*) males were crossed with *GFP-str-2*-tagged *srf-6* hermaphrodites. After a week from the initial plating, however, there were the same number of males as initially put onto the plate, suggesting that no mating occurred. After repeating this same process twice, it was concluded that the *CX4998* male strain is unable to mate. As such, another mechanism for creating the *nsy-1/srf-6* heterozygote was required.

Another potential male strain option was *nsy-1* (*ok593*). However, the mutant *nsy-1 (ok593)* worms were rather sickly, so successful mating between *VC390* males and a *srf-6* mutant was thought to be unlikely.

The primary crosses completed for the complementation test can be seen in figure 7 below. For the first cross, *srf-6 (yj13)* males were crossed with *GFP-str2;unc-4 srf-6* hermaphrodites. After a week, the resulting F1 males from the cross were then mated with *nsy-1 (ok593)* hermaphrodites to create the *nsy1/srf6* heterozygote worms.

The primary reason for including the *unc-4* mutant allele as part of the hermaphrodite genotype, and thus for executing the first cross, is that this allowed non-Unc outcross progeny to be distinguished from Unc self progeny. The F1 males from the first cross were *GFP-str-2/ +*; *unc-4 srf-6/+ srf-6*. F1 males on the crossing plate were almost guaranteed to be cross progeny, barring random X-chromosome non-disjunction, so no differentiation techniques were required. The males would express the 2AWC+ phenotype and not display the *unc-4* phenotype.

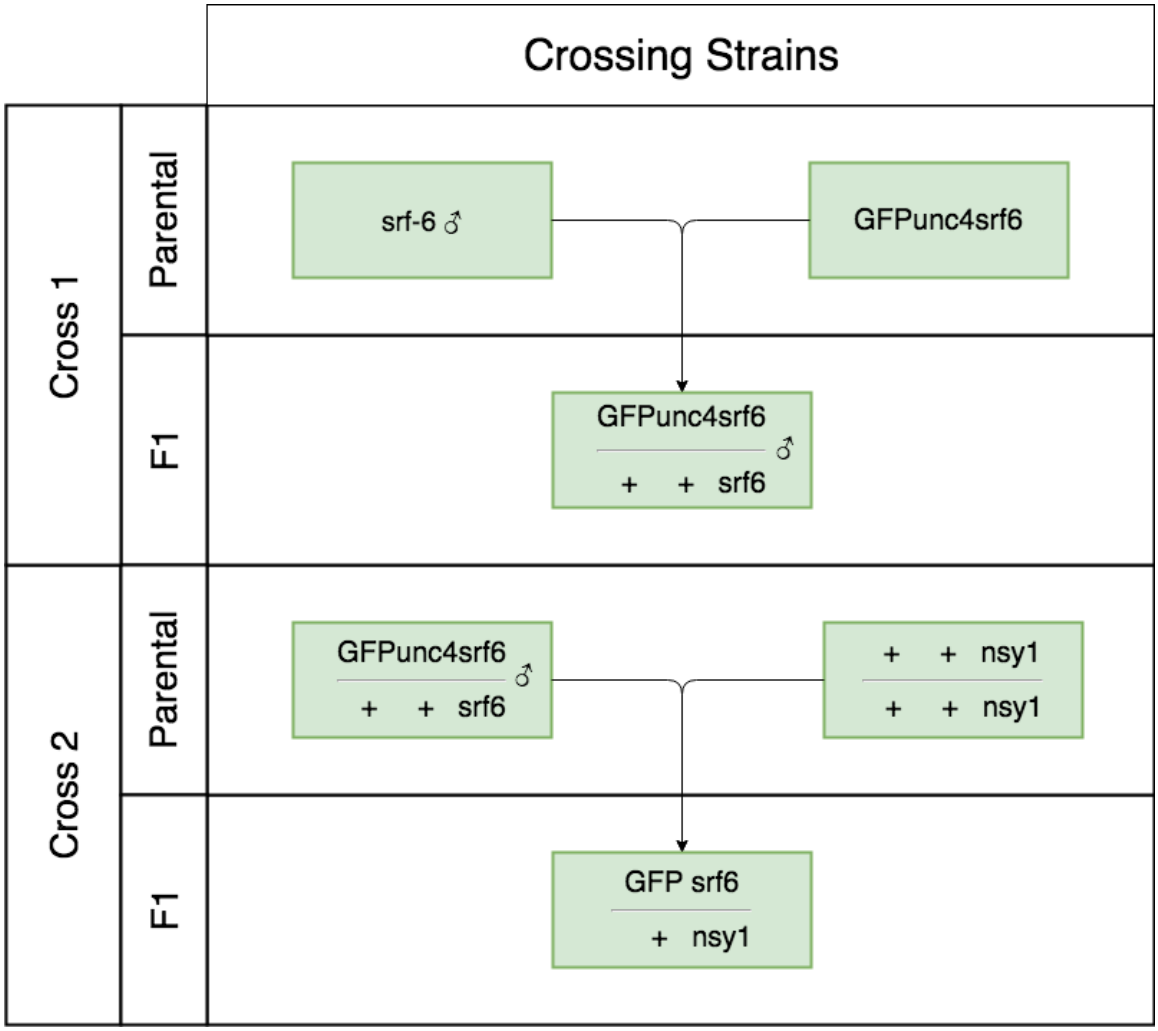

Figure 7: This is the crossing scheme used to create a *nsy-1/srf-6* heterozygote. Two crosses were carried out: the first to create males with a GFP marker with sufficient mating abilities, and the second to create the *nsy-1/srf-6* heterozygote.

All of the F1s in the second cross would be trans doubly heterozygous for *nsy-1* and *srf-6*. Due to the heterozygosity of *GFP* and *unc-4* in the *srf-6* males, half of the F1s would express *GFP*. The *unc-4* phenotype would not be expressed, because it is a recessive mutation. None of the *nsy-1* self-progeny would express *GFP*, so in theory, fluorescence would serve as a sufficient tool for distinguishing cross- and self-progeny.

Results

Complementation crosses were performed as described in the methodology section. Figure 8 shows the AWC fluorescence in the F1 males. Of the *srf-6/nsy-1* doubly heterozygous males examined, 28/35 expressed 2AWC+ (80%), 4/35 expressed 1AWC+ (11.4%), and 3 expressed no fluorescence (8.6%).

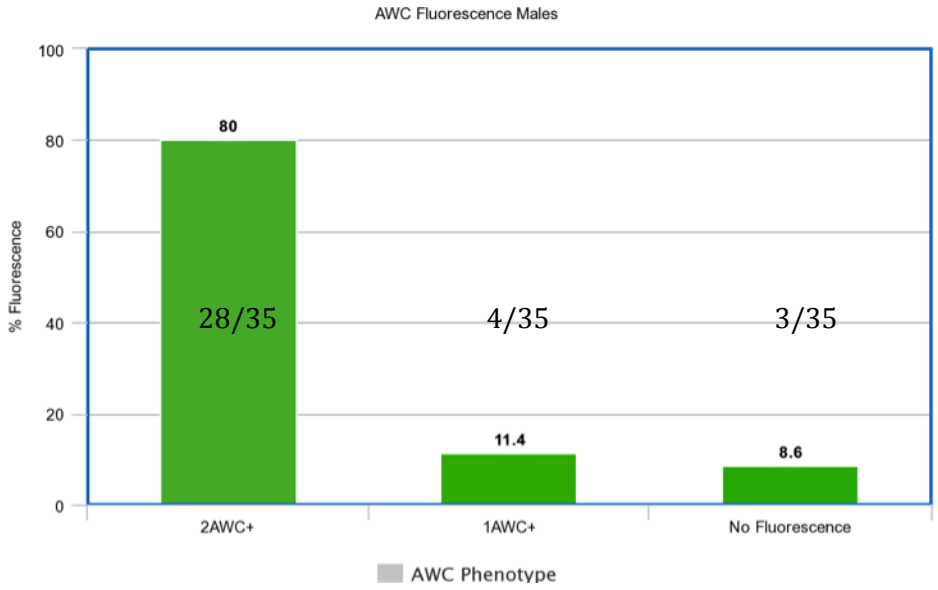

Figure 8: AWC Fluorescence in F1 Males. Of the males examined, a large fraction of males were 2AWC+ (80%). 11.4% were 1AWC+, and 8.6% did not express fluorescence.

There was a disparity between the male and hermaphrodite data. Significantly fewer hermaphrodites expressed fluorescence than males did. Figure 9 shows the AWC fluorescence in the hermaphrodites. 3/60 expressed 2AWC+ (5%), 0/60 expressed 1AWC+, and 57/60 expressed no fluorescence (95%).

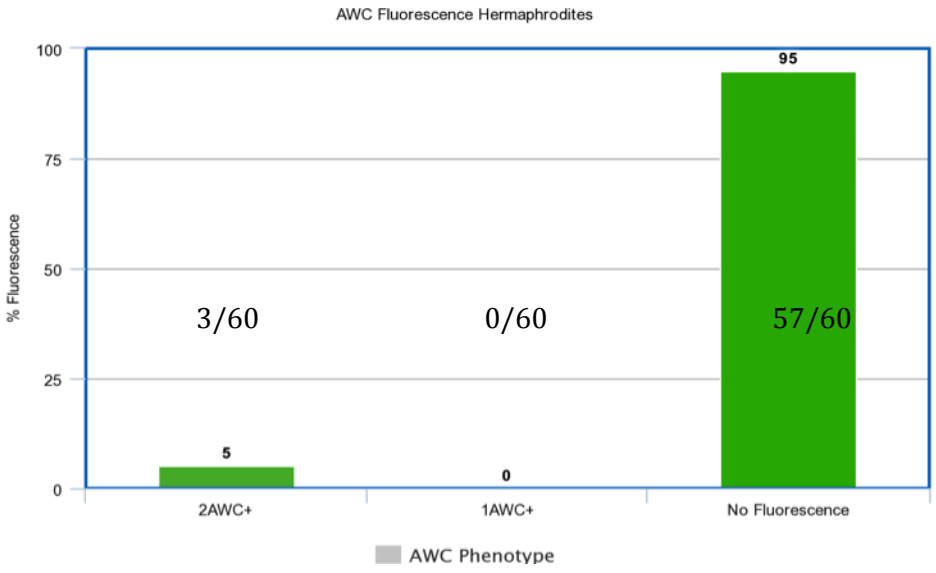

Figure 9: AWC Fluorescence in F1 Hermaphrodites. Of the hermaphrodites examined, 5% were 2AWC+, 0% were 1AWC+, and 95% did not express fluorescence.

Discussion

In mammals, the *ASK-1* gene encodes a protein that requires dimerization to become active (Hayakawa *et al.,* 2006). Because *nsy-1* is closely related to its *ASK-1* mammalian homolog, it is very possible that *nsy-1* requires dimerization to create a functional protein as well. This raises the possibility that *nsy-1* and *srf-6* mutations might complement intragenically, due to the dimerization of two proteins whose defects are mutually offset.

The results of the complementation test we describe here argue against intragenic complementation. Of the worms that were fluorescent, 88.6% expressed the 2AWC+ mutant phenotype. These results are consistent with the hypothesis that *nsy-1* and *srf-6* failed to complement. The failure to complement suggests that the *nsy-1* and *srf-6* mutations affect the same genetic function. The simplest explanation of this would be that *nsy-1* and *srf-6* are the same gene.

The males observed when determining fluorescence were guaranteed to be cross-progeny. As such, only 50% of the cross progeny were expected to be fluorescent. However, a disproportionate 91.4% of the males expressed either 2AWC+ or 1AWC+ fluorescence.

A plausible explanation for the observed lack of fluorescence in the F1 hermaphrodites is that the majority of the nonfluorescent hermaphrodites were *nsy-1 (ok593)* self-progeny. In order to have a perfect 1:1 ratio of fluorescent to nonfluorescent progeny, every hermaphrodite would have to have mated with a male. A few hermaphrodites that did fluoresce expressed the 2AWC phenotype. AWC+ fluorescent hermaphrodites were guaranteed to be cross-progeny.

To confirm the results obtained in males, a method for testing complementation of *nsy-1* and *srf*-6 in hermaphrodites should be developed. One way to do this would be to construct a strain containing *GFP str-2; nsy-1(ok593).* This strain could be mated with *srf-6(yj13)* males to construct the desired double heterozygote.

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