Induction of the *qa-y* and *qa-1F* Genes in *Neurospora crassa* at Differing Times of Quinic Acid Exposure

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ABSTRACT

Neurospora crassa, a model filamentous fungus, contains the quinic acid (*qa*) gene cluster, allowing the organism to effectively metabolize quinic acid as an alternative carbon source. In the presence of quinic acid, the *qa* gene cluster is significantly up-regulated, showing an increased expression of both regulatory proteins as well as the structural proteins required for the utilization of quinic acid as an energy source. Of these structural genes, *qa-2*, *qa-3*, and *qa-4* encode for the enzymes required for the conversion of quinic acid to protocathechuic acid while *qa-y* encodes for a quinate permease and the function of *qa-x* remains unknown.

The present study aims to elucidate the expression pattern of *qa-y*, the quinate permease-producing gene required for efficient uptake and utilization of quinic acid in *N. crassa*, as well as *qa-1F*, the activator protein of the *qa* gene cluster, at differing times of quinic acid exposure. Quantitative real-time PCR (RT-qPCR) using reverse transcriptase was first used to determine Histone-3 as the optimal housekeeping reference gene. This gene was then used to accurately quantify the expression of both *qa-y* and *qa-1F* at differing times of exposure to quinic acid (0min, 15min, 30min, 1hr, 2hr, and 3hr).

The expression of *qa-y* was observed to steadily increase for the first 30 minutes, showing up to a 17-fold change in expression after only 30 minutes following introduction of quinic acid. A prompt response of the *qa-1F* gene was also observed. At the 15-minute time point, a 3-fold increase in expression of the *qa-1F* occurred, presumably to initiate activation of transcription of the genes of the *qa* gene cluster, including itself. When combined, these data allow for a comprehensive representation of the timing and method of regulation of the *qa* gene cluster in *N. crassa*.

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CHAPTER 1

LITERATURE REVIEW

I. *NEUROSPORA CRASSA*

While many organisms have proven to be effective model organisms for gene expression studies, *Neurospora crassa* has shown to be one of the most useful models of representation in the research of systems involving complex eukaryotic biochemical pathways. First described by Payen in 1843 as a contaminant in French bakeries, this "champignons rouges du pain" (translated: red-bread mold) was not classified as the new genus *Neurospora* until 1927, following the observance of a complete sexual cycle as well as the presence of grooved ascospores (Perkins, 1992). The work of Beadle and Tatum in 1941 further advanced the role of *N. crassa* as a model organism. Beadle and Tatum utilized *N. crassa* as a model organism to observe how specific genes controlled biochemical reactions. The results from this work strongly supported the "one gene-one enzyme" hypothesis and further promoted the use of *N. crassa* as a model enzyme for biochemical genetics (Beadle and Tatum, 1941).

With the emergence of the gram-negative bacterium *Escherichia coli* as the new model organism for biochemical genetics in the mid-1900s, the role of *N. crassa* was slightly diminished. While *E. coli* is an attractive model organism (rapid growth rate, simple nutritional requirements, well-established genetics and completed genomic sequence) for linear biochemical pathways, it cannot accurately represent complex eukaryotic pathways, as can *N. crassa*. Finding its niche, *N. crassa* is

currently recognized as the model filamentous fungus, representing the eukaryotic processes such as mitochondrial inheritance, meiotic recombination, gene conversion, meiotic drive, metabolic pathway regulation, cellular development and differentiation, gene silencing and DNA methylation, circadian rhythms, and photobiology (Roche et al., 2014).

In 2003, *Neurospora crassa* became the first filamentous fungi to have its genome completely sequenced (Galagan et al., 2003). The genome of *N. crassa* consists of 41.04 Mb and contains 10,082 protein-coding genes arranged across seven linkage groups. (I-VII). Interestingly, at the time of this research, 5,805 (57%) of the identified *Neurospora* proteins had no significant matches to genes in either of two common yeast model organisms (*Saccharomyces cerevisiae* or *Schizosaccharomyces pombe*), further supporting the niche of *Neurospora* as a preferred model organism for complex eukaryotic pathways (Galagan et al., 2003).

II. Fungal Gene Clusters

Within the genome of many fungal species, there are certain clusters of coordinately regulated genes that participate in a common metabolic or developmental pathway. These eukaryotic gene clusters resemble prokaryotic operons and may have arisen through horizontal gene transfer. The purpose of gene clusters deals with the conservation of energy at times when certain enzymes are not necessary for the viability of the organism or cell. Specifically, fungi possess numerous pathways that have been termed as "dispensable metabolic pathways". This term describes metabolic pathways that are not required for sustainability and

growth under optimal cellular conditions. When exposed to suboptimal growth conditions, these pathways are activated depending on the needs of the cell (Keller and Hohn, 1997).

Within many fungal genomes, there exists some combination of four primary gene clusters that have been extensively studied: the quinic acid gene cluster, the ethanol gene cluster, the proline gene cluster, and the nitrate utilization gene cluster (Keller and Hohn, 1997). The quinic acid gene cluster and the ethanol gene cluster are activated in the absence of a preferred carbon source, such as glucose, and the presence of either quinic acid or ethanol, respectively. The proline cluster differs from the quinic acid and ethanol clusters in that it can also be utilized as an alternative nitrogen source in addition to an alternative carbon source. The nitrate utilization pathway is activated for nitrate assimilation to produce ammonium for utilization by the cell. Other than the four listed, many other gene clusters exist within the fungal kingdom and help to provide metabolic versatility and flexibility to many fungal species to grow and develop in various locations and conditions (Keller and Hohn, 1997).

III. Quinic Acid Gene Cluster

In *Neurospora crassa*, the quinic acid (*qa*) gene cluster is composed of five structural genes and two regulatory genes spanning a 17.3 kb region of DNA. Three of the structural genes, *qa-2*, *qa-3*, and *qa-4* encode for enzymes required for the conversion of quinic acid to protocathechuic acid (**Figure 1**), while *qa-y* encodes for a quinate permease and the function of *qa-x* remains unknown (Giles et al., 1985).

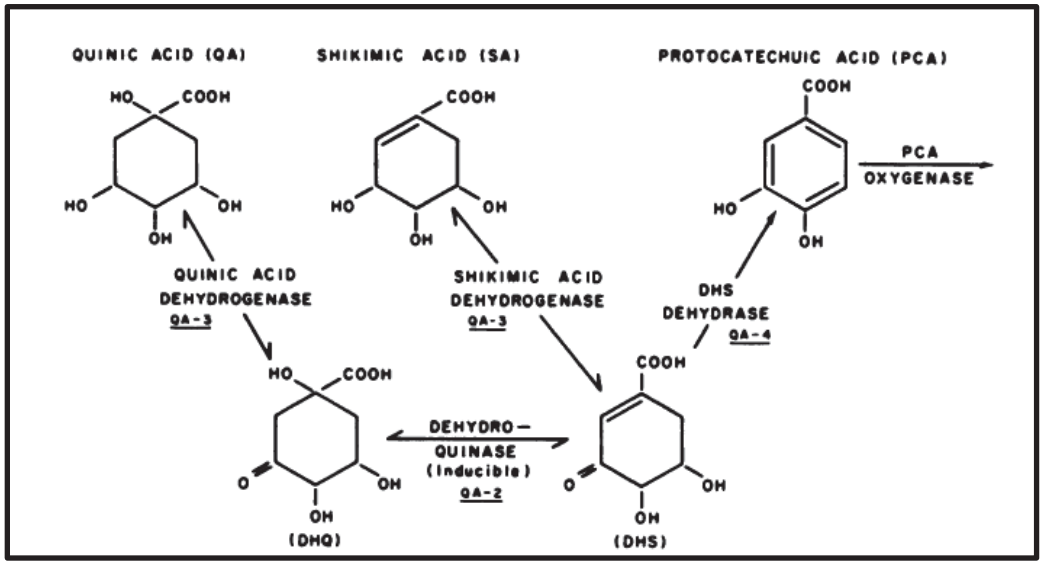

Figure 1: Biological pathway depicting the conversion of quinic acid to protocathechuic acid using protein products of *qa* gene cluster genes. (Giles et al. 1985)

The genes are arranged in three divergently transcribed pairs, with the single unpaired gene, *qa-y*, separating the regulatory genes from the remainder of the *qa* genes (**Figure 2**) (Geever et al., 1989).

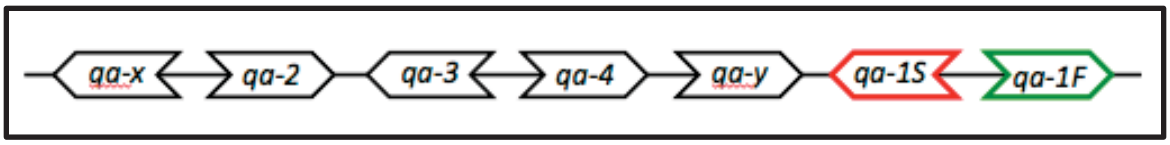

Figure 2: Organization of the *qa* gene cluster in *N. crassa*. The structural genes (*qa-2, qa-3,* and *qa*-4) are separated from the regulatory genes by the *qa-y* gene. Figure adapted from Geever et al. 1989.

Initial studies of the *qa* gene cluster by Rines in 1965 (Ph.D thesis) demonstrated that *qa-2* mutants lacked catabolic 3-dehydroquinate dehydratase (C-DHQase) activity. These studies confirmed the existence of *qa-2* as a structural

gene, producing a protein product responsible for the conversion of 5-dehydroquinate (DHQ) to 5-dehydroshikimate (DHS). This protein, composed of 12 identical subunits, consists a total of 173 amino acids and has a molecular weight of 18.3 kDa (Giles et al., 1985).

The bifunctional enzyme QA/SA dehydrogenase (QDHase), coded for by the *qa-3* gene, exists as a 35.2 kDa, 321 amino acid monomer. This NAD-linked dehydrogenase has the ability to recognize and bind both quinic acid and shikimic acid substrates within its single active site, resulting in their conversion to dehydroquinate and dehydroshikimate, respectively (Chaleff, 1974).

Through mutational analysis, the structural gene *qa-4* was determined to encode dehydroskikimate dehydratase (DHS dehydratase), the final structural enzyme required for the conversion of quinic acid to protocathechuic acid. This 40.5 kDA, monomeric enzyme is composed of 359 amino acids and primarily functions to catalyze the conversion of dehydroshikimate to protocathechuic acid (Giles et al., 1985).

The two additional structural genes, *qa-x* and *qa-y* were initially characterized through DNA/RNA hybridization studies and later confirmed through DNA sequencing data. Both genes produce quinic acid-inducible polyadenylated RNAs that are distinct from those encoded by *qa-2*, *qa-3*, *qa4*, and the two regulatory genes (Patel et al., 1981). The protein produced by *qa-x* contains 340 amino acids and has a molecular weight of 37.3 kDa, while the *qa-y* polypeptide contains 537 amino acids and has a molecular weight of 60.1 kDa (Giles et al., 1985).

Initially, the *qa* gene cluster was thought to be regulated by a single, positively acting regulatory gene containing two functionally distinct regions (Reinert et al., 1981), but DNA-RNA hybridization studies later demonstrated the presence of two distinct regulatory genes, *qa-1S* and *qa-1F*. The results of this study clearly indicated the presence of two non-overlapping DNA fragments, as well as the hybridization of distinctly sized mRNA species (Huiet, 1984). These two regulatory genes, *qa-1S* and *qa-1F*, encode a repressor and activator protein, respectively. The repressor protein is composed of 925 amino acids and has a molecular weight of 100,650, while the activator protein contains 816 amino acids and has a molecular weight of 88,960 (Giles et al., 1985).

IV. Regulation of the Quinic Acid (*qa*) Gene Cluster in *Neurospora crassa*

The expression of the quinic acid (*qa*) gene cluster in *Neurospora crassa* is regulated at the transcriptional level by two distinct mechanisms. The primary level of control allows for increased expression of the activator gene, *qa-1F*, in the presence of quinic acid. As a result, the *qa* gene cluster is up-regulated, increasing the expression of all structural genes, as well as the regulatory genes of the cluster (**Figure 3a**). The secondary regulatory mechanism is by carbon catabolite repression, which returns the expression of the *qa* gene cluster to basal levels in the presence of a preferred carbon source (Giles et al., 1985).

In the absence of quinic acid, it is hypothesized that the repressor protein, *qa-1S*, binds to the activation domain of *qa-1F*, thus blocking its interaction with the transcription machinery and therefore, inhibiting transcription of the *qa* gene

cluster (Geever et al., 1989). When quinic acid is introduced, it is hypothesized to bind to the repressor protein, resulting in its release from the activation domain and ultimately allowing for transcription of the *qa* gene cluster (**Figure 3b**) (Giles et al., 1985).

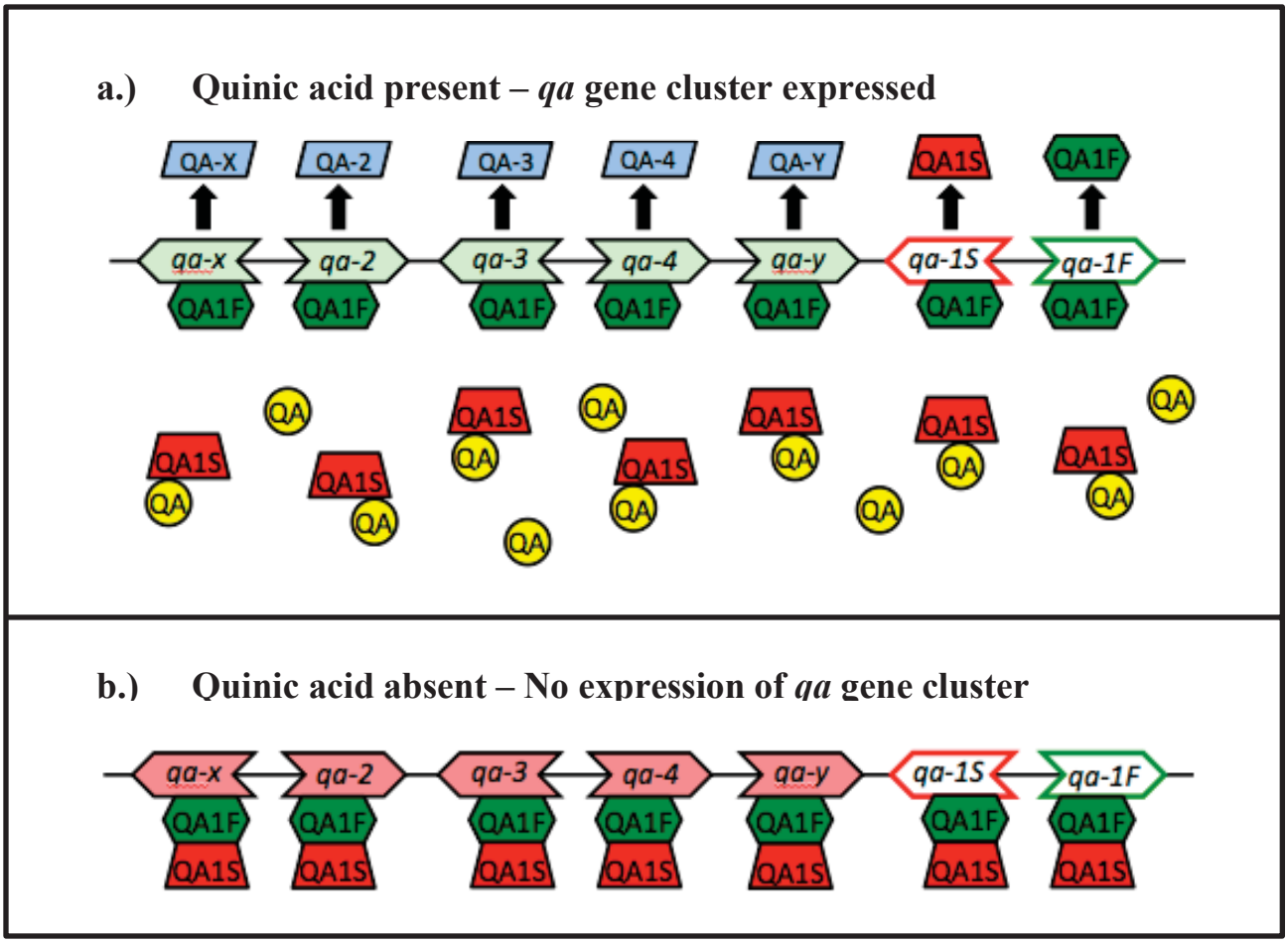

Figure 3: Proposed method of regulation of the *qa* gene cluster in *N. crassa*. Regulation is controlled by two regulatory genes, *qa-1S* and *qa-1F.* **Figure 3a** depicts the system in the presence of quinic acid (QA), which is proposed to release the repressor protein from the activator protein, therefore enabling transcription to occur. **Figure 3b** shows the system in the absence of quinic acid, resulting in no gene product due to the binding of the repressor protein (QA-1S) to the activator protein (QA-1F).

While the exact mechanism of catabolite repression in *N. crassa* is not completely clear, it has been shown that in the presence of a preferred carbon source, such as glucose, *qa* genes are expressed at approximately 10% of the level as when quinic acid is the sole carbon source (Giles et al. 1991). It was first proposed that the structural gene, *qa-x* played a direct role in catabolite repression due to its severely decreased expression when compared to the other *qa* genes in the presence of glucose (Giles et al. 1991). Recent studies have since discredited that hypothesis, as catabolite repression was observed to occur in strains lacking the *qa-x* gene (Arnett et al., 2009). Three alternative methods of catabolite repression have since been proposed. The first model suggests the interaction of trans-acting repressors with cis-acting DNA sequences 5' to the *qa* genes in the presence of a preferred carbon source, therefore blocking transcription. A second hypothesis proposes the repression of transcription of the *qa-1F* gene, leading to repression of the other *qa* genes due to a lack of activator protein. A final hypothesis suggests the repression of quinate permease (encoded by the *qa-y* gene), resulting in a decreased amount of inducer present within the cell and therefore inhibiting the transcription of the *qa* gene cluster (Arnett et al., 2009).

V. QA-Y: QUINATE PERMEASE

In the presence of a preferred carbon source, such as glucose, four of the seven *qa* genes (*qa-x*, *qa-y*, *qa-3,* and *qa-1F*) are expressed at lower levels than when in the presence of an alternative carbon source. Through RNA blot quantitation, a ten-fold repression of the *qa-y* gene was observed in the presence of glucose as

compared to quinic acid (Arnett et al., 2009). This was the most drastically effected gene of the four, suggesting that the inhibition of the expression of the *qa-y* gene may play a pivotal role in catabolite repression. Inhibition of the *qa-y* gene would result in a decreased amount of quinate permease available within the cell and therefore decreased expression of the *qa* gene cluster due to a limited amount of inducer (quinate) entering the cell.

Subsequently, comparison of DNA sequences in two species closely related to *N. crassa* (*N. africana* and *N. terricola*) revealed a highly conserved region upstream of the *qa-y* gene. This data possibly suggests the existence of a catabolite repression mediator binding site, which could possibly possess the ability to regulate the expression of the *qa-y* gene product and therefore the amount of quinate available within the cell (Arnett et al., 2009).

VI. Quantitative Reverse Transcription PCR (RT-QPCR)

The real-time, quantitative polymerase chain reaction using reverse transcriptase (RT-qPCR) is one of the most useful tools for accurate quantitation of gene expression. This technique utilizes a variety of different fluorescent chemistries to collect data from the PCR process as it occurs at each amplification step. RT-qPCR is an extremely useful technique that can produce quantitative data, in the form of a threshold cycle (C_t) value, with an accurate dynamic range of 7 to 8 log orders of magnitude and is sensitive enough to detect even a single copy of a specific transcript. In contrast, the major disadvantages to real-time PCR is the great cost of the instrumentation and reagents, as well as the complexity of the

experimental design and normalization techniques (Heid et al., 1996).

In general, there are two methods of quantitation used following RT-qPCR. In the absolute quantitation method, serial dilutions of standards of known concentrations are used to generate a standard curve. This produces a linear relationship between the Ct and amount of starting RNA, from which the unknown samples can then be interpolated. The other method, relative quantitation, calculates gene expression based on the expression of a reference gene, which should remain constant across all experimental conditions (Wong and Medrano, 2005).

CHAPTER 2

MATERIALS & METHODS

I. TISSUE GROWTH

Wild-type *Neurospora crassa* 74A (FGSC #2489) strain was obtained from laboratory stock collection originating from the Fungal Genetics Stock Center. Samples were grown on Vogel's minimal media (1X Vogel's minimal media (**Tables 1-3**), 2% sucrose, 1.5% agar, and 50mL DI water) for two days at 30°C. Following incubation, the samples were kept at room temperature under fluorescent lights for approximately two weeks. Mature conidia were harvested by adding liquid Vogel's media (1X) to the flask, gently swirling, and then filtering through sterile cheesecloth into a new Erlenmeyer flask. The solution was transferred, in equal volumes, into two flasks containing 50mL of 1X Vogel's + sucrose (2%) and shaken (180 RPM) at 30°C overnight. The resulting mycelia were collected through vacuum filtration using cellulose filter paper (Whatman), washed with sterile water, and vacuum filtrated once again. The washed mycelia were then harvested into flasks containing 50 mL Vogel's + quinic acid (0.3%) and shaken (180 RPM) at 30°C for the specified time points: 0 min, 15 min, 30 min, 1 hr, 2 hr, and 3 hr. Following incubation, the mycelia were vacuum filtrated and the tissue was harvested and stored at -80°C.

TABLE 1: 50X VOGEL'S MINIMAL MEDIA (1 L)

Na_3 citrate, 5.5 H_2O	150 g
KH_2PO_4, anhydrous	250 g
NH_4NO_3, anhydrous	100 g
$MgSO_4$, 7 H_2O	10 g
$CaCl_2$, 2 H_2O	5 g
Trace Element Solution (**Table 2**)	5 ml
Biotin Solution (**Table 3**)	2.5 ml
Distilled H_2O	Bring to final volume of 1 L

TABLE 2: TRACE ELEMENT SOLUTION (1 L)

Citric acid, 1 H_2O	5 g
$ZnSO_4$, 7 H_2O	5 g
$Fe(NH_4)_2(SO_4)_2$, 6 H_2O	1 g
$CuSO_4$, 5 H_2O	0.25 g
$MnSO_4$, 1 H_2O	0.05 g
H_3BO_3, anhydrous	0.05 g
Na_2MoO_4, 2 H_2O	0.05 g
Distilled H_2O	Bring to final volume of 1 L
Chloroform (Preservative)	2 ml

TABLE 3: BIOTIN SOLUTION (50 ML)

Biotin	5 mg
Distilled H_2O	Bring to final volume of 50 ml

II. RNA ISOLATION

A mortar and pestle were used to grind frozen tissue samples into a fine powder using liquid nitrogen. The ground tissue was divided equally into four 1.5 mL Eppendorf tubes and 1.4 mL Ribozol (Amresco) was added to each tube and vortexed. All samples were centrifuged at 12,000 RPM for 10 min, incubated at room temperature for 5 min, and the supernatant was transferred to a new Eppendorf tube. To each tube, 200 µL of chloroform was added and mixed by inversion. Following incubation for 3 minutes at room temperature, samples were centrifuged for 15 min at 12,000 RPM. Following centrifugation, 3 distinct phases should be visible. The clear, upper phase was carefully pipetted into a new Eppendorf tube, being sure not to disrupt the mid-phase. To precipitate the RNA, 500 µL isopropanol was added and the samples were centrifuged at 12,000 RPM for 10 min. The supernatant was discarded, leaving the RNA pellet. Two ethanol (75%) washes of the pellet were performed in the centrifuge at 7,500 RPM for 5 min. Following ethanol washes, supernatant was discarded and the pellets were air-dried. Once sufficiently dry, resulting pellet was resuspended in 200 µL RNase-free water (Amresco) and concentration was determined using the NanoDrop2000 (Thermo Scientific).

To confirm successful RNA isolation, gel electrophoresis was performed using a 1.5% agarose gel. RNA samples (10 µL) were heated for five minutes at 65°C, put on ice, and combined with 3 µL Agarose Gel Loading Dye (6X, Amresco). Samples were loaded into the wells and the gel was run at 60 volts (400 amps) for 90 minutes in TPE buffer (1X). Upon completion, the gel was removed, soaked in a

solution of diluted ethidium bromide (~1/5000) for twenty minutes and visualized using UV light.

III. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

In order to confirm primer specificity, OneStep RT-PCR was performed according to QIAGEN's protocol. In an Eppendorf tube, 10 µL 5x Buffer, 2 µL dNTP, 2 µL Enzyme Mix, 2 µL forward primer, 1 µL reverse primer, and 1.5 µL RNA sample (0.6 µg/µL) were combined and diluted final volume of 50 µL using RNase-free water (Amresco). The samples were placed in the MJ Mini personal thermal cycler (Bio-Rad). Reverse transcriptase was first activated at 50°C for 30 min, followed by the activation of the HotStarTaq DNA Polymerase at 95°C for 15 min. Next, a 3-step cycle was performed for 40 cycles. The cycle consisted of a denaturation step at 95°C for 30 sec, the annealing of primers at 50°C for 30 sec, and extension of the newly formed cDNA strand at 72°C for 1 min. Following 40 cycles, a final extension step at 72°C for 7 min was performed and the samples were held at 4°C. Gel electrophoresis confirmed the amplification of a single transcript for each primer pair tested (**Table 4**).

TABLE 4: PRIMER SEQUENCES

PCR PRIMERS		
GENE	**SEQUENCE**	**DIRECTION**
ACTIN	5’ GCC GAG CGC GAA ATC GTT 3'	Forward
	5’ GCT CTG GGC AGC GGT CTG A 3'	Reverse
HISTONE-3	5' AGA TTC GTC GCT ACC AGA AG 3'	Forward
	5' CGG AGG TCG GAC TTG AAG 3'	Reverse
BETA-TUBULIN	5’ TGG CCG GAA GGT CCG GGT 3'	Forward
	5’ AGT AGC GAC GGG CGG TGT TG 3'	Reverse
QA-Y	5' GGT ATC AAT GCC ATC AAC TAT TAC 3'	Forward
	5' GCC ACA GAA GCC AGA TAA TG 3'	Reverse
QA-1F	5’ TGC CGG GGC AAA GAC ATC CG 3’	Forward
	5’ CCG GGC CTG GTA GAG TGC G 3’	Reverse

IV. QUANTITATIVE REVERSE-TRANSCRIPTION PCR (RT-QPCR)

Quantitative reverse transcription PCR was performed using the iTaq™ Universal SYBR® Green One Step Kit (Bio-Rad). The PCR reaction consisted of 2 µL of RNA (diluted to 100 ng/µL) combined with 18 µL of primer-specific master mix (10 µL iTaq universal SYBR® Green reaction mix (2x), 0.25 µL iScript™ reverse transcriptase, 2 µL of each forward and reverse primer, nuclease-free water up to 20 µL). Reaction components were combined in 96-well plates and real time PCR amplification was completed using an iQ5 Real-Time PCR Detection System (Bio-Rad). The cycling program consisted of denaturation (95 °C for 15 min), followed by amplification and quantitation (45 cycles of 95°C for 30 sec, and 60°C for 30 seconds) followed by a single fluorescence measurement at the end of each cycle and a final extension period at 72°C for 5 min. Upon completion of the protocol, the genes of interest (*qa-y* and *qa-1F*) was analyzed using relative quantitation method with Histone-3 as a reference gene. The comparative threshold cycle ($2^{-\Delta\Delta Ct}$) method

was utilized according to the Applied Biosystems Real-Time Quantitative PCR manual in order to achieve a normalized fold change in expression of each gene of interest compared to the reference gene at the time points of quinic acid exposure being observed.

CHAPTER 3

INDUCTION OF THE *QA-Y* AND *QA-1F* GENES IN *NEUROSPORA CRASSA* AT DIFFERING TIMES OF QUINIC ACID EXPOSURE

I. INTRODUCTION

Depending on the resources that are present within the environment, microorganisms have the ability to regulate their transcriptomes in order to utilize the available carbon sources in the most efficient manner. In the presence of a preferred carbon source such as sucrose or dextrose, many alternative metabolic pathways are repressed to conserve energy. Such a pathway is the quinic acid pathway in *Neurospora crassa*. In conditions where quinic acid exists as the sole carbon source, the appropriate genes of the quinic acid (*qa*) gene cluster must be activated for efficient metabolism to occur (N H Giles, 1991).

The *qa* gene cluster is comprised of seven genes, five structural and two regulatory, that span a 17.3 kB region of DNA located on linkage group VII (**Figure 4**). Of the five structural genes, *qa-2*, *qa-3*, and *qa-4* encode for proteins required for the conversion of quinic acid to protocathechuic acid, while *qa-y* encodes for a quinate permease protein and the function of the *qa-x* protein product is still not completely understood. The regulatory genes *qa-1F* and *qa-1S* encode for an activator and repressor protein, respectively (Geever et al., 1989). DNase I footprinting analyses have previously shown direct binding of the activator protein

QA-IF to the DNA, while it is postulated that the repressor protein binds directly to the activator protein (J A Baum, 1987).

Figure 4: Organization of the *qa* gene cluster in *N. crassa*. The structural genes (*qa-2, qa-3,* and *qa*-4) are separated from the regulatory genes by the *qa-y* gene. Figure adapted from Geever et al. 1989.

The currently accepted model of regulation of the *qa* gene cluster suggests that in the absence of quinic acid, the *qa-1S* repressor protein remains bound to the *qa-1F* activator protein, therefore inhibiting transcriptional machinery to bind to the DNA (**Figure 5a**) (Geever et al., 1989). When quinic acid enters the cell, it is believed to bind to the *qa*-1S repressor protein, thereby releasing it from the activation domain of *qa-1F* and promoting the initiation of transcription (**Figure 5b**) (Giles et al., 1985).

A second regulatory mechanism, carbon catabolite repression, is believed to control the expression of the *qa* gene cluster in the presence of a preferred carbon source. The precise mechanism by which carbon catabolite repression suppresses the expression of the *qa* gene cluster is not yet completely understood. Arnett et al. suggested that a trans-acting repressors may interact with cis-acting DNA sequences that are upstream of the *qa* gene cluster, preventing transcription. Another possibility is the specific repression of either the *qa-1F* gene or the *qa-y* gene. Repression of the *qa-1F* gene would result in less activator protein present, and therefore less transcription of all *qa* genes, while repression of the *qa-y* gene would

result in decreased amounts of permease protein available and therefore affect how much inducer (quinic acid) could enter the cell (Arnett et al., 2009).

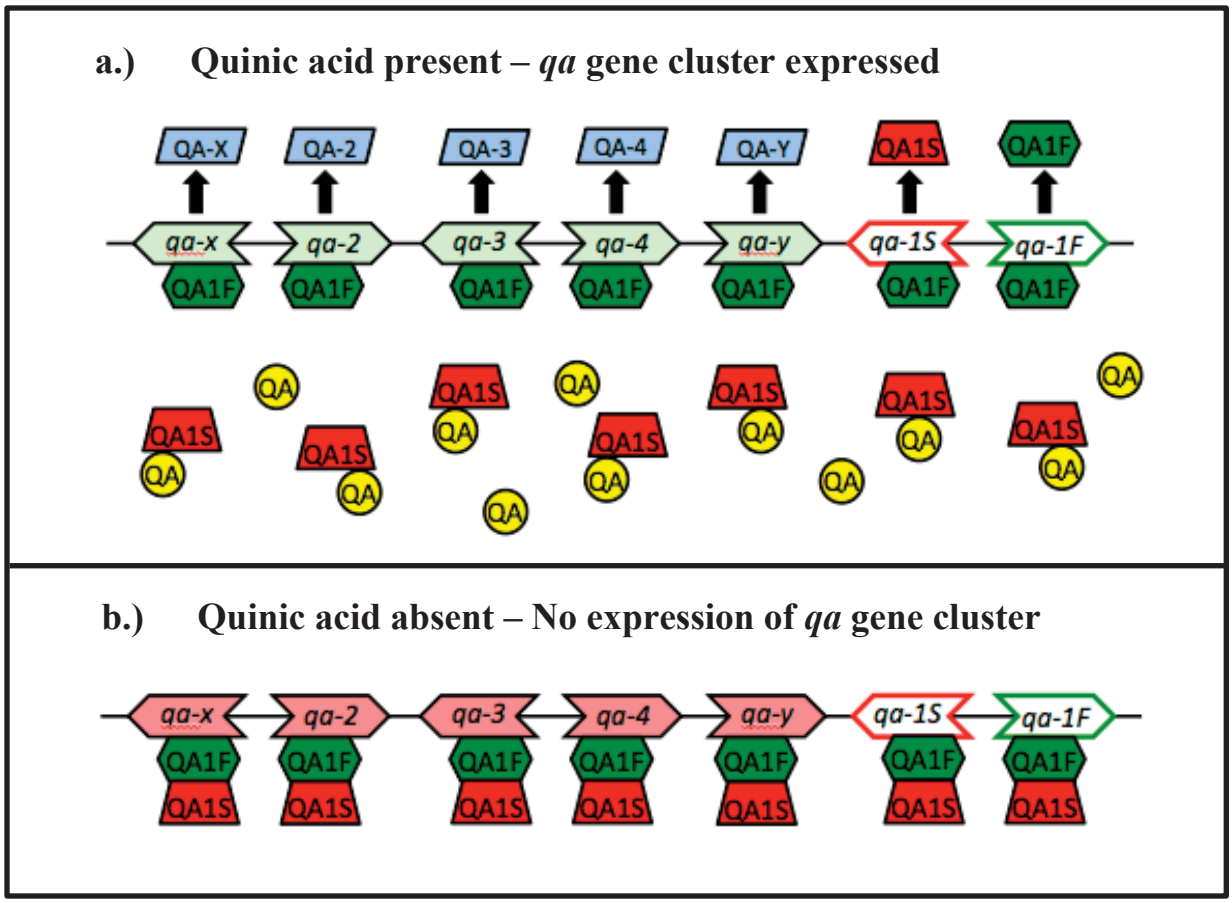

Figure 5: Proposed method of regulation of the *qa* gene cluster in *N. crassa*. Regulation is controlled by two regulatory genes, *qa-1S* and *qa-1F*. **Figure 5a** depicts the system in the presence of quinic acid (QA), which is proposed to release the repressor protein from the activator protein, therefore enabling transcription to occur. **Figure 5b** shows the system in the absence of quinic acid, resulting in no gene product due to the binding of the repressor protein (QA-1S) to the activator protein (QA-1F).

The present study examines how quickly the *qa-1F* and the *qa-y* genes are transcribed in the presence of quinic acid. Logically, these two genes must be the first to be expressed in order for quinic acid to be metabolized. The QA-1F protein is needed to activate transcription of all *qa* genes, including itself, while the QA-Y protein is needed quinic acid to enter into the cell. Observing levels of transcription

at multiple times points following a shift from sucrose to quinic acid allows for a more complete insight to the mechanisms and timing of the utilization of alternative carbon sources in *N. crassa*.

II. MATERIALS & METHODS

I. TISSUE GROWTH

Wild-type *Neurospora crassa* 74A (FGSC #2489) strain was obtained from the laboratory stock collection and grown on Vogel's minimal media, 2% sucrose, and 1.5% agar for two days at 30°C before being placed under fluorescent lights for approximately two weeks at room temperature. Mature conidia were harvested using liquid Vogel's media (1X) and samples were then grown overnight in Vogel's + sucrose (2%). The washed mycelia were then harvested using vacuum filtration, rinsed, and placed into flasks containing 50 mL Vogel's + quinic acid (0.3%) and shaken (180 RPM) at 30°C for the specified time points: 0 min, 15 min, 30 min, 1 hr, 2 hr, and 3 hr. Tissue was then harvested and stored at -70°C.

II. RNA ISOLATION

RNA was isolated using Ribozol (VWR-Amresco) as described by the manufacturer. RNA pellet was resuspended in RNase-free water (Amresco) and concentration was determined using the NanoDrop2000 (Thermo Scientific). Agarose gel electrophoresis (1.5%) was also performed to confirm successful RNA isolation.

III. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

In order to confirm primer specificity, reverse transcription PCR was performed using Quiagen's OneStep RT-PCR kit according to the manufacturer's protocol. Gel electrophoresis confirmed the amplification of a single transcript for each primer pair tested. Primers for *Histone-3*, *qa-y* and *qa-1F* are shown in **Table 5.**

Table 5: Primer Sequences

PCR Primers		
Gene	**Sequence**	**Direction**
Histone-3	5' AGA TTC GTC GCT ACC AGA AG 3'	Forward
	5' CGG AGG TCG GAC TTG AAG 3'	Reverse
qa-y	5' GGT ATC AAT GCC ATC AAC TAT TAC 3'	Forward
	5' GCC ACA GAA GCC AGA TAA TG 3'	Reverse
qa-1F	5' TGC CGG GGC AAA GAC ATC CG 3'	Forward
	5' CCG GGC CTG GTA GAG TGC G 3'	Reverse

IV. Quantitative Reverse-Transcription PCR (RT-QPCR)

Quantitative reverse transcription PCR was performed using the iTaq™ Universal SYBR® Green One Step Kit (Bio-Rad). Upon completion of the protocol, the genes of interest (*qa-y* and *qa-1F*) were analyzed using the relative quantitation method with *Histone-3* as a reference gene. The comparative threshold cycle ($2^{-\Delta\Delta Ct}$) method was utilized according to the Applied Biosystems Real-Time Quantitative PCR manual in order to achieve a normalized fold change in expression of each gene of interest compared to the reference gene at the time points of quinic acid exposure being observed.

III. Results

I. Determination of a Stable Reference Gene

In order to accurately quantify the expression of the *qa-y* and *qa-1F* genes, a reference gene that is stable throughout the experimental conditions had to be established. The expression of three common housekeeping genes (actin, beta-tubulin, and histone-3) was compared at differing times of quinic acid exposure in *Neurospora crassa* to determine which was the most reliable reference gene for the present study.

Using quantitative reverse-transcription PCR (RT-qPCR), average threshold cycle (C_t) values were calculated for each potential reference gene at 0 minutes, 1 hour, and 3 hours exposure time to quinic acid. A one-way analysis of variance (ANOVA) was performed to test for statistically significant data. It is important to note that p-value ≥ 0.05 indicates that the difference between the mean C_t values at each time point were not statistically significant from one another, indicating a more stable reference gene. Of the three housekeeping genes observed, histone-3 showed the least variance between time points, exhibiting a p-value of 0.084, and was therefore selected as the optimal reference gene for this experiment (**Figure 6**). Actin was also determined to be a stable reference gene (p=0.059), while the difference of the mean C_t values of the beta tubulin gene were observed to be statistically different (p=0.047).

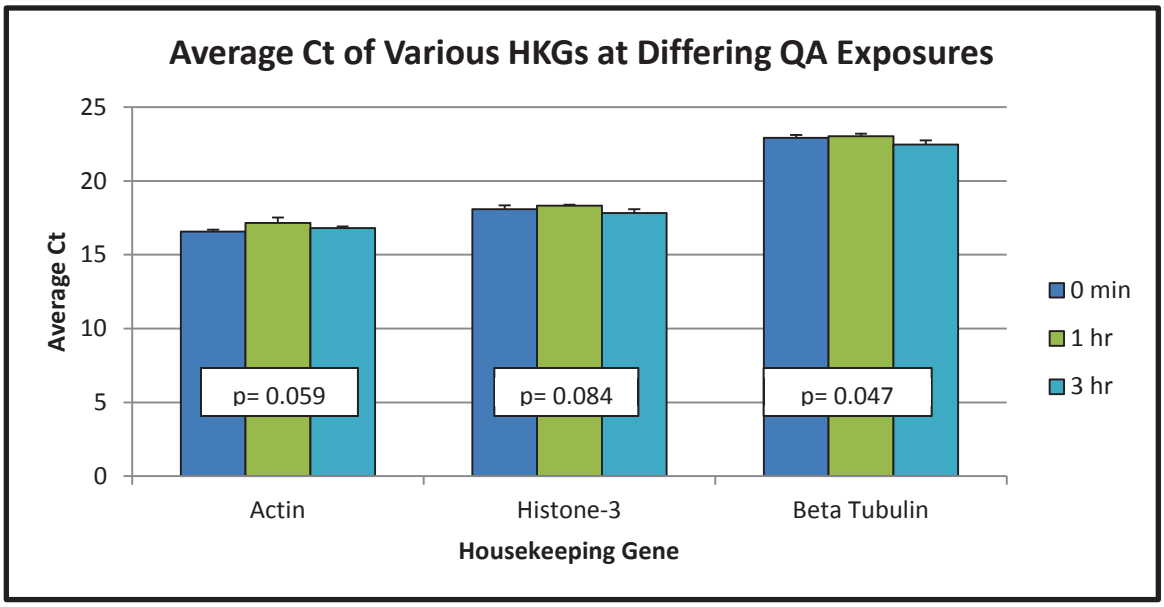

Figure 6: Average threshold cycle (Ct) of three housekeeping genes (HKGs) in *N. crassa* tissue subjected to a quinic acid (0.3%) medium shift for the time periods specified. The ANOVA function of StatPlus:mac was used to obtain p-values. A p-value > 0.05 indicates consistent expression throughout experimental conditions.

II. *QA-Y* Expression Profile

The *qa-y* gene in *N. crassa* encodes for a quinate permease protein that allows for quinic acid to enter the cell when necessary for growth and metabolism. In this study, expression of the *qa-y* gene was observed at a variety of time points following the introduction of quinic acid and the removal of sucrose. After 15 minutes, a 9.46 fold change in expression of the *qa-y* gene was observed in relation to time zero. A sharp increase in expression then occurs after 30 minutes of quinic acid exposure, with the *qa-y* gene being expressed at a 17.56-fold greater level than when quinic acid was not present. Following the sharp increase, expression values of the *qa*-y gene decrease to 4.72 times greater than time zero, but then steadily increase at both 2 and 3 hours (6.34-fold and 11.36-fold, respectively) (**Figure 7**).

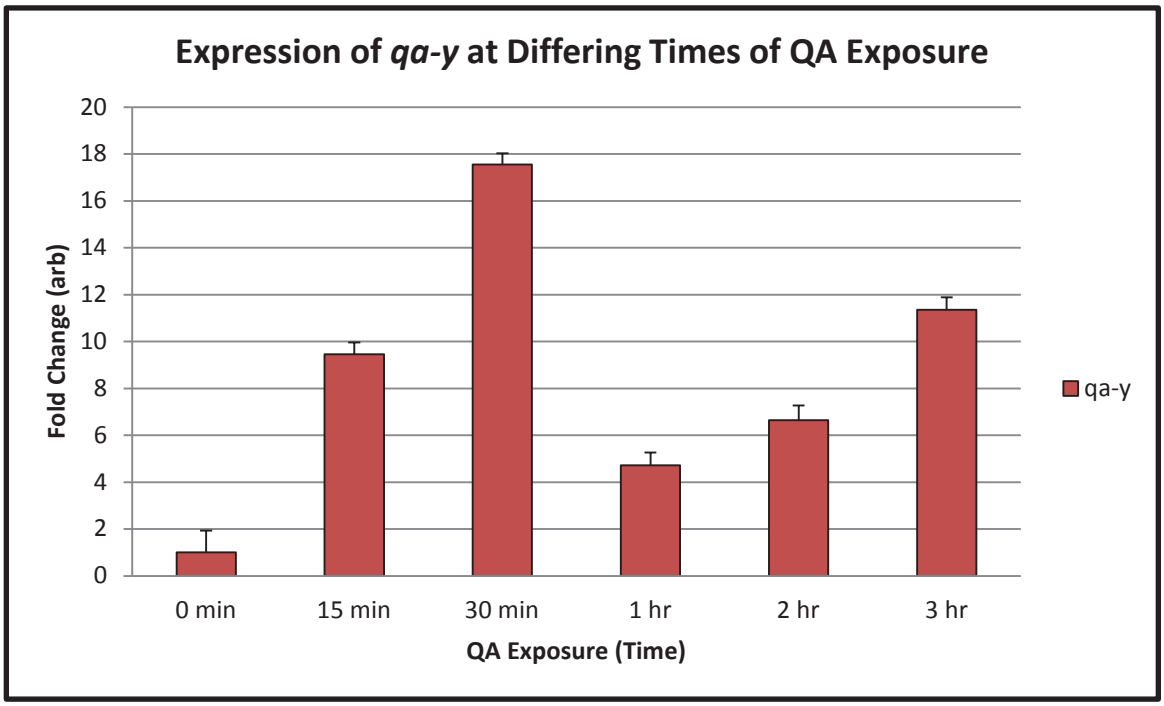

Figure 7: Expression profile of the *qa-y* gene at differing time of quinic acid exposure. The *qa-y* gene encodes for a quinate permease protein that allows quinic acid to be transported into the cell to be metabolized. Expression data was obtained using RT-qPCR and was quantified using the comparative threshold cycle method of relative quantitation and is expressed as a fold-change in expression relative to the 0 min time point.

III. *QA-1F* Expression Profile

The *qa-1F* gene encodes for the activator protein QA-1F of the *qa* gene cluster in *N. crassa*. The transcription of this gene, as well as all other *qa* cluster genes, has been shown to increase when quinic acid is present as the sole carbon source (Arnett et al., 2009). The present study aims to elucidate as to the timing and pattern of this increased expression following a shift to quinic acid as a sole carbon source.

An initial, sharp increase in expression (2.98-fold change) of the *qa-1F* gene is observed after just 15 minutes following the nutrient shift. At 30 minutes, expression levels return to pre-shift levels, but then approximately double and

remain stable for the next hour and a half. After 3 hours, a slight decrease in expression (0.78-fold change) is observed when compared to the 0 minute time point (**Figure 8**).

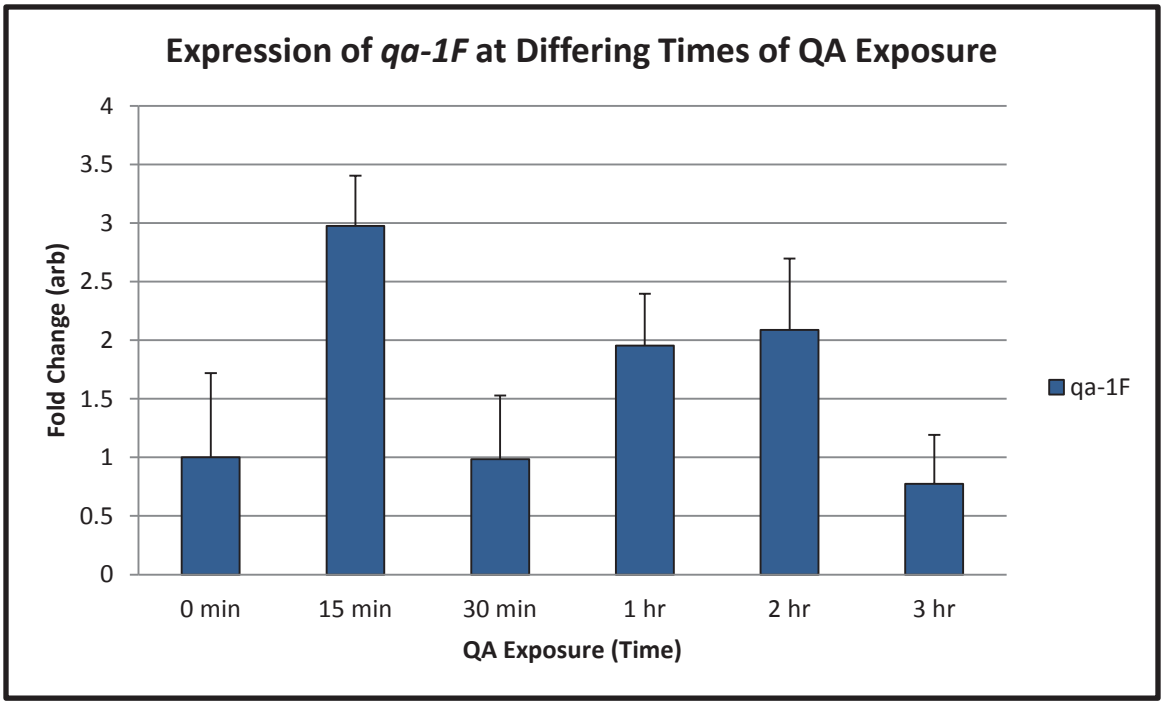

Figure 8: Expression profile of the *qa-1F* gene at differing time of quinic acid exposure. The *qa-1F* gene encodes for the activator protein of the *qa* gene cluster. Expression data was obtained using RT-qPCR and was quantified using the comparative threshold cycle method of relative quantitation and is expressed as a fold-change in expression relative to the 0 min time point.

IV. DISCUSSION

When combined, the expression data of *qa-y* and *qa-1F* provide molecular insight as to the timing and mechanisms of quinic acid metabolism in *N. crassa*. Previous studies have shown increased levels of transcription following a quinic acid shift (Arnett et al., 2009; N H Giles, 1991; Reinert et al., 1981), but no research has been published to date that examines how quickly these genes respond to the nutrient shift.

As can be seen at the 15-minute time point in **Figure 9**, transcription of both genes initially increases. This increase of *qa-y* transcription is necessary to produce the additional quinate permease proteins that are necessary for quinic acid to enter the cell and be metabolized. The initial increase in transcription of the *qa-1F* gene is essential in order to activate and account for the increased transcription of itself as well as the *qa-y* gene. Once transcription of the *qa* gene cluster is sufficiently activated, the focus of the cell turns to producing quinate permease protein as quickly and efficiently as possible. This can be observed at the 30-minute time point, where *qa-y* expression is significantly increased while the expression of *qa-1F* decreases to below basal levels.

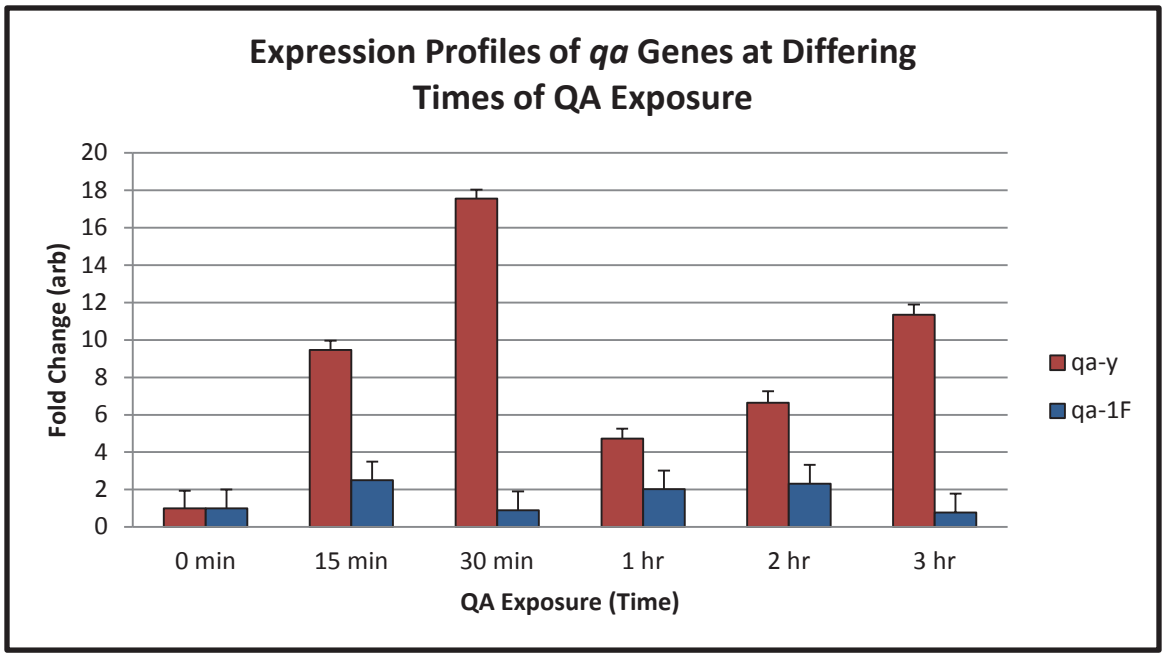

Figure 9: Expression profile of both *qa-y* and *qa-1F* genes of the quinic acid gene cluster. These genes encode for a quinate permease protein and a *qa* gene cluster activator protein, respectively. Expression data was obtained using RT-qPCR and was quantified using the comparative threshold cycle method of relative quantitation.

Once quinic acid is present in large amounts within the cell, it needs to be metabolized as quickly as possible in order for the cell to grow and survive. As can be seen at the 1- and 2-hour time points, *qa-1F* is expressed at approximately double the level that it is immediately following the nutrient shift, while *qa-y* gene expression is significantly reduced from the peak expression levels observed at the 30-minute time point. We hypothesize that the three metabolic structural genes (*qa-2*, *qa-3*, and *qa-4*) would see a significant increase in expression at the 1- and 2-hour time points in a direct response to the increased QA-1F activator protein present within the cell. All three of these genes contain an upstream DNA sequence within their promoter regions that has the ability to directly bind the activator protein with high affinities, therefore allowing for the prompt recognition and activation of transcription for each specific gene (J A Baum, 1987).

A very similar ratio of expression between *qa-y* and *qa-1F* is observed at the 3-hour time point when compared to the 30-minute time point. As quinic acid is not believed to be a limited resource in this experimental setup, it can be hypothesized that the cell might have utilized a majority of the quinic acid that rushed into the cell at the 30-minute time point and now needs to allow for another influx of quinic acid to be metabolized. Additional experimentation with a longer exposure to quinic acid would be necessary to confirm this hypothesis.

Overall, this study alone cannot describe the transcriptional pattern of the entire *qa* gene cluster, but it gives tremendous insight as to the timing and mechanism of the induction and activation of the *qa* gene cluster through analysis of the *qa-y* and *qa-1F* genes. A large influx of quinic acid is proposed to occur at

sometime between 15 minutes and 1 hour following a nutrient shift from a preferred carbon source to quinic acid. Shortly after that (as soon as 1 hour post-nutrient shift), transcription of the genes producing the metabolic enzymes required for the metabolism of quinic acid is activated, presumably resulting in the efficient utilization of quinic acid as a sole carbon source in *N. crassa*.

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CHAPTER 4

DISCUSSION

Choosing a suitable and stable reference gene for the specific experimental conditions is of the utmost importance whenever RT-qPCR is being employed within an experimental design. One common flaw of RT-qPCR studies is that reference genes are often selected to be used for relative quantification without *a priori* conformation that expression levels do not vary under the specific experimental conditions being investigated (Cusick et al., 2014). To date, minimal experimentation has been completed to assess the stability of reference genes following a nutrient shift from a preferred carbon source to a non-preferred carbon source in *N. crassa*. The current study examines the stability of three housekeeping genes (actin, beta-tubulin, and histone-3) at three different time points following a nutrient shift from sucrose (preferred carbon source) to quinic acid (non-preferred carbon source).

Using quantitative reverse-transcription PCR (RT-qPCR), average threshold cycle (C_t) values were calculated for each potential reference gene at 0 minutes, 1 hour, and 3 hours exposure time to quinic acid. A one-way analysis of variance (ANOVA) was performed to test for statistically significant data. It is important to note that p-value ≥ 0.05 indicates that the difference between the mean C_t values at each time point were not statistically significant from one another, indicating a more stable reference gene. Of the three housekeeping genes observed, histone-3 showed the least variance between time points, exhibiting a p-value of 0.084, and was

therefore selected as the optimal reference gene for this experiment (**Figure 6**). Actin was also determined to be a stable reference gene (p=0.059), while the difference of the mean C_t values of the beta tubulin gene were observed to be statistically different (p=0.047).

Following the selection of histone-3 as a suitable reference gene, a validation experiment was performed in order to ensure that the amplification efficiencies were comparable between the reference gene and the experimental genes. For a valid $\Delta\Delta C_T$ calculation, the efficiency of the target amplification and the efficiency of the reference amplification must be approximately equal (Applied Biosystems, 2004). The validation experiment confirmed that the *qa-y* gene exhibited a very similar amplification efficiency to histone-3, whereas the efficiency of the *qa-1F* gene was not as comparable (See **Appendix Figures 1-2**). This variation in amplification efficiencies between histone-3 and *qa-1F* can possibly be explained by the relatively low amount of starting transcript for *qa-1F* (a regulatory gene) compared to that of histone-3 (a constitutively expressed housekeeping gene). Although not ideal, the primer efficiencies of both genes were suitable to proceed with the study.

The expressional analysis of the *qa-y* and *qa-1F* genes in this study provide a novel, quantitative perspective to the timing and mechanisms of quinic acid metabolism in *N. crassa*. Shortly following a nutrient shift from the preferred carbon source sucrose to the non-preferred carbon source quinic acid, a sharp increase in expression is observed for both *qa-y* and *qa-1F* genes (**Figure 9**). The initial increase of *qa-y* transcription is necessary to produce the additional quinate permease

proteins that are necessary for quinic acid to enter the cell and be metabolized, while the initial increase in transcription of the *qa-1F* gene is essential in order to activate and account for the increased transcription of itself as well as the *qa-y* gene. When transcription of the *qa* gene cluster is sufficiently activated, the focus of the cell turns to producing quinate permease protein as quickly and efficiently as possible, as evidenced by a significant increase in *qa-y* expression paired with a decrease of *qa-1F* expression to below basal levels.

Once copious amounts of quinic acid have been transported into the cell, it must be metabolized as quickly as possible in order for the cell to grow and survive. Expression of the *qa-1F* gene is approximately doubled at the 1- and 2-hour time points when compared to immediately following the nutrient shift, while expression of the *qa-y* gene significantly reduced from the peak expression levels observed at the 30-minute time point. We hypothesize that the three metabolic structural genes (*qa-2*, *qa-3*, and *qa-4*) would see a significant increase in expression at the 1- and 2-hour time points in a direct response to the increased QA-1F activator protein present within the cell. All three of these genes contain an upstream DNA sequence within its promoter region that has the ability to directly bind the activator protein with high affinities, therefore allowing for the prompt recognition and activation of transcription for each specific gene (J A Baum, 1987). In total, there have been 13 activator-binding sites identified within the *qa* gene cluster DNA. It can be reasonably hypothesized that those sites with the highest binding affinities would correspond to the genes that are transcribed first in response to quinic acid. Further experimentation would be necessary in order to observe if any correlation between

the timing of *qa* gene expression and the published activator-binding site affinities exists (J A Baum, 1987).

A very similar ratio of expression between *qa-y* and *qa-1F* is observed at the 3-hour time point when compared to the 30-minute time point. As quinic acid is not believed to be a limited resource in this experimental setup, it can be hypothesized that the cell might have utilized a majority of the quinic acid that rushed into the cell at the 30-minute time point and now needs to allow for another influx of quinic acid to be metabolized. Additional experimentation with a longer exposure to quinic acid would be necessary to confirm this hypothesis.

Overall, this study alone cannot describe the transcriptional pattern of the entire *qa* gene cluster, but it gives tremendous insight as to the timing and mechanism of the induction and activation of the *qa* gene cluster through analysis of the *qa-y* and *qa-1F* genes. A large influx of quinic acid is proposed to occur at sometime between 15 minutes and 1 hour following a nutrient shift from a preferred carbon source to quinic acid. Shortly after that (as soon as 1 hour post-nutrient shift), transcription of the genes producing the metabolic enzymes required for the metabolism of quinic acid is activated, presumably resulting in the efficient utilization of quinic acid as a sole carbon source in *N. crassa*.

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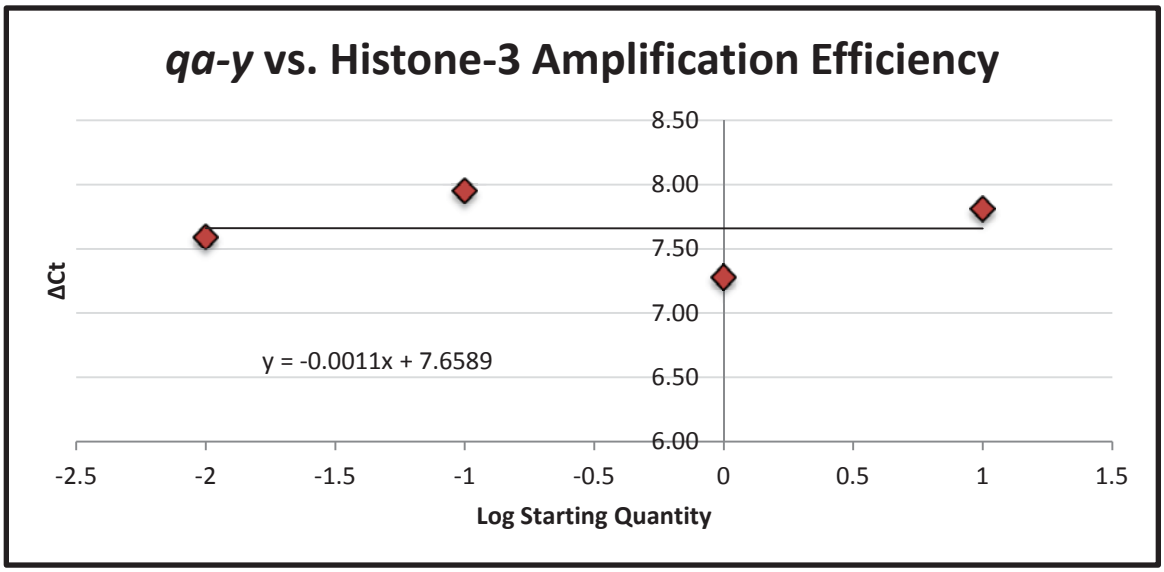
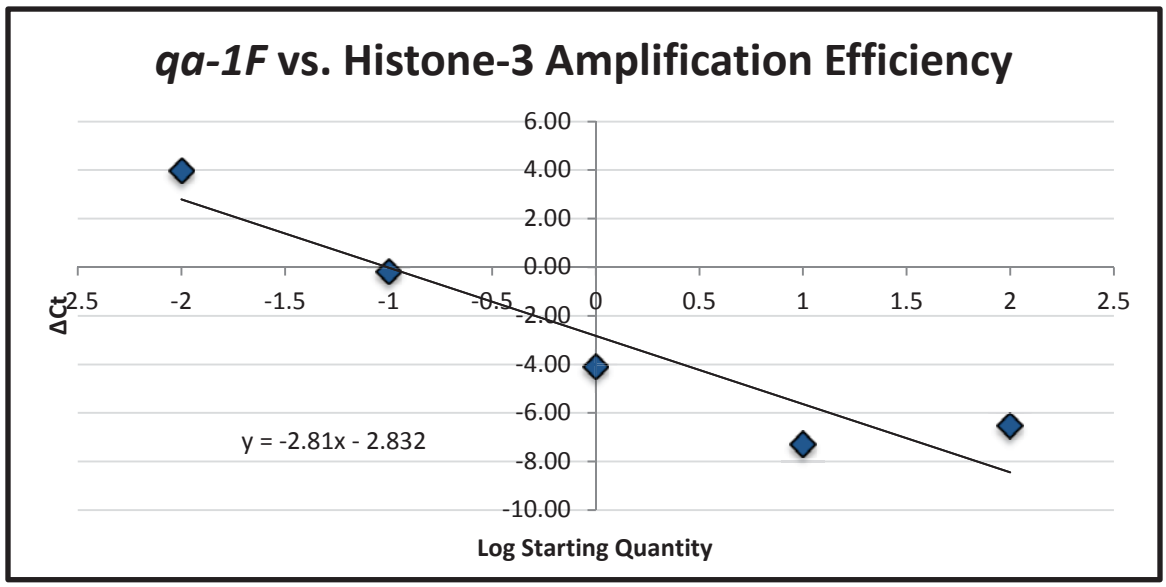
APPENDIX



Appendix Figure 1: Comparison of amplification efficiencies of the experimental gene *qa-y* and the reference housekeeping gene histone-3 following amplification using RT-qPCR. A slope of less than 0.1 indicates statistically comparable efficiencies.



Appendix Figure 1: Comparison of amplification efficiencies of the experimental gene *qa-1F* and the reference housekeeping gene histone-3 following amplification using RT-qPCR. A slope of less than 0.1 indicates statistically comparable efficiencies.