STUDIES ON QUINIC ACID (*QA*) GENE CLUSTER IN VARIOUS STRAINS OF *NEUROSPORA CRASSA*

By

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ABSTRACT

Neurospora crassa belongs to the kingdom of fungi, phylum ascomycota and is eukaryotic. *Neurospora* has the ability to adapt and grow in environments with a variety of carbon sources. Gene systems involved in this adaptation in *N.crassa* such as the quinic acid (*qa*) gene cluster have been studied for many years. We are utilizing a strain of *Neurospora* which has the *qa-1S* repressor gene deleted (ΔS) in it and is used for the study of carbon repression of *qa* gene cluster. In order to determine the levels of *qa* gene expression, RNA is isolated from the *Neurospora* (ΔS) tissue grown in dextrose and quinic acid and one step RT-PCR is carried out. qRT-PCR is used to quantitate the amount of *qa* gene expression. *qa-x*$^{-}$ strain which has *qa-x* gene deleted is grown in dextrose and quinic acid and a combination of both, a brown pigment is separated and phenotype and physical characteristic studies are carried out. Bioinformatic analysis is carried out on *qa-x* gene present in the quinic acid gene cluster of *Neurospora crassa* in order to find the function of the enzyme encoded by *qa-x* gene.

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CHAPTER I

INTRODUCTION

1.1 Description and significance:

Neurospora crassa is a filamentous fungus of phylum ascomycota and is eukaryotic in nature. *N.crassa* in its natural environment can be found growing on dead plant matter predominantly in tropical and subtropical regions. *Neuropsora* is highly valued for research purposes. This is true for the following reasons. First, it is easy to grow. Second, its simple life cycle makes it easy to study genetics and map genes. *Neuropsora* is considered a model organism because its characteristics make it ideal for scientific research (Davis R.H 2002).

Neuropsora contains approximately 10,000 genes (Borkivich and Krystofova 2003). A great deal of genetic research on *Neurospora* has been done at the molecular level. *N.crassa* has the ability to adapt to a variety of carbon sources. Some researchers have used the genetic structure of *Neurospora* to better understand biological processes (Drainas C. 1980).

1.2 Cell structure and metabolism:

Neurospora cell structures share many common characteristics with other fungi. The cytoplasm is enclosed in a plasma membrane, and cell walls are typically composed of chitin. There are at least twelve specialized cell types. The three most common are hyphal, condidium, and ascospore cells. The way *Neurospora* grows is indicative of what

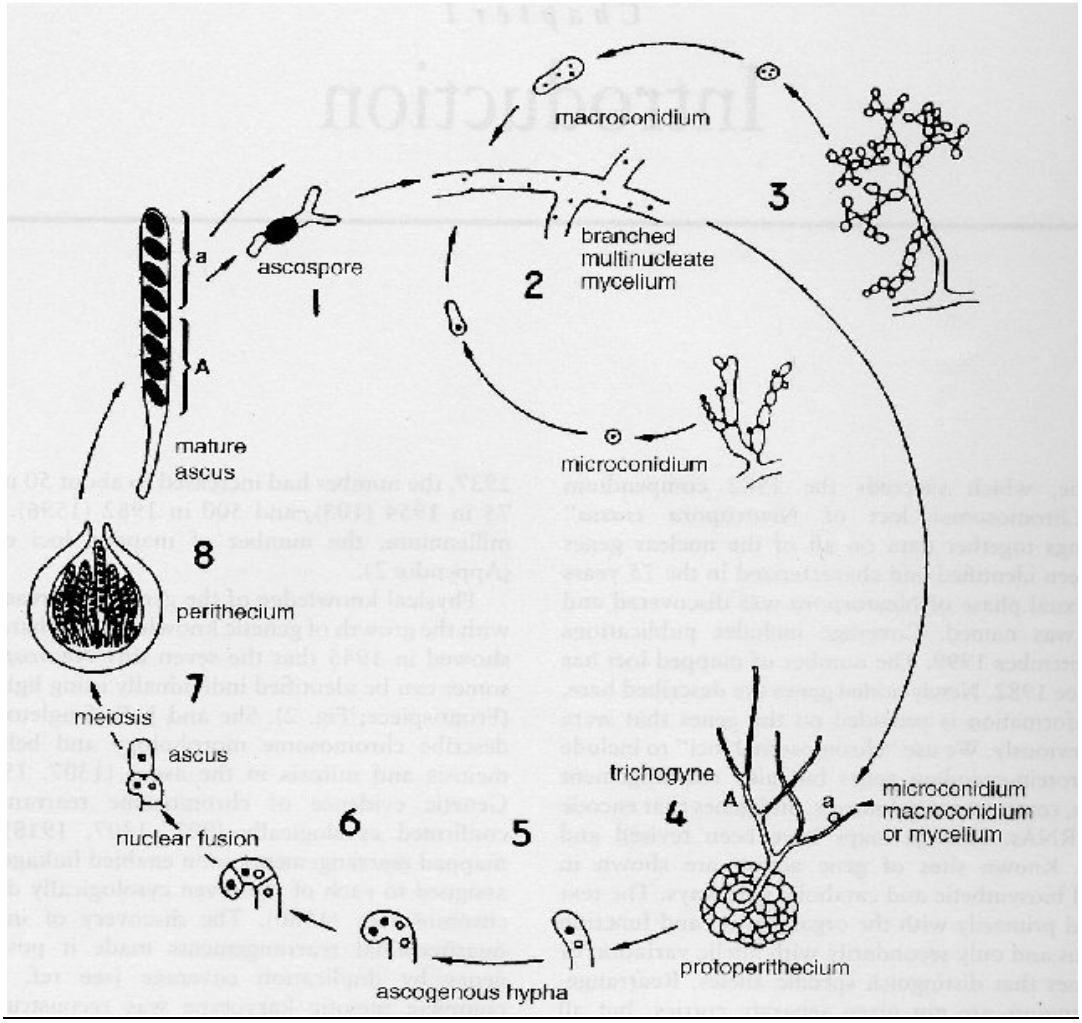
type of organism it is. Yeasts grow through cell division, while filamentous fungi grow by tip extension and branching (Perkins 2000). *Neurospora* is one of the fastest-growing filamentous fungi, with a rate of about ten centimeters per day. It has been shown that *Neurospora* has a circadian rhythm, called the sporulation rhythm (Ruoff P, 2001). If at some point, mycelia is transferred from a high-light and high-nutrient environment, to a low-light, low-nutrient one, the circadian oscillator will begin. These circadian rhythms are caused by compensation mechanisms within cells (Aronson *et al,* 1994 Dunlap *et al* 1993). They are the physiological clocks of organisms.

1.3 Life cycle:

Neurospora being a member of the Ascomycota reproduces both sexually and asexually. *Neurospora* spends most of its life as a haploid organism. There are three different types of sexual life cycles: heterothallic, homothallic, and pseudohomothallic. In a heterothallic life cycle, *Neurospora* has two mating types termed A and a. These mating types are determined by alternate DNA sequences at a given chromosomal locus. Mating can only occur between individuals of different mating types, which results in diploid cells in long sacs. Meiosis produces four haploid cells. These haploid cells undergo mitosis within the ascus, forming eight spores (Nelson *et al* 1997 Dolan *et al* 2000). *N. crassa* is such a heterothallic species. In homothallic reproduction, an individual haploid strain can undergo sexual reproduction without pairing with another strain. To undergo meiosis, a diploid nucleus forms by the fusion of two haploid nuclei. *N. galapagoensis, N. africana* and *N. terricola* are homothallic species. In pseudohomothallic reproduction, a spore will grow into a dual mating type mixture of nuclei. It can then go through the sexual cycle

without having to pair. *N. tetrasperma* is a pseduohomothallic species. The sexual cycle is complete when spores germinate and form mycelia, produce aerial hyphae which produce sexual conidia.

Figure 1.1: Life cycle of *Neurospora crassa* (adapted from Seale 1973)


macroconidium
3
branched
multinucleate
mycelium
2
ascospore
1
a
A
mature
ascus
microconidium
8
perithecium
7
meiosis
ascus
nuclear fusion
6
5
trichogyne
4
A
a
microconidium
macroconidium
or mycelium
protoperithecium
ascogenous hypha

1.4 Gene regulation in eukaryotes:

Like prokaryotes, eukaryotic organisms do not express all of their genes all of the time. Given the complexity of multi-cellular eukaryotes, gene regulation in these organisms needs to be very complex. Eukaryotes need to regulate their genes for different reasons than prokaryotes. In prokaryotes, gene regulation allowed them to respond to their environment efficiently and economically. While eukaryotes can respond to their environment, the main reason higher eukaryotes need to regulate their genes is cell specialization. Whereas prokaryotes are simple, unicellular organisms, multi-cellular eukaryotes consist of hundreds of different cell types, each differentiated to serve a different specialized function. Each cell type differentiates by activating a different subset of genes. The regulation of gene expression required to bring about such differentiation is necessarily complex due to multitude of cell types. This complexity is demonstrated is in multiple levels of regulation of gene expression.

The basic mechanisms of gene regulation are the same in both eukaryotes and bacteria. As the environment changes or as growth and development proceed, gene expression is suitably regulated to cope with the new environment. Hormones, vitamins, minerals, chemicals and pathogens may induce or repress certain genes. This would cause production of certain proteins and inhibit the formation of other proteins, thereby initiating new or inhibition of existing metabolic pathways. Catabolism of quinic acid in *N. crassa* serves as an insight into the gene regulation in eukaryotic filamentous fungi.

1.5 Quinic acid:

Quinic acid is a prominent component of dead vegetation and leaf matter, and it is no surprise that *N.crassa* and other fungi have evolved a pathway to use it as a carbon source as *Neurospora* grows primarily on the dead plant matter. This pathway was first detected in *N.crassa* by the appearance of inducible dehydroquinase activity that has made it impossible to select mutants for the same activity in aromatic amino acid biosynthesis (Davis R.H 2000).

The quinate catabolic pathway runs parallel to the similar reactions in third and fourth steps of aromatic amino acid pathway. Thus, the enzyme ARO-9 in the aromatic amino acid pathway can be replaced with QA-2 (Rhines 1969) as both genes produce the same enzyme.

1.6 Quinic acid gene cluster of *N.crassa*:

Gene cloning technology has allowed a closer examination of the molecular level of regulation and organization of quinic acid genes (Huiet and Case 1985). Four distinct genes are involved with the catabolism of quinic acid and it was determined that these genes occurred in a tightly linked cluster, the *qa* gene cluster. Simultaneous biochemical studies established that three of the *qa* genes (*qa*-2*, qa-3, qa- 4*) encoded inducible enzymes which catalyze the conversion of quinic acid to protocatechuic acid in *N. crassa* (Figure 1.3). These early studies also provided evidence for a presumptive regulatory gene controlling the synthesis of the three enzymes. During this period, based on then available genetic and biochemical data, a hypothesis was proposed which postulated that

the product of a positive regulatory gene, together with the inducer - quinic acid, regulated expression of the *qa* enzymes. The development of recombinant deoxyribonucleic acid (DNA) technology, which made possible the cloning of genes from various organisms into expression vectors, indicated clearly that the cloning of the *qa* gene cluster would permit a detailed analysis of the organization and regulation of this system at the molecular level. The initial experiments on cloning and expression of one of the *qa* genes *(qa-2)* in *E.coli* were successful (Giles N.H 1985). Larger segments of DNA flanking the *qa*-2+ gene were cloned by using cosmid technique and a 42kb of *N.crassa* DNA centered around the *qa*-2+ gene was obtained and subsequent transformation experiments led to the mapping of the *qa-3* and *qa-4* genes. (Case M.E 1976).

Subsequent experiments revealed that the *qa* gene cluster consists of five structural and two regulatory genes (Geever 1989). Each of the genes in the cluster is transcribed at basal levels in the presence of any carbon source, but if quinic acid is substituted as the carbon source, these transcription levels dramatically increase (Geever 1989). The *qa* gene cluster of *N. crassa* is located on approximately 17.2 kb of DNA on linkage group VI1 (Giles *et al.* 1985, Geever*et al*. 1989). Three of the structural genes, *qa-2, qa-3* and *qa-4* are involved in the first three steps of quinic acid catabolism (Figure 2) (Giles 1985). A fourth gene, *qa-y,* encodes quinic acid permease that facilitates the uptake of quinic acid. The function of other structural gene *qa-x* is unknown (Patel et al 1981). The other two genes of the *qa* cluster *(qa-IF* and *qa-IS)* code for regulatory proteins on the basis of genetic and molecular analysis (Giles.1985, Geever1989). The *qa-1F* and *qa-1S* genes control the transcription of various genes in the quinic acid gene

cluster. The *qa-1S* gene encodes a repressor protein. In the absence of the inducer (quinic acid), the repressor inhibits the transcription of genes from the *qa* gene cluster. The other regulatory gene *qa-1F* encodes the activator protein that exerts a positive control on transcription of the *qa* gene cluster by binding the specific nucleotide sequence (GGGTAARYRYTTAYCC) present in the 5'end of each *qa* gene cluster (Baum J. 1987).

The activator protein (QA-1F) contains 816 amino acids and has at least two domains. One of these interacts with the repressor protein and the other interacts with the DNA sequence. The repressor binds to the C-terminal domain of the activator. The repressor protein (QA-1S) contains 918 amino acids and all mutant alleles described so far contain mutations in the region containing N-terminal 300 amino acids (GILES 1987). The present hypothesis states that in the absence of quinic acid the repressor protein binds the C-terminal region of the activator protein blocking it's interaction with the transcriptional apparatus. In the presence of quinic acid, the inhibition of the activator protein is relieved.

1.7 Significance of ΔS strain:

Mutants in the *qa-1S* repressor gene had been isolated in the 1980s. These mutants were always constitutive for the *qa* gene cluster since they lacked a functional repressor. In these strains the genes of the *qa* gene cluster were transcribed even if quinic acid was not present (Geever 1989). A strain containing a deletion of the *qa-1S* repressor gene was constructed. This strain is termed delta S (ΔS). To construct this strain a plasmid construct, *pΔqa-1S* which retained the function of *qa-y* and *qa-1F* genes was

used to transform a strain containing *qa-1F*, *aro-9* mutations to obtain strain in which the *qa-1S* gene is deleted by using targeted transformation. These transformants then were crossed with a *met-7* strain of *Neurospora* in order to obtain homokaryotic progeny (Case 1992). Transformants containing the *Δqa-1S* deletion have constitutive levels of expression of *qa-2*, *qa-1F* and *qa-x* genes as determined by the mRNA analysis indicating that *qa-1S* product is a repressor molecule (Case 1992).

Figure 1.2: Metabolic pathway of quinic acid and shikimic acid via qa gene cluster (Arnett 2000 MS Thesis)

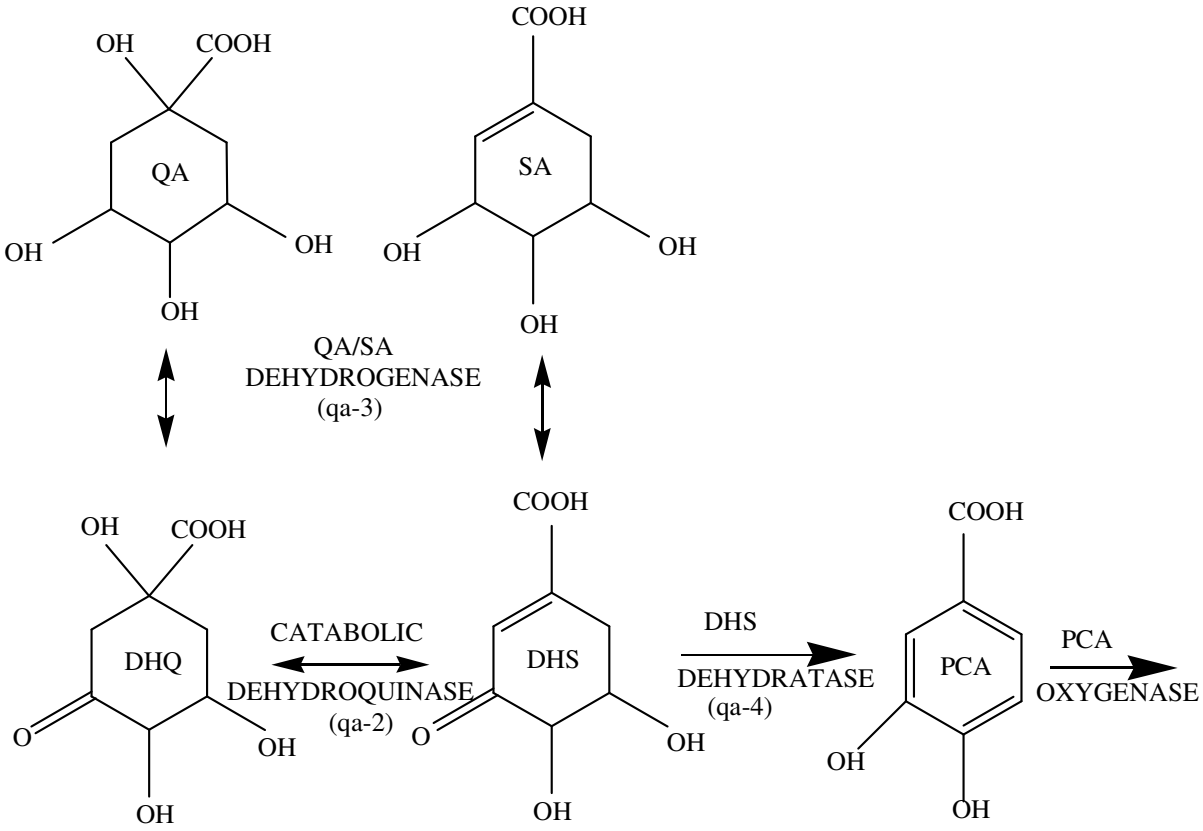
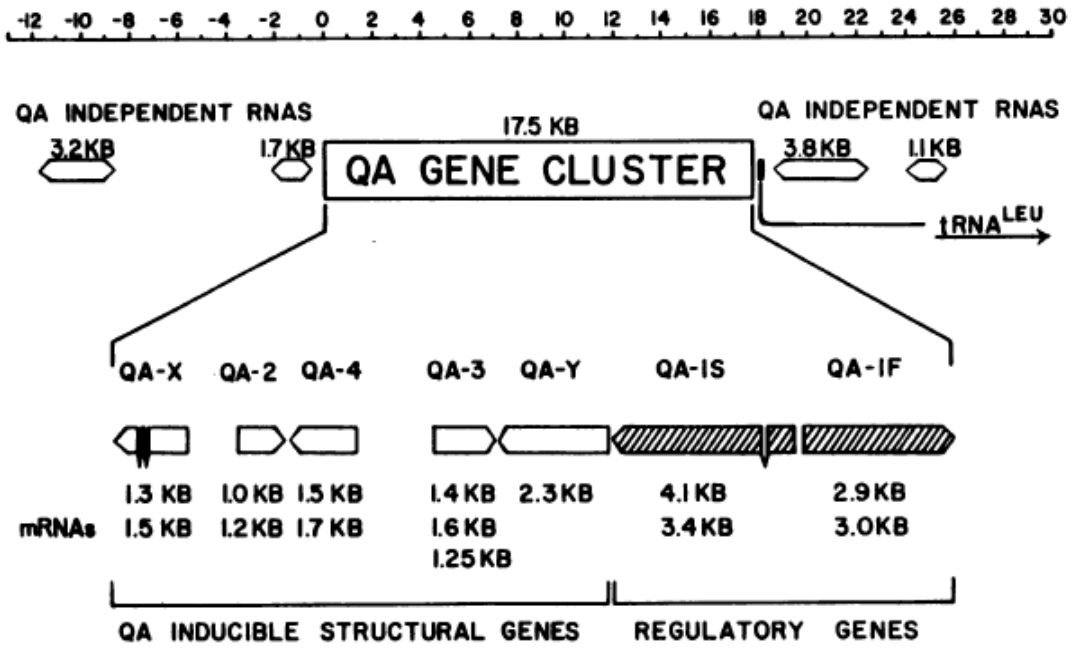

OH
COOH
QA
OH
OH
OH
COOH
SA
OH
OH
OH
QA/SA
DEHYDROGENASE
(qa-3)
OH
COOH
DHQ
O
OH
OH
CATABOLIC
DEHYDROQUINASE
(qa-2)
COOH
DHS
O
OH
OH
DHS
DEHYDRATASE
(qa-4)
COOH
PCA
OH
OH
PCA
OXYGENASE

Figure 1.3: Quinic acid gene cluster of *Neurospora crassa* (adapted from Giles1985)


-12 -10 -8 -6 -4 -2 0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
QA INDEPENDENT RNAS
3.2KB
1.7KB
17.5 KB
QA GENE CLUSTER
QA INDEPENDENT RNAS
3.8KB
1.1KB
tRNA LEU
QA-X
QA-2
QA-4
QA-3
QA-Y
QA-IS
QA-IF
1.3 KB
1.0 KB
1.5 KB
1.4 KB
2.3 KB
4.1 KB
2.9 KB
mRNAs
1.5 KB
1.2 KB
1.7 KB
1.6 KB
3.4 KB
3.0 KB
1.25 KB
QA INDUCIBLE STRUCTURAL GENES
REGULATORY GENES

1.8 Catabolite repression under various growth conditions:

Genetic analysis demonstrated that mutants of *Neurospora crassa* could be obtained with apparent defects in glucose repression (Ebbole D. J. 1998). However, in contrast to the advanced level of understanding of the regulatory systems governing nitrogen, phosphorus, and sulfur utilization, little is known about the molecular basis of carbon catabolite repression in *N. crassa.* In *N. crassa,* understanding carbon regulation is also important for understanding signals that regulate development since conidiation is induced by carbon starvation. Several genes have been characterized that highlight the interaction between conidiation and glucose repression pathways and provide insight into the signaling pathways that control carbon catabolite repression and development in *N. crassa* (Ebbole D. J. 1998). Carbon catabolite repression refers to the lower level of gene product activity observed in response to an abundance of a preferred carbon source, such as glucose. Transcriptional repression is often found to be responsible for carbon catabolite repression. Although a wealth of information has been integrated to generate a complex (but still evolving) model for glucose regulation in *Saccharomyces cerevisiae* (Gancedo 1998, Klein 1998), our understanding of this process in filamentous fungi is more limited. As we stated earlier, the genes in the quinic acid utilization gene cluster are induced by quinic acid through the activity of the QA-1F transcriptional activator (Geever 1989, Case 1992). QA-1S is a negative regulator thought to bind to QA-1F to block activation in the absence of quinic acid. Quinic acid binding to QA-1S is proposed to release the QA-1F activator. The induction by quinic acid is largely blocked by glucose in the medium. However, *qa-1S* mutants are derepressed for *qa-2, qa-x,* and *qa-1F* even in the presence of glucose, suggesting that glucose repression does not occur

directly at each of these *qa*genes (Case 1992). It appears more likely that glucose inhibits transport of quinic acid into cells. The RNA blot data showed that inducer exclusion may be an important mechanism in glucose regulation of quinic acid utilization and that catabolite repression may be the direct inhibition of the transcription of quinic acid permease gene, *qa-y* (Arnett 2009). Quantitative RT-PCR is the most sensitive technique in quantifying mRNA levels when compared to Northern blot analysis. Therefore qRT-PCR is the technique that is proposed to use to confirm the expression and quantitation of *qa* gene levels by Northern blot technique.

1.9 *qa-x* gene in Quinic acid (*qa)* gene cluster:

qa-x is located near the *qa-2* gene in the *qa* gene cluster. In order to elucidate the function of *qa*-x gene, gene replacement transformation was carried out. A plasmid pRD10 which is a *qa-2*$^{+}$ is constructed having the *qa-x* disrupted by filling in a *HindIII* site, creating a *NheI* site in the amino-terminal end of the *qa-x* protein disrupting the coding sequence (Case 1992). The strain containing the disrupted *qa-x* gene *(qa-x*$^{-}$*)* showed the accumulation of unidentified brown pigment when grown in the presence of quinic acid as a sole carbon source (Case 1992). No other phenotype for this mutation has yet to be uncovered. It was believed that QA-X protein might be involved in mediating the slight repression of the *qa-1F* activator gene but is not required for the repression of the *qa-y* gene in the presence of glucose (Arnett 2009).

Figure 1.4: The *qa* gene cluster after transformation with *PΔqa-1S* plasmids

B

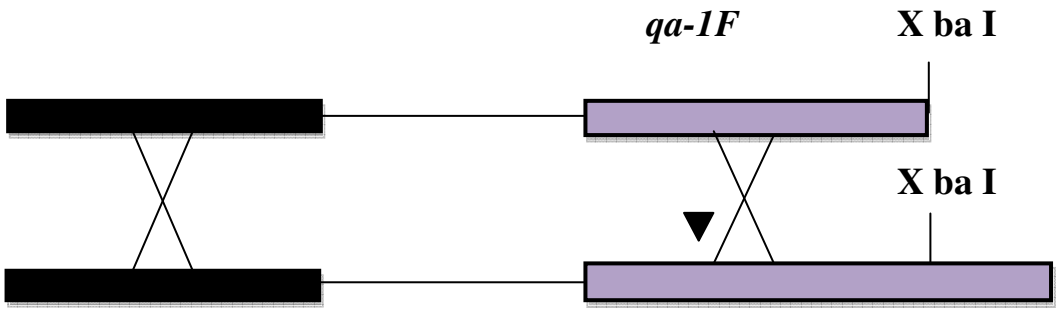
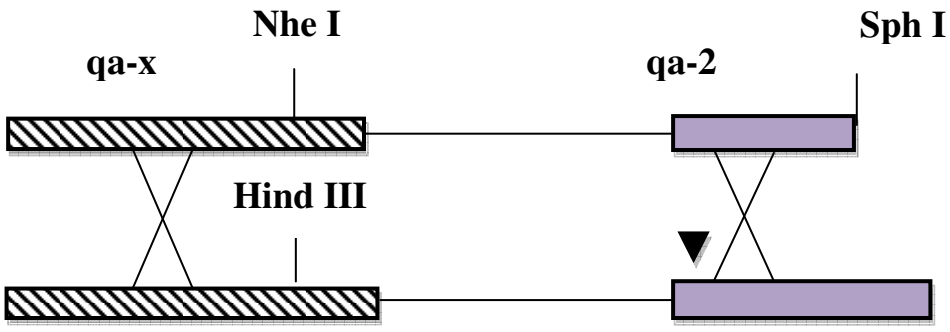


Figure 1.5: Homologous recombination event to produce pRD10 which contains the disruption of *qa-x* gene (Case 1992)

A



CHAPTER II

GOALS OF THE PROJECT

The goals of the project include:

- Quantitation of mRNA levels of quinic acid gene cluster in ΔS strain of *Neurospora crassa.*
- Studies on the brown pigment produced by *qa-x*$^{-}$ strain of *Neurospora crassa.*
- Bioinformatic analysis of *qa-x* gene present in the quinic acid gene cluster of *Neurospora crassa.*

CHAPTER III

MATERIALS AND METHODS

3.1 Materials:

Neurospora crassa wild type strain 74A, *Neurospora crassa* strain containing *qa-x*$^{-}$ mutation strain, *Neurospora crassa* strain containing the ΔS mutation strain, 1X vogels media (Vogels 1956) , sucrose, agar, 2% dextrose, 0.3% quinic acid, liquid nitrogen, sand, RIBOSOL RNA extraction reagent, chloroform, isoproponol, 75% ethanol, DEPC water, oligo nucleotide primers (integrated DNA Technologies), Qiagen one- step RT-PCR kit, agarose, 1X TPE buffer, ethidium bromide, Distilled water, 1X external well factors solution, Concentrated HCl, silica paper, 2-butanol, acetic acid, pyridine, toluene, D_2O, DMSO, PREP SEP solid phase extraction reverse phase column with silica, methanol, shaker, vacuum filtration apparatus, eppendroff tubes, mortor and pestle, Sorvall RT 6000B refrigerated centrifuge, Amicon ultra centrifugal filter tubes, BIORAD smart spec plus spectrophotometer, incubator, cuvettes, BIO-RAD S1000 thermocycler, agarose gel electrophoresis equipment, digital camera, UV deterctor, 96 well plates, micro film, BIO-RAD icycler, 0.45 micron dialysis tube, pH meter, lyophilizer, BRUKER Ultrashield 400 NMR instrument.

3.2 Preparation of the **Δ**S strain of *Neurospora crassa*:

All strains of *Neurospora crassa* are grown in 1X Vogels media with 2% sucrose, 1.5% agar and incubated at 30°C for 2 days and kept under white light for 2 weeks at room temperature.

3.3 Preparation of the Sample for Quinic acid shifts:

Conidia from a strain of *N.crassa* containing the ΔS mutation is re-suspended in 25 mL of 1X Vogels and 2% dextrose, swirled and poured back to 125 mL flask through sterile cheese cloth. The conidial suspension (liquid) was added into 250 mL flask containing 1X Vogels and 2% dextrose and grown at 25°-30°C while shaking for 24 hours. This tissue is then vacuum filtrated and the mycelial pad is transferred to 250 mL flask with 50 mL 1X Vogels and 2% dextrose or a flask containing 50 mL 1X Vogels and 0.3% quinic acid and incubated with shaking for three hours at 25°-30°C. The mycelia are again filtered wrapped in a foil and are stored at -70°C.

3.4 Preparation of the *Neurospora* tissue for Isolation of RNA:

Frozen tissue of the **Δ**S strain of *N.crassa* was crushed using liquid nitrogen approximately 0.5g of sand in a mortor and pestle. The ground tissue is transferred into eppendorff tubes. Ribosol Extraction reagent is added to the tissue and the sample and is homogenized. The samples are allowed to stand for 5-10 minutes. After 10 minutes 200 µL of chloroform per 1 mL of Ribozol Extraction reagent is added and this is incubated for 2-3 minutes then centrifuged for 15min at 12,000xg, 4°C. Of the three phases visible,

a lower red phase consists of chloroform, a white interface and the upper portion of sample which consists of RNA is pipetted out into fresh eppendorff tubes and is treated with iso-proponol to precipitate the RNA. The eppendorff tubes are incubated for 10 minutes at room temperature and centrifuged for 10 minutes at 12,000xg, 4°C to collect the pelleted RNA. The supernatant is removed after centrifugation and the pellet is washed with 1 mL of 75% Ethanol at -70°C. The sample is then centrifuged for 5 minutes at 4°C and the Ethanol is removed from the sample. Fifty µL of DEPC water is added to each eppendorff and is allowed to stand for 15 minutes at room temperature to dissolve RNA. Diethylpyrocarbonate (DEPC) treatment is the most commonly used method for eliminating RNase contamination from water, buffers, and other solutions. DEPC water is prepared by adding 0.1% DEPC; inoculating at room temperature and then autoclaving.

3.5 Finding the Absorbances of RNA Samples:

The isolated RNA sample is diluted 200 fold and the absorbance readings are taken in BIORAD Smart Spec Plus Spectrometer. The ratio of absorbances at $A_{260}/A_{280} >$ 1.5 signifies the presence of RNA. The concentrations and the A_{260}/A_{280} ratios for each sample are calculated.

3.6 Primers used for running RT-PCR:

It is generally accepted that the optimal length of PCR primers is 18-22 bp. This length is long enough for adequate specificity, and short enough for primers to bind easily to the template at the annealing temperature. Primers with melting temperatures in the range of 52-58^{o}C generally produce the best results. Both forward and reverse primers

are required for the RT-PCR reaction. Histone gene sequences are used as a standard in RT-PCR reaction along with primers for the *qa-y* and *qa-2* genes.

Table 3.1: Primers used in RT-PCR Reaction

primer name	Length (bp)	Sequence	Tm (50mM NaCl)	MW
H3F2 forward primer	20	5^1- AGA TTC GTC GCT ACC AGA AG- 3^1	54.1°C	6,126.00
H3R2 reverse primer	18	5^1- CGG AGG TCG GAC TTG AAG- 3^1	55.1°C	5,604.70
qa-2F1 forward primer	19	5^1- TCA ACA ACC CTC CAT GAC A- 3^1	53.8°C	5,685.80
qa-2R1 reverse primer	20	5^1- CAC ATG AAC CTC CAC AAA CG- 3^1	54.2°C	6,024.00
qa-yF1 forward primer	24	5^1- GGT ATC AAT GCC ATC AAC TAT TAC- 3^1	51.7°C	7,295.00
qa-yR1 reverse primer	20	5^1- GCC ACA GAA GCC AGA TAA TG- 3^1	53.6°C	6,144.10

3.7 RT-PCR on RNA isolated from the ΔS strain of *N.crassa*:

The RT-PCR is carried out using various primers at a concentration of 0.6 µM. The primers initiate the Polymerase chain reaction. The RNA sample is diluted such that the concentration of the sample is between 1pg-2µg/reaction. Very little concentration of RNA is sufficient based on the model and sensitivity of the RT-PCR technique. Qiagen RT-PCR kit is used for one step RT-PCR. The Qiagen RT-PCR kit contains DEPC water, a reverse transcriptase enzyme, an enzyme mix with DNA polymerase enzyme, dNTP's and RNAse free buffer. 5 µL of 10X PCR buffer, 1 µL of dNTP mix, 1.5 µL of forward primer, and 1.5 µL of reverse primer, 0.25 µL of polymerase and 1 µL of template at the above concentration is used to run the reaction. The total volume of each sample should be adjusted to 50 µL with DEPC water.

3.8 Program for RT-PCR:

The RT-PCR is carried out in a BIO-RAD S1000 thermo cycler. The reaction is set at 37°C for 30 min for reverse transcriptase, then at 95°C for 15 min to deactivate reverse transcriptase. PCR is then done at 94°C for 45 sec helps denature DNA, then at 56°C for 45 sec for annealing of the primers and then at 72°C for 1 min, for the polymerase reaction, These steps are repeated 32 cycles and final extension is carried out at 72°C for 10 min and the sample is stored at 4°C.

3.9 Performing Gel Electrophoresis on the RT-PCR product:

The RT- PCR product is run on 1.5% agarose gel in 1XTPE (0.09M Tris-Phosphate - 0.002M EDTA) buffer, stained with ethidium bromide, visualized under UV light.

3.10 Preparation of RNA dilutions for qRT-PCR:

RNA isolated from the ΔS strain of *Neurospora crassa* grown on either 2% dextrose or 0.3% quinic acid is thawed and four dilutions of RNA are prepared (9.9 ng/µL, 0.99 ng/µL, 0.099 ng/µL and 0.0099 ng/µL) with distilled water. Sample volumes are adjusted to 25 µL and prepared in triplicate for each dilution.

3.11 Primers used for running qRT-PCR:

Upon experiment 18SrRNA as standard primers gave more sensitive results than Histone primers. Therefore along with qa-2 and qa-y primers in the RT-PCR reaction, 18SrRNA is used as a control (Table 3.2).

3.12 Calibration of qRT-PCR:

BIO–RAD iCycler iQ External well factor solution is provided by the manufacturer. 1X external well factor solution is prepared by dilution of 10X external well factor solution with water. One plate is filled with 1X external well factor solution in all the wells, 25 µL in each well. A second plate used with 25 µL 1X external well factor

solution into 8 wells of 96-well plate. A third plate is sealed empty for background calibration. The calibration of BIO-RAD iCycler qRT-PCR is carried out in three steps. A mask calibration is carried out by taking an exposure of the mask of the plate with all wells filled with 1X well factor solution and optimizing it. The background calibration is carried out with the sealed empty plate. The well factors calibration is done with the plate having eight wells of 1X well factor solution and persistent well factors are collected.

Table 3.2: Primers used in qRT-PCR Reaction

primer name	Length (bp)	Sequence	Tm (50mM NaCl)	MW
18SrRNA forward primer	18	51- TGG CCG GAA GGT CCG GGT- 31	64.9°C	5,596.40
18SrRNA reverse primer	20	51- AGT AGC GAC GGG CGG TGT GT- 31	63.8°C	6,254.10
qa-2F1 forward primer	19	51- TCA ACA ACC CTC CAT GAC A- 31	53.8°C	5,685.80
qa-2R1 reverse primer	20	51- CAC ATG AAC CTC CAC AAA CG- 31	54.2°C	6,024.00
qa-yF1 forward primer	24	51- GGT ATC AAT GCC ATC AAC TAT TAC- 31	51.7°C	7,295.00
qa-yR1 reverse primer	20	51- GCC ACA GAA GCC AGA TAA TG- 31	53.6°C	6,144.10

3.13 Preparation and Selection of sample plate:

The sample plate is setup using plate setup editor in BIO-RAD software depending on the requirement for number of samples to be run on one plate.

3.14 Setting the program for qRT-PCR:

A protocol is obtained from the protocol template and is then edited. The dwell time is set and the set point temperatures are given (Table 3.3). A melt curve step is added in order to obtain the melt curve and a final step to store the sample at 4°C.

3.15 Preparation of the media for *qa-x*$^{-}$ strain and *74A* strain in *Neurospora crassa*:

50 mL of 1X Vogels is prepared, autoclaved and either 2% dextrose or 0.3% quinic acid is added. Media is then inoculated with strains of *N.crassa* containing the *qa-x*$^{-}$ mutation or the wild-type strain *74A*. Strains are incubated at 30°C for two days and moved to be grown under white light at room temperature for about 6 to 8 weeks. The sample obtained is vacuum filtrated and the filtrate is stored at 4°C.

The filtrate is dialyzed using 0.45 micron tube for 3 days in 50ml of 1X TE (100mM Tris-HCl and EDTA) buffer (pH 7.5). The dialyzed sample is acidified with concentrated HCl to pH 4. The absorbance of the sample is recorded using spectrophotometer.

Table 3.3: Protocol for qRT-PCR reaction

Cycle	Repeats	Step	Dwell time	Setpoint	Temp change	End point
1	1					
		1	3:00	95		
2	40					
		1	0:10	95		
		2	0:30	60		
3	81					
		1	1:00	55	0.5	95
4	1					
		1		4		

3.16 Thin layer chromatography of brown pigment obtained from *qa-x*$^-$ strain of *Neurospora crassa:*

The brown pigment is obtained from the *qa-x*$^-$strain of *Neurospora crassa* is subject to centrifugation with the help of Amicon ultra centrifugal filter devices with 5,000Da, 10,000Da, 30,000Da, and 100,000Da at 4°C for two days. Four samples obtained from the centrifugation are spotted on the silica plate 1cm apart from each other and is placed in elution solvent (2-butanol : acetic acid : Pyridine) (2:1:2) for approximately 45 minutes. The combination for the elution solvent is chosen depending upon the solubility of the sample. The silica plate is then sprayed with iodine and is allowed to dry with the help of a blow dryer and the silica plate is checked for florescence under ultraviolet light. The combination of elution solvent is changed to (toluene: acetic acid: 2-butanol) and the procedure is performed once again.

3.17 Lyophilization of the brown pigment obtained from *qa-x*$^-$ strain of *Neurospora crassa:*

The brown pigment obtained from *qa-x*$^-$ strain is dialyzed using a 0.45 micron dialysis tube with 1X TBE buffer (pH 8) for 3 days and lyophilized.

3.18 1H NMR of the above sample:

The brown pigment is lyophilized three times using D_2O as a solvent in order to remove as much as water possible. The final sample is prepared with D_2O +DMSO; a 1H NMR spectrum was collected.

3.19 Reverse phase column chromatography of dialyzed brown pigment:

The lyophilized brown pigment is dissolved in a small amount of distilled water and methanol is added to the solution. The PREP SEP solid phase extraction reverse phase column consists of silica, since the brown pigment is water soluble; methanol is selected as an elution solvent for the column. Methanol is initially run through the silica column followed by the pigment and the sample is eluted by using methanol. The eluted sample is lyophilized and Heteronuclear Single Quantum Coherence (HSQC) is performed for 23 minutes, 16 hours and 48 hours.

3.20 Bioinformatic analysis of *qa-x* gene of *Neurospora crassa*:

The primary sequence of the *qa-x* in the quinic acid gene cluster is retrieved from the NCBI website and basic local alignment search tool (BLAST) is used to search for the sequences that are similar to the protein sequence of *qa-x*. The primary sequences of the eight other related organisms having a protein sequence similar to *qa-x* is retrieved and is used to perform the multiple sequence alignment using ClustalW software. The phylogenetic analysis of the QA-X protein is performed on the basis of the similarity obtained by multiple sequence alignment. The possible neighboring genes are determined by using NCBI genome table, Joint genome institute (JGI). The Pfam database is used to do the structural analysis of IMPase protein. Structural classification of the proteins (Scop) is used to classify the IMPases. SignalP3.0 is used to predict the protein property of the QA-X.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Performing RT-PCR on RNA isolated from the ΔS strain of *Neurospora crassa*:

In order to determine the quality of RNA samples and the primers that were selected, RNA was isolated from the strain of *Neurospora crassa* containing the ΔS mutation grown with either quinic acid or dextrose as a carbon source. RNA showing the absorbances ratio of A260/A280 > 1.5 were used for RT-PCR.

Histone primers are used to detect the histone mRNA which acts like a standard for the cDNA that is being produced. RT-PCR is carried out to detect the mRNAs of the *qa-y* and *qa-2* genes in the ΔS strain of *Neurospora crassa*. Therefore, based on the sequences of the *qa-y* and *qa-2* genes the primers for *qa-y* and *qa-2* are designed.

RT-PCR was performed as described earlier to detect the mRNAs for the *qa-2*, *qa-y* and histone genes. The cDNA samples produced from these RT-PCR reactions were electrophoresised on a 1% agarose gel with a 100 bp DNA ladder on the side in order to determine size of the bands produced.

Figure 4.1 shows that histone primers produced bands of the predicted size in RNA of ΔS strain of *N.crassa* grown in either quinic acid or dextrose, thereby proving that histone can be a primer for acting as a standard on RNA from tissue grown under these conditions.

Figure 4.1: Agarose Gel Electrophoresis of histone cDNA from the RNA samples prepared from **Δ**S strain of *Neurospora crassa*

From left to right: Lane 1 **Δ**S RNA quinic acid with Histone3&4 primers, Lane 2 **Δ**S RNA Dextrose with Histone3&4 primers, Lane 3 100 bp DNA ladder

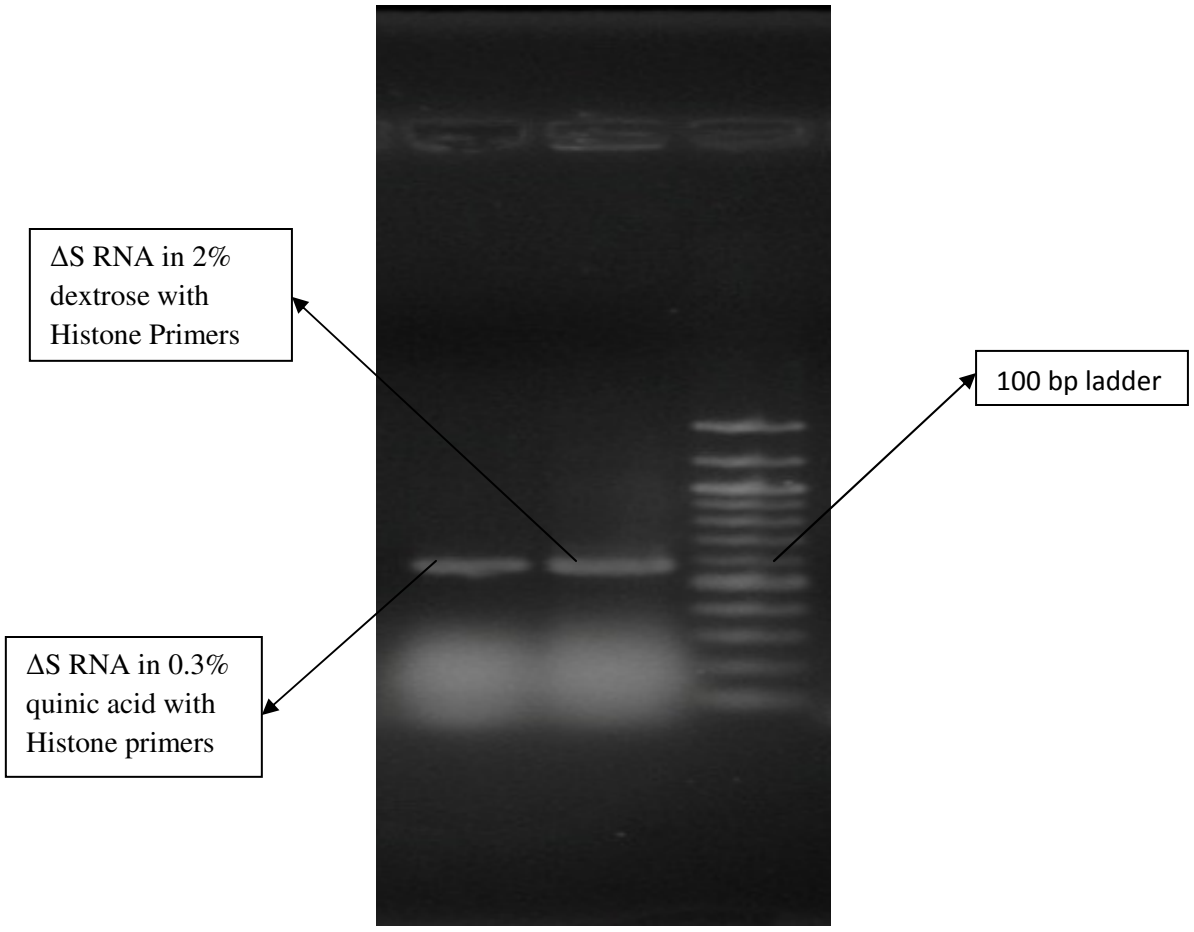
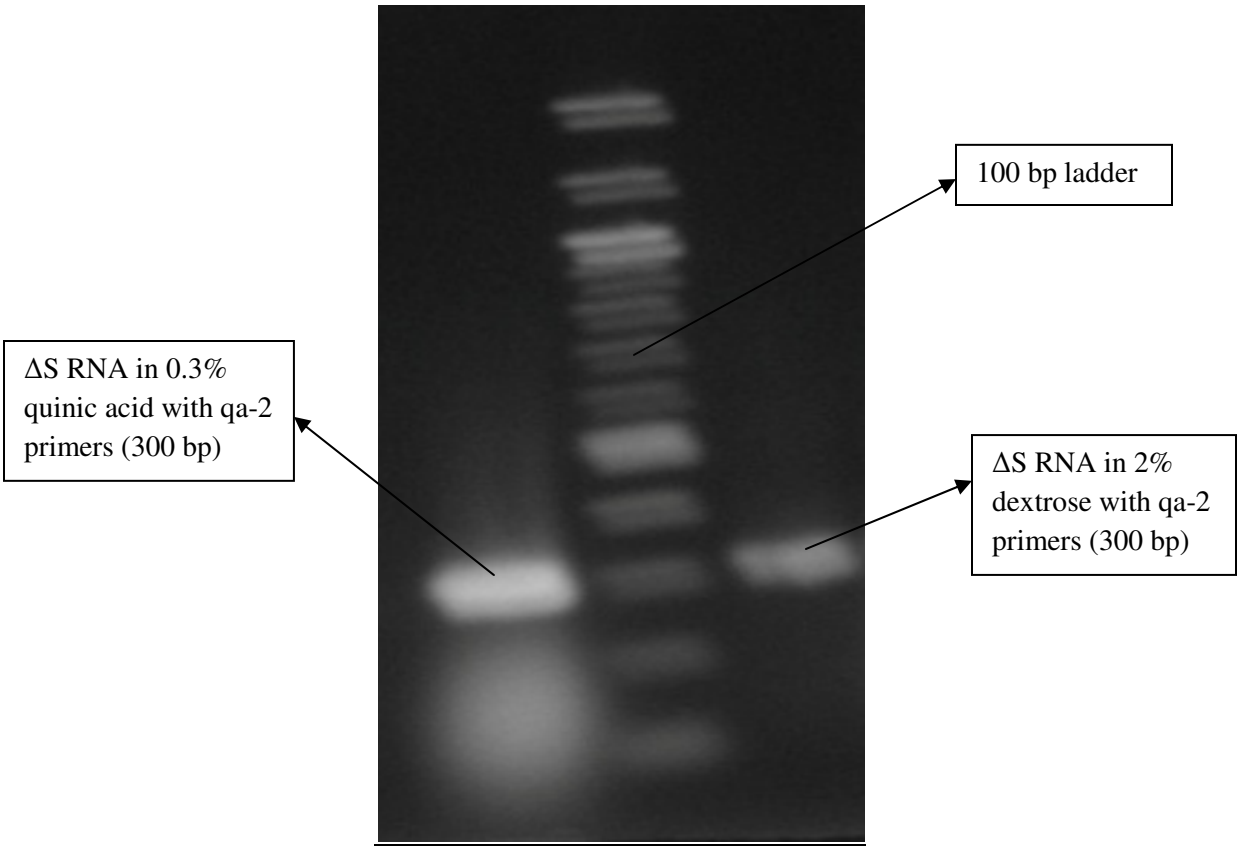

ΔS RNA in 2% dextrose with Histone Primers
100 bp ladder
ΔS RNA in 0.3% quinic acid with Histone primers

Figure 4.2: Agarose Gel Electrophoresis of *qa-2* cDNA from the RNA samples prepared from **Δ**S strain of *Neurospora crassa*

From left to right: Lane 1 **Δ**S RNA quinic acid with qa-2 primers, Lane 2 100 bp DNA ladder, Lane 3 ΔS RNA dextrose with qa-2 primers.


100 bp ladder
ΔS RNA in 0.3% quinic acid with qa-2 primers (300 bp)
ΔS RNA in 2% dextrose with qa-2 primers (300 bp)

To determine the effectiveness of the primer designed to amplify the *qa-2* RNA, RT-PCR was performed on RNA from the ΔS strain of *N.crassa* grown in the presence of either quinic acid or dextrose. Figure 4.2 shows that *qa-2* RNA can be detected in the presence of both dextrose and quinic acid RNA of ΔS strain of *Neurospora crassa*. This confirms the results of Northern blot analysis that in the absence of the repressor (*qa-1S*) *qa-2* gene product is being expressed in both dextrose and quinic acid (Arnett 2009).

To determine the effectiveness of our primers designed to detect RNA from the qa-y gene, RT-PCR was performed on RNA isolated from the ΔS strain grown as described earlier. Figure 4.3 shows that *qa-y* RNA can be detected in RNA from tissue grown in the presence of quinic acid but cannot be detected in the RNA from ΔS tissue grown in the presence of dextrose. This confirms the Northern blot results that show that the *qa-y* gene is not being expressed in dextrose in the ΔS strain of *N.crassa* (Arnett 2009).

4.2 qPCR on RNA from the ΔS strain of *N.crassa*:

In order to obtain a better quantitation of mRNA levels of the *qa-2* and *qa-y* transcripts in the strain of *N.crassa* containing the ΔS mutation, the qRT-PCR is performed. RNA was isolated from the ΔS strain after growth on either 0.3% quinic acid or 2% dextrose. Dilutions of this RNA were used for qRT-PCR reactions. The qRT-PCR reactions were performed as described earlier (See materials and methods).

Earlier work in our laboratory had suggested that the 18S rRNA gene on *N.crassa* would be a more constant internal control under the growth conditions that we had been

using than the (his-3) gene that we had been using (M.Fleeger, MS Thesis 2010). Therefore we began using the 18S rRNA as the internal control in our qRT-PCR experiments.

In qRT-PCR the threshold cycle (C_T value) serves as a tool for calculation of the starting template amount in each sample. This is the cycle in which there is the first detectable significant increase in fluorescence. The graph is plotted using the BIO-RAD qRT-PCR software taking log of amount of RNA taken in triplicate Vs threshold cycle thereby giving the efficiency of 18S rRNA primer on dextrose grown ΔS RNA.

The mean c_T values obtained from the qRT-PCR are taken on y-axis and 4 dilutions made for each ΔS RNA sample is taken on x-axis and is plotted. The higher the R^2 value is close to 1 the better the dilutions are made.

Figure 4.3: Gel Electrophoresis of *qa-y* cDNA from the RNA samples prepared from **ΔS** strain of *Neurospora crassa*

From left to right: Lane 1 **ΔS** RNA quinic acid with qa-y primers, Lane 2 100 bp DNA ladder, Lane 3 **ΔS** RNA dextrose with qa-y primers.

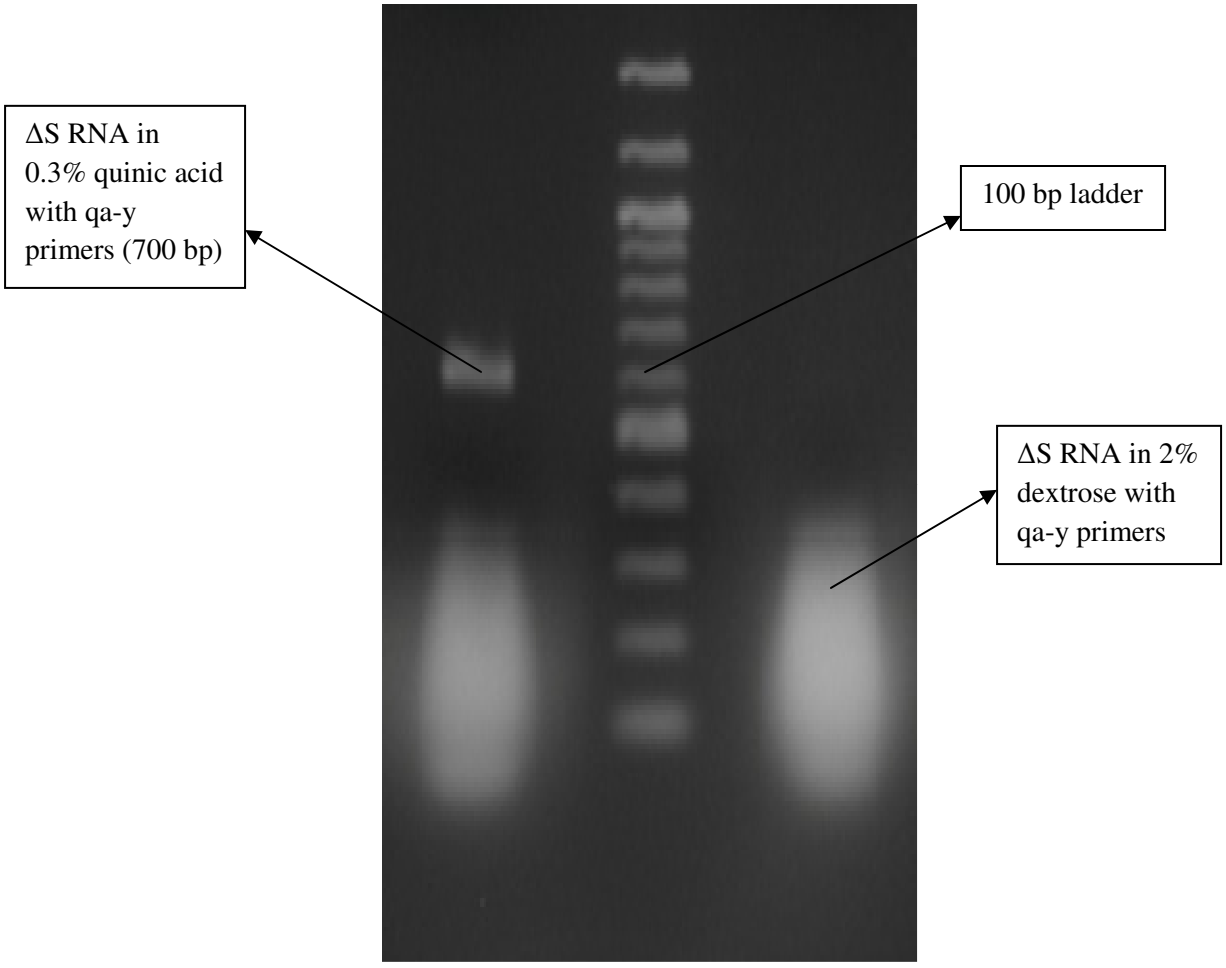
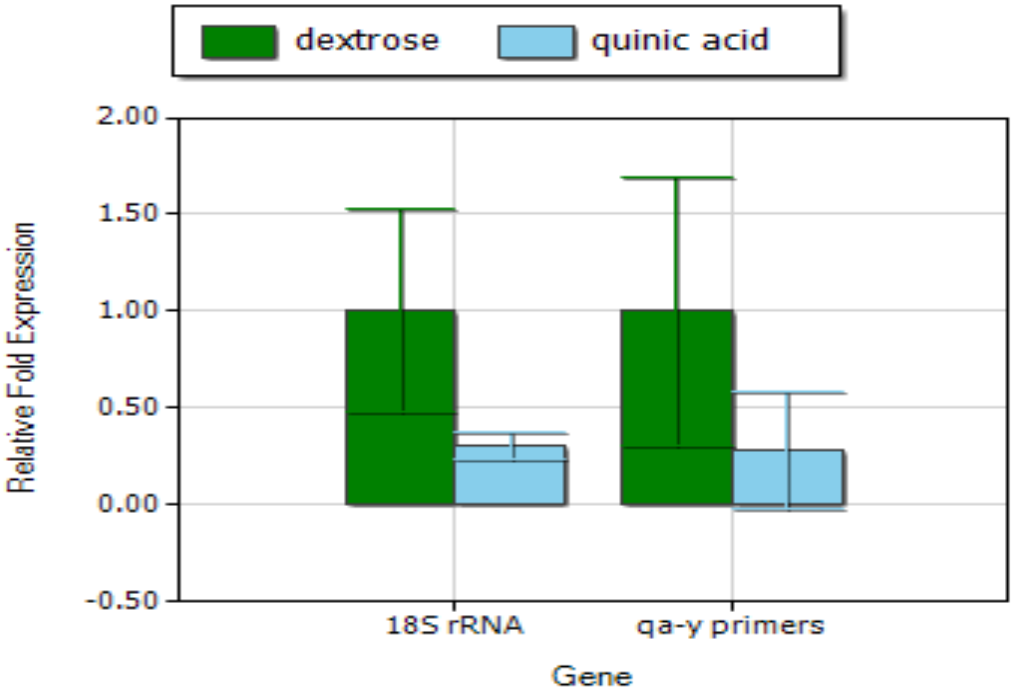

ΔS RNA in 0.3% quinic acid with qa-y primers (700 bp)
100 bp ladder
ΔS RNA in 2% dextrose with qa-y primers

Figure 4.4 represents the gene expression of *qa-y* gene using 18S rRNA as a control. Both the 18S rRNA primers and the qa-y primers in 2% dextrose are set to a fold expression of 1 is taken as standard. In the presence of 0.3% quinic acid 18S rRNA showed a fold expression of 0.4 and *qa-y* gene showed the fold expression of 0.6. An unwanted bump has been observed in the melt curve of 18S rRNA with *qa-y* gene. That might be a reason for a lesser fold expression of 18S rRNA.

Figure 4.5 represents the gene expression of *qa-2* gene using 18S rRNA as a control. Both the 18S rRNA primers and the qa-2 primers in 2% dextrose are set to a fold expression of 1 is taken as standard. In the presence of 0.3% quinic acid 18S rRNA showed a fold expression of 0.8 and *qa-2* gene showed the fold expression of 1. This suggests that both 18S rRNA and *qa-2* expressed the same amount in the presence of 0.3% quinic acid.

Figure 4.4: Graph relative to zero for the 9.9 ng dilution of RNA with *qa-y* primers

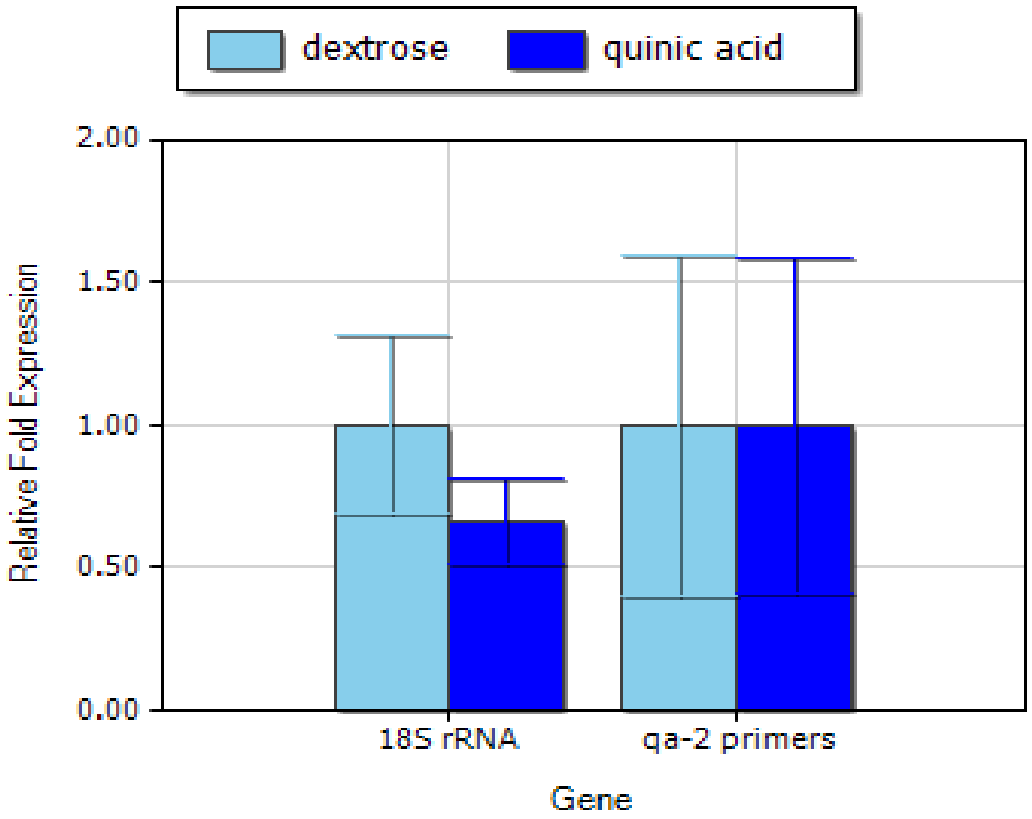
The below graph represent the comparison of expression of 18S rRNA and *qa-y* gene of 9.9 ng of ΔS strain RNA in the presence of dextrose and quinic acid.



Gene Expression : VRJ 2010-05-11 1159 qa-y,2.opd

Figure 4.5: Graph relative to zero for the 9.9 ng dilution of RNA with *qa-2* primer

The below graph represent the comparison of expression of 18S rRNA and *qa-2* gene of 9.9 ng of ΔS RNA in the presence of dextrose and quinic acid.



Gene Expression : vrj 2010-03-31 1231 qay-2.opd

4.3 Phenotype of the strain of *N.crassa* containing the *qa-x*$^{-}$ mutation under various growth conditions:

It had been observed that the only phenotype of the strain of *N.crassa* containing the *qa-x*$^{-}$ mutation was the production of a brown pigment when grown on quinic acid as a carbon source (Case 1992). In order to determine if the pigment is produced under other conditions both the wild-type strain of *N.crassa* and the strain containing the *qa-x*$^{-}$ mutation were inoculated in Vogel media containing either dextrose alone, quinic acid alone or quinic acid and dextrose together as carbon sources. The results are shown in Table 4.1.

Table 4.1: Production of brown pigment of *qa-x*$^{-}$ strain and wild-type 74A strain of *N.crassa* grown under various conditions

S.No	Strain of *N.crassa*	2% Dextrose	0.3% quinic acid	color obatined
1	*qa-x*⁻ strain	yes	no	colorless
2	*qa-x*⁻ strain	no	yes	deep brown
3	*qa-x*⁻ strain	yes	yes	light brown
4	wild type *74A*	yes	no	colorless
5	wild type *74A*	no	yes	colorless
6	wild type *74A*	yes	yes	colorless

4.4 Physical characteristics of brown pigment obtained from the *qa-x*$^{-}$ strain of *N.crassa*:

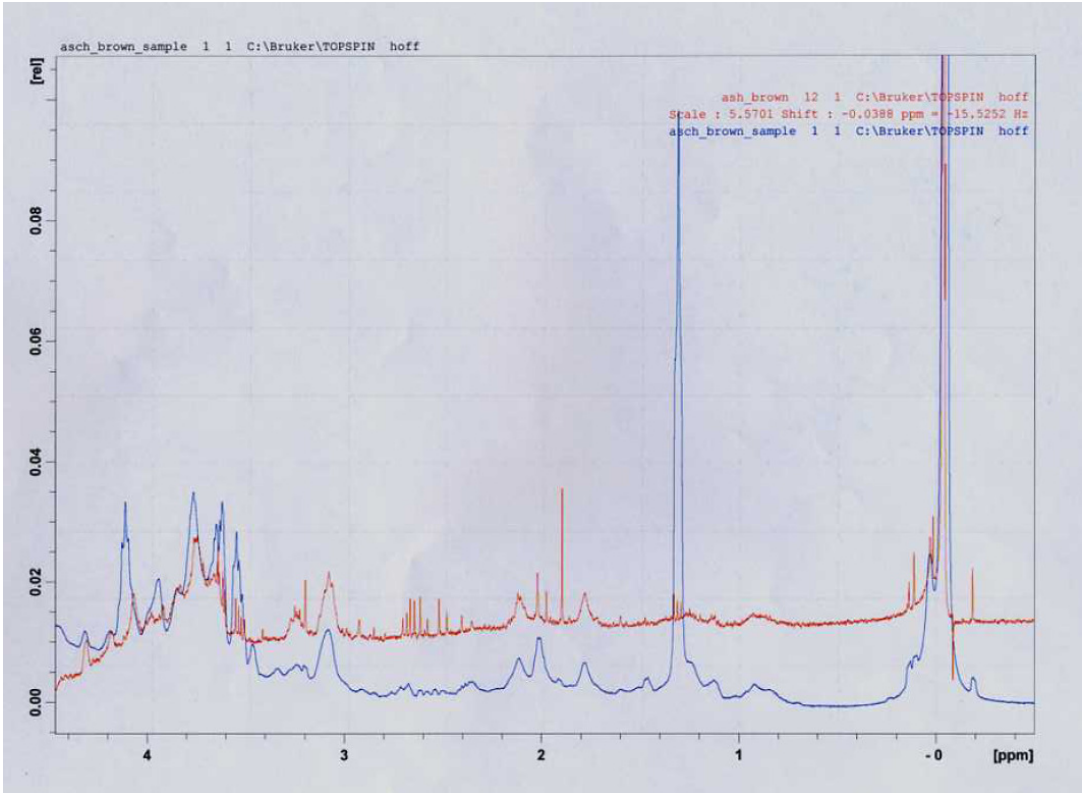
In order to further characterize the phenotype of the *qa-x*$^{-}$ mutation, the brown pigment obtained from the *qa-x*$^{-}$ strain of *N.crassa* was dialyzed against 1X TBE buffer (pH 8) using 0.45micron dialysis tubing for 3 days, the brown pigment was found to stay in the dialysis tube thereby suggesting that the molecular weight of the compound might be greater than 10,000 Da.

The brown pigment was found to be stable to both boiling and freezing up to -70°C. It was found to precipitate when it is acidified with Concentrated HCl to pH less than 4. It can be lyophilized to a crystal/powder form which can in turn be re-dissolved in water. It is soluble in water at a pH range from 6.5 to 7.5 at temperatures from 0°C to 100°C. It also shows maximum absorbance at 320nm.

When centrifuged on 5,000Da and 10,000Da filter tubes overnight, the sample is found to stay in the tube also suggesting that the molecular weight of the compound is greater than 10,000Da. The brown pigment when centrifuged through 30,000Da and 100,000Da filter tubes, showed a slight brown color from 30,000Da tube and thick brown color solution from 100,000Da tube. This suggests that varieties of compounds are present in the brown solution that is being analyzed.

The dots spotted on thin layer chromatographic plate did not move when developed with a variety of organic solvents under UV light or with Iodoform spray.

Figure 4.6: 1H NMR spectra of brown pigment from *qa-x*$^{-}$ strain of *N.crassa*


asch_brown_sample 1 1 C:\Bruker\TOPSPIN hoff
ash_brown 12 1 C:\Bruker\TOPSPIN hoff
Scale : 5.5701 Shift : -0.0388 ppm = -15.5252 Hz
asch_brown_sample 1 1 C:\Bruker\TOPSPIN hoff
[rel]
0.08
0.06
0.04
0.02
0.00
4
3
2
1
-0
[ppm]

1H NMR data obtained from the brown pigment of *qa-x*$^{-}$ strain of *N.crassa* before running a reverse phase silica column (in red) and also after running the reverse phase silica column (in blue) (Figure 4.6) contained peaks that are complicated to identify the compound. The 1H NMR spectra in blue represents the 1H NMR performed after running the brown sample through reverse phase silica column.

4.5 Bioinformatic results of *qa-x* gene present in the *qa* gene cluster of *Neurospora crassa:*

In order to further determine the functions of the *qa-x* gene and its relationship to the quinic acid gene cluster in both *N.crassa* and other fungi, a bioinformatic analysis of the *qa-x* gene of *N.crassa* was undertaken. The National Center of Biotechnology Information (NCBI) website is used to retrieve the primary sequence of *qa-x* gene

Basic local alignment sequence tool (BLAST) tool was used to find the similarity of the QA-X protein sequence with other fungi. The percent similarity with *qa-x* sequence in other fungi is shown in figure 4.7.

4.5.1 Multiple Sequence Alignment:

The primary sequences of the related organisms were retrieved multiple sequence alignment is performed using ClustalW software. The results are shown in Table 4.3

Figure 4.7: BLAST search sequence of product of *qa-x* gene of *Neurospora crassa*

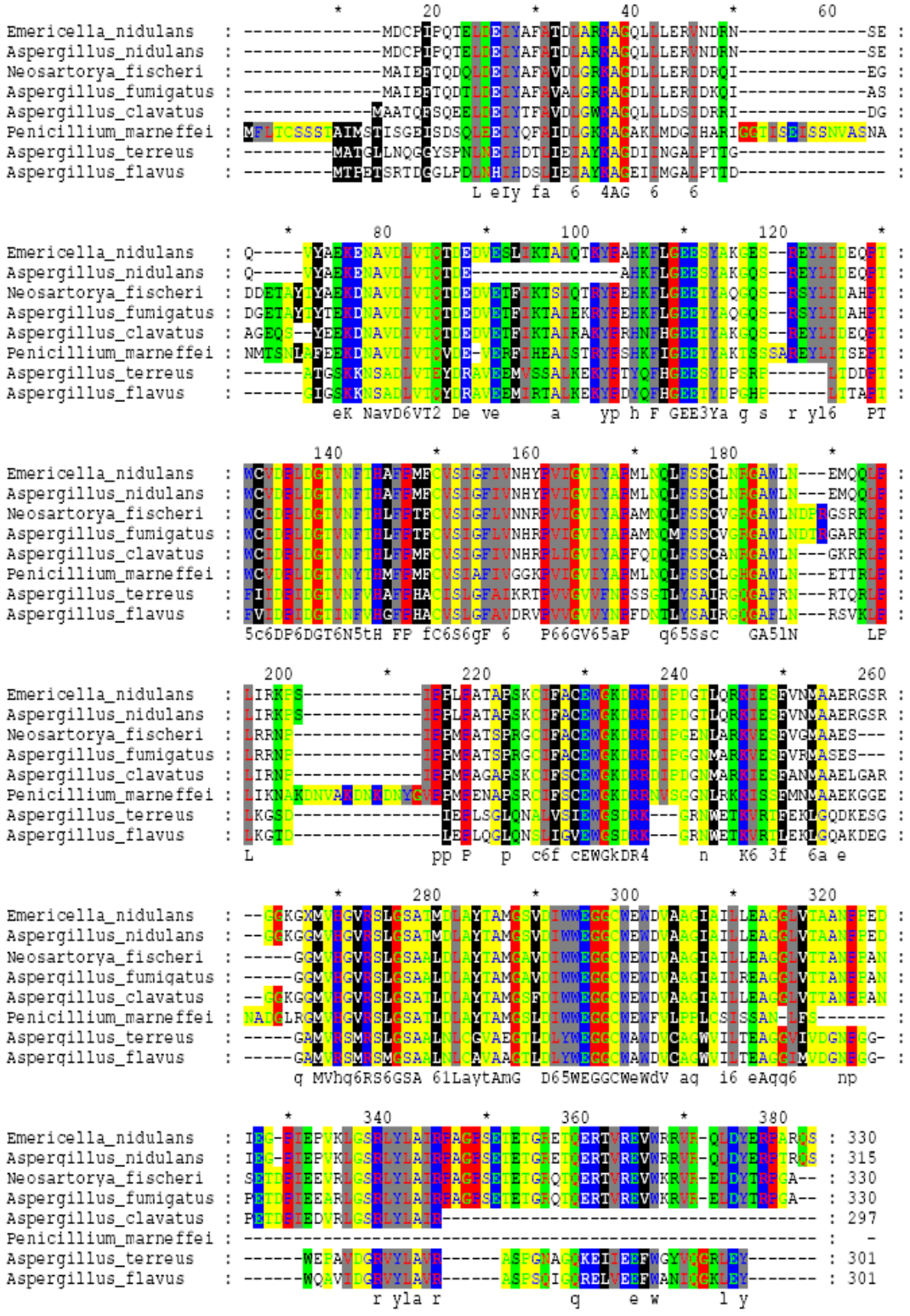
>gi|295928|emb|CAA32748.1| qa-x [*Neurospora crassa*]

MTSRTTTATELDEIYTFAVQLGKDAGNLLMEAARLRFSNNNANHDKESTTQEFT
EKDSAVDIVTQTDEDVEAFIKSAINTRYPSHDFIGEETYAKSSQSTRPYLVTHTTP
TWVVDPLDGTVNYTHLFPMFCVSIAFLVDGTPVIGVICAPMLGQLFTACKGRGA
WLNETQRLPLVRQPMPKSAPGGCVFSCEWGKDRKDRPEGNLYRKVESFVNMA
AEVGGRGGKGGMVHGVRSLGSATLDLAYTAMGSFDIWWEGGCWEWDVAAGI
AILQEAGGLITSANPPEDWATAEIPDVKLGSRLYLVVRPAGPSEGETAREGQERTI
REVWRRVRALDYTRPGA

Table 4.2: Organisms that contain similar sequence as the *qa-x* gene of *N.crassa*

ORGANISM	Similarity with qa-x in *N.crassa*
Aspergillus flavus	67%
Emericella nidulans	67%
Neosartorya fischeri	65%
Aspergillus fumigatus	64%
Aspergillus nidulans	64%
Aspergillus clavatus	65%
Pencillium marneffei	62%

Table 4.3: Multiple sequence alignment of QA-X from *qa-x* gene of *N.crassa* and related species obtained from BLAST search



The “*” symbol represents no conservation at all.

Based on the BLAST search and multiple sequence alignment between the related species, approximately 65% similarity of other fungal organisms with QA-X protein sequence shows that the enzyme that is encoded by *qa-x* gene has great similarity to inositol monophosphatase (IMPase). Inositol monophosphatase is found to dephosphorylate inositol phosphate to inositol. IMPase is also involved in recycling and *denovo* synthesis of glucose to inositol.

4.5.2 Phylogenetic analysis of *qa-x* gene in *Neurosporacrassa*:

The relationship between the fungal organisms is sorted out by MEGA4 (Molecular evolutionary genetic analysis) software. The phylogenetic tree for the choosen organisms from the BLAST search is shown in Figure 4.8

4.5.3 Results for the neighboring genes of IMPase in all the related species of *Neurospora crassa*:

Joint genome institute (JGI), MIT broad institute and NCBI genome table provided information on the clustering and the neighboring genes of all the aspergillus species related to *Neurospora crassa*. Quinate- 5 dehydrogenase, quinatedehydratase, dehydrogenase are some of the neighbors to the IMPase.

Figure 4.8: Phylogenetic tree of *N.crassa* with the related species

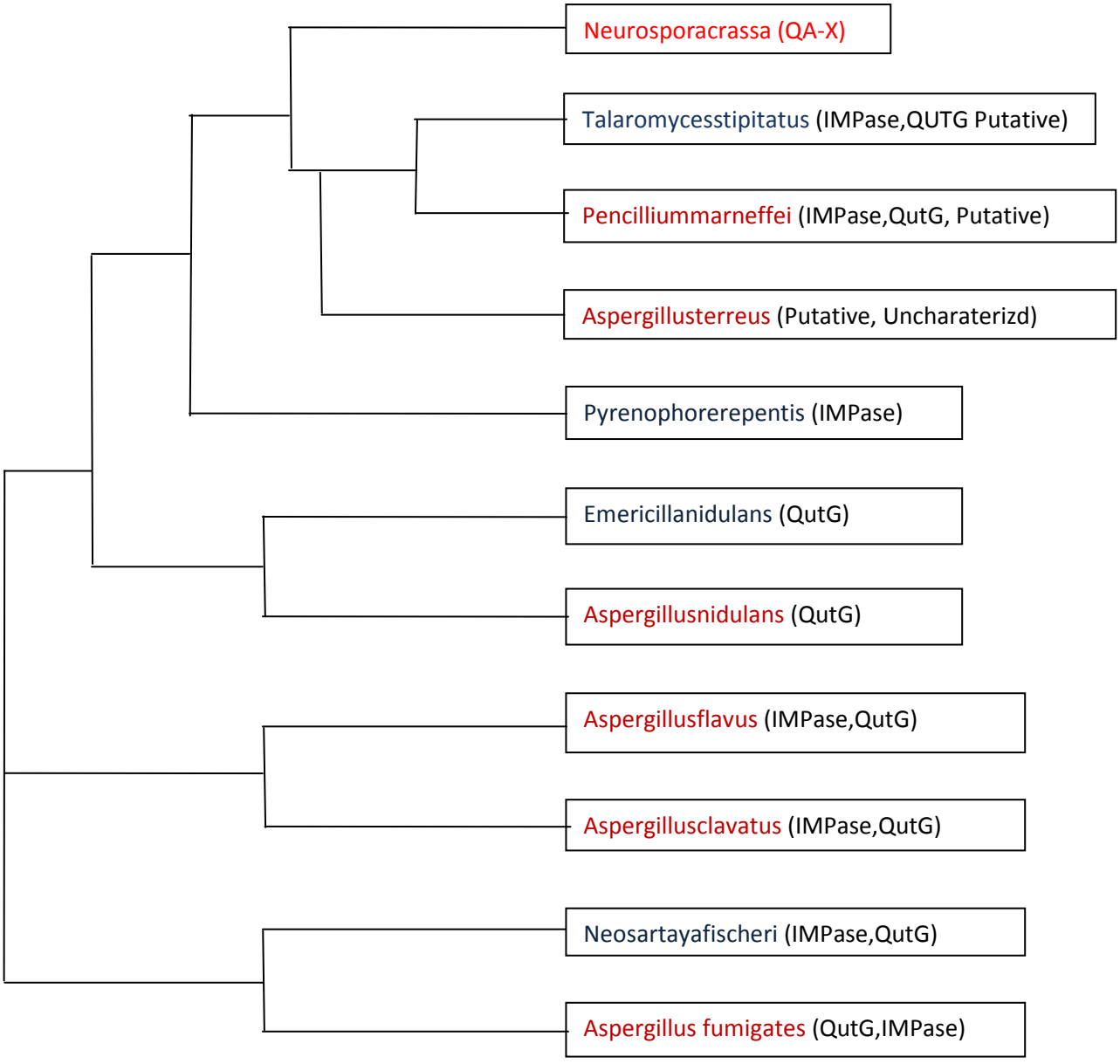

Neurosporacrassa (QA-X)
Talaromycesstipitatus (IMPase,QUTG Putative)
Pencilliummarneffei (IMPase,QutG, Putative)
Aspergillusterreus (Putative, Uncharaterizd)
Pyrenophorerepentis (IMPase)
Emericillanidulans (QutG)
Aspergillusnidulans (QutG)
Aspergillusflavus (IMPase,QutG)
Aspergillusclavatus (IMPase,QutG)
Neosartayafischeri (IMPase,QutG)
Aspergillus fumigates (QutG,IMPase)

Figure 4.9: The neighboring genes of *Aspergillus flavus* that are related to QA-X function

A.Flavus: [57.9812 bitscore]

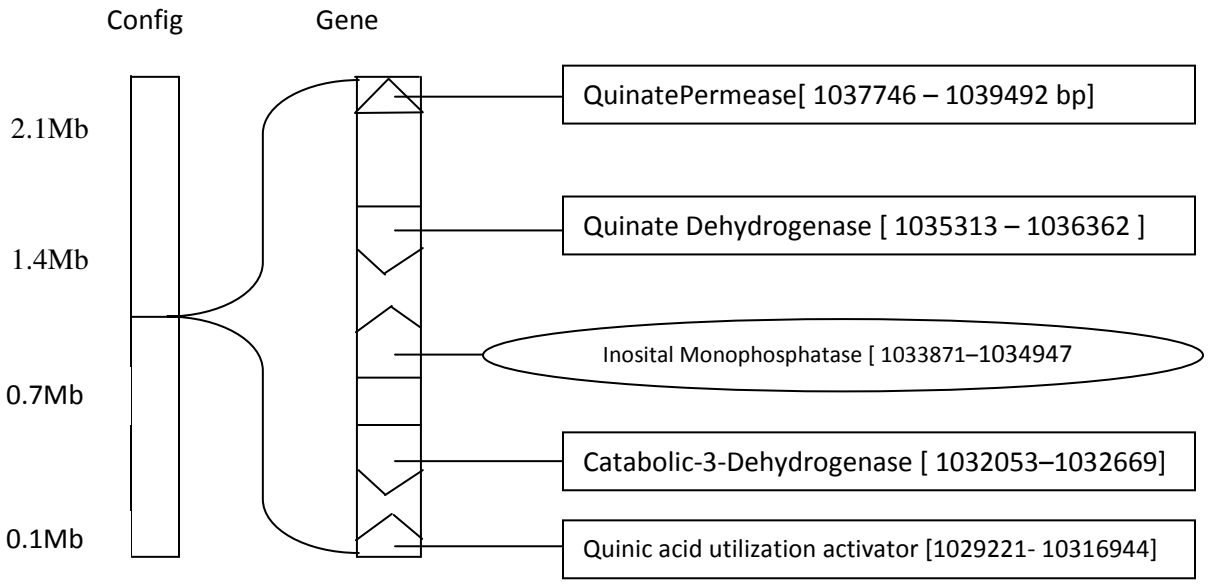


Figure 4.10: The neighboring genes of *Aspergillus fumigatus* that are related to QA-X function

A.Fumigatus: [50.0517 score]

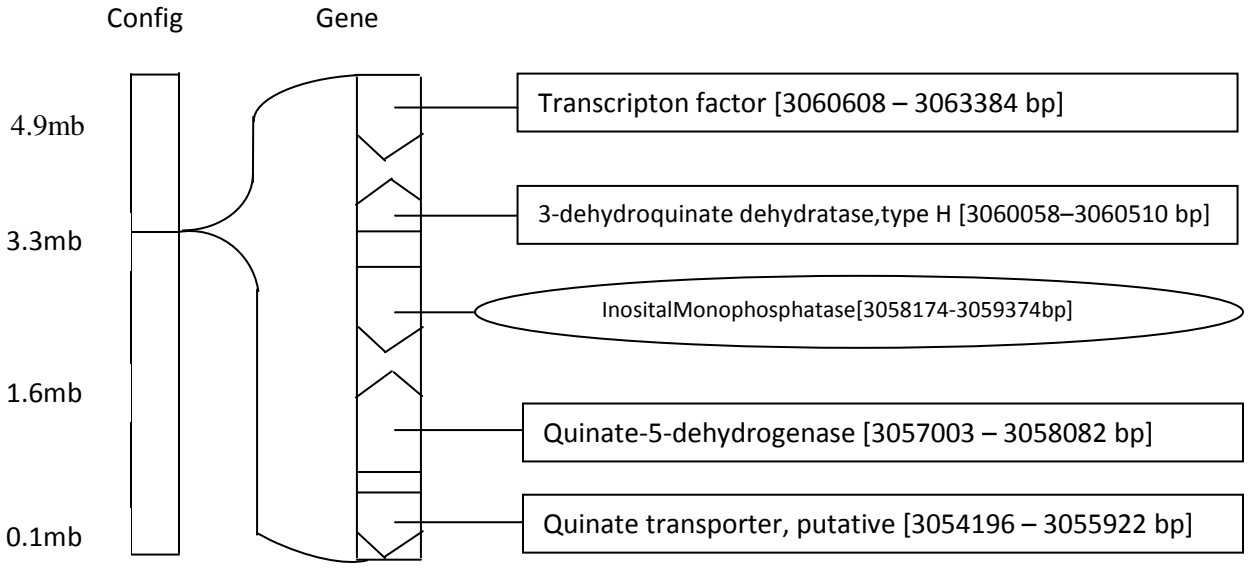


Figure 4.11: The neighboring genes of N*eosartorya fischeri* that are related to QA-X function

N.Fischeri: [50.0517 score]

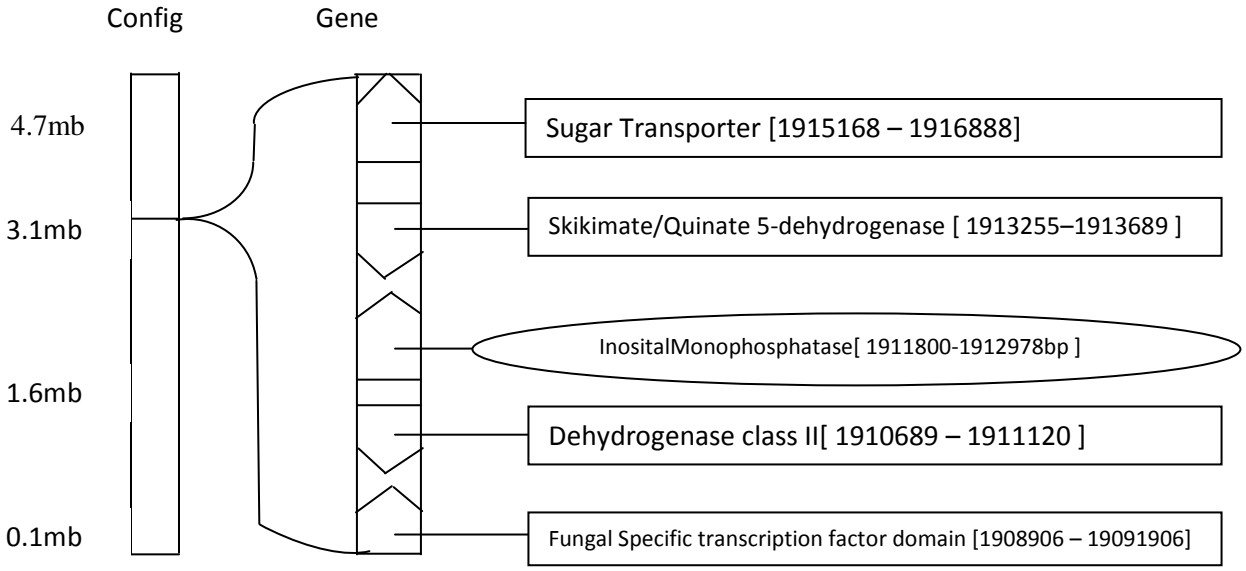


Figure 4.12: The neighboring genes of *Aspergillus* n*idulans* that are related to QA-X function

A. Nidulans: Linkage group VIII [87.7165 score]

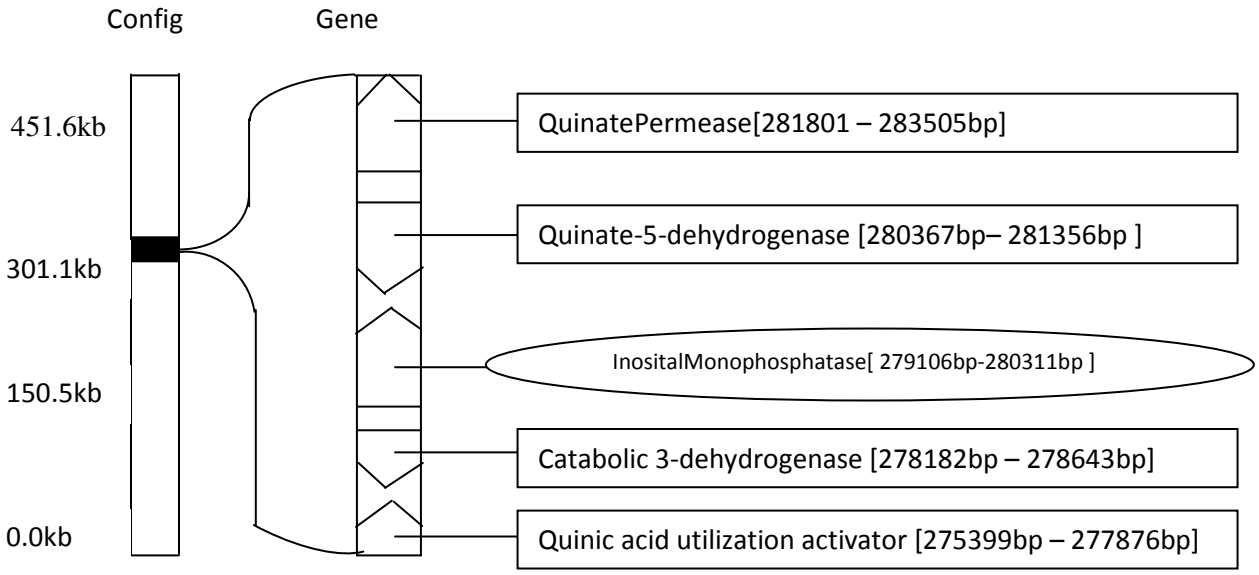
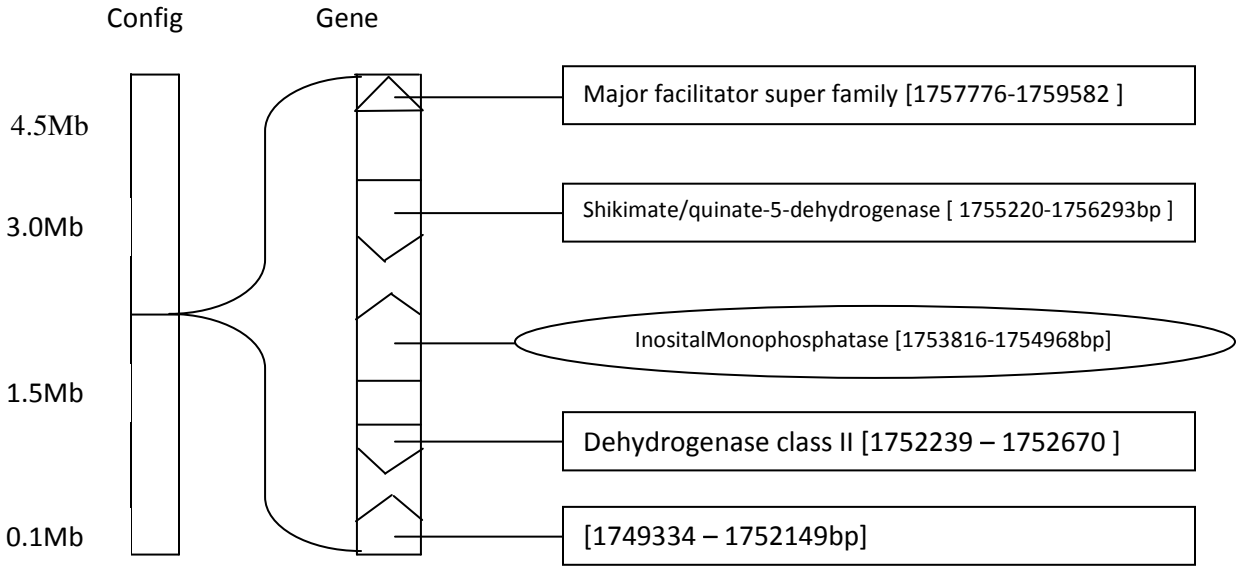


Figure 4.13: The neighboring genes of *Aspergillus clavatus* that are related to QA-X function

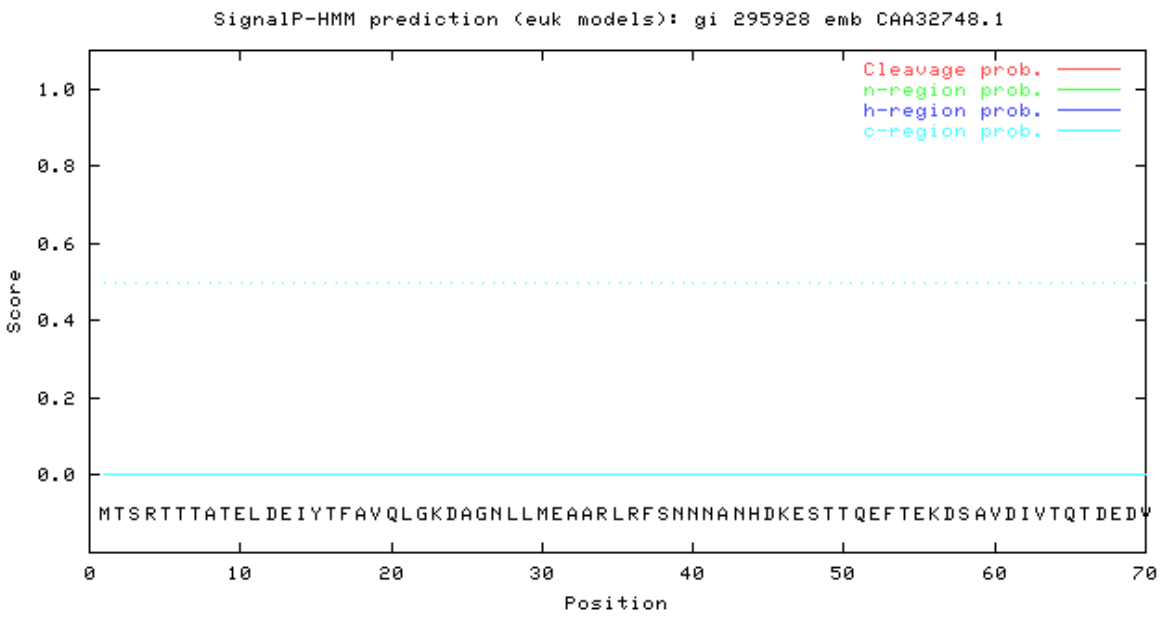
A.Clavatus: [69.8753 score]



4.5.4 Results for the structural analysis and properties of IMPase protein:

The **Pfam** database provided the information that the structural analysis of IMPase proteins has revealed a common core of 155 residues, which includes residues essential for metal binding and catalysis. An interesting property of the enzymes of IMPase family is their sensitivity to Li^+. The targets and mechanism of action of Li^+ are yet unknown. The **Scop** (Structural classification of proteins) revealed that IMPases belong to multi domain proteins (alpha and beta), one domain being IMPase and has a carbohydrate phosphatase fold and belongs to the family of inositol monophosphatase/ fructose 1-6 bis phosphatase like family. The **SignalP 3.0** server is used to predict if the protein is secretory or not. SignalP used both Neural networks (NN) and Hidden markov models (HMM) to predict the protein property. Figure 4.14 plot suggests that the QA-X protein encoded by *qa-x* gene in the quinic acid gene cluster is a non-secretory protein.

Figure 4.14: SignalP prediction for the sequence of QA-X protein encoded by *qa-x* gene of *N.crassa*



>gi_295928_emb_CAA32748.1_

Prediction: Non-secretory protein

Signal peptide probability: 0.000

Signal anchor probability: 0.000

Max cleavage site probability: 0.000 between pos. 25 and 26

CHAPTER V

CONCLUSIONS AND FUTURE WORK

RT-PCR successfully confirmed the efficiency of our primers, RNA expression of quinic acid (*qa*) genes in **Δ**S strain of *Neurospora crassa* grown in 2% dextrose and/ or 0.3% quinic acid. This result confirmed earlier data obtained by Northern blot (Arnett 2009).

Quantitation of mRNA levels of *qa-y* and *qa-2* genes in the quinic acid (*qa*) gene cluster is proved to be successful using 18S rRNA as the control although the efficiency values are not seen as expected. Both the 18S rRNA primers, qa-y primers and qa-2 primers in 2% dextrose are set to a fold expression of 1 is taken as standard. In the presence of 0.3% quinic acid 18S rRNA showed a fold expression of 0.4 and *qa-y* gene showed the fold expression of 0.6. In the presence of 0.3% quinic acid 18S rRNA showed a fold expression of 0.8 and *qa-2* gene showed the fold expression of 1. Since the efficiency values of most of the primers used are greater than 100%, the dilution of RNA might give desired results with the efficiency values. Trying a different kind of control might also help the efficiency of the qRT-PCR reaction. All the dilutions for each sample are found to be within three cycles of the qRT-PCR reaction.

The phenotype of the *qa-x*$^{-}$ strain of the *Neurospora crassa* under 2% dextrose and/or 0.3% quinic acid was determined. Interestingly *qa-x*$^{-}$ strain in 2% dextrose did not make the brown pigment but the *qa-x*$^{-}$ strain with 0.3% quinic acid and 2% dextrose did make the brown pigment. Analysis of the brown pigment produced by the *qa-x*$^{-}$ strain of

Neuropsora crassa in 0.3% quinic acid using 1H NMR graph showed numerous peaks indicating the presence of various functional and aromatic groups that are complicated to interpret. The future work might include using a liquid chromatography-Mass spectrometry (LC-MS) on the brown pigment to find out the actual molecular weight of the substance and to see if any compounds have similar molecular weight in the tyrosine to melanin synthesis pathway.

The bioinformatic analysis proved that the *qa-x* gene might have Inositol mono-phosphatase (IMPase) activity. Inositol mono-phosphatase dephosphorylates inositol phosphate to inositol. IMPase is also involved in recycling and *denovo* synthesis of glucose to inositol. The future work can focus on investigation of how the IMPase and the melanin pathway are related. Obtaining a 3D structure for the IMPase might yield more information on IMPase activity in *qa-x*$^{-}$ strain of *Neurospora crassa*.

CHAPTER VI

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