Expression kinetics of the quinic acid (qa) gene cluster in *Neurospora crassa*

by

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Abstract

Eukaryotic genes are tightly regulated through a highly complex system with a number of checks and balances. When environmental conditions change, organisms need to adapt. Part of this reaction may be a shift in gene expression based on the regulation of that particular gene or gene cluster. The quinic acid (*qa)* gene cluster of *Neurospora crassa* is such a system. The up-regulation in gene expression for the *qa* cluster is triggered by the carbon source quinic acid. When the fungus is grown on quinic acid as a sole carbon source, the *qa* genes are expressed at high levels. However, when a preferred carbon source, such as dextrose, is used, the *qa* genes are repressed. This study will quantify the effects of changing carbon source over a three hour time period in the wild type strain of *N. crassa*, 74A.

The focus of this study is the kinetics of induction of the various quinic acid genes as the environment changes. RNA was isolated from *N. crassa* grown under various conditions. Transcript levels of the various genes are detected by SYBR Green using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). Results are normalized to the internal control gene, *18s rRNA*, and analyzed with Bio-Rad iQ5 software. These results reveal that the up-regulation in expression of the *qa* genes is detectable within 15 minutes of incubation with quinic acid and reaches peak levels, higher than previously thought, within 3 hours.

Acknowledgements

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Introduction

I. Kingdom Fungi

Kingdom Fungi is one of the six kingdoms in current classification. All fungi possess a number of key characteristics separating them from other groups. Fungi are eukaryotic with a cellular wall that frequently contains chitin. They form hyphae for nutrient absorption, making them heterotrophs. Also, fungi can reproduce either sexually or asexually via the production of spores (Deacon, 2006). This kingdom is very diverse with approximately 1.5 million known species.

This group, with a great deal of diversity, has become one of the most important groups known to humans. Some fungi are relatively easy to produce and alter making them efficient tools for the production of biomolecules. These biomolecules continue to be developed and are invaluable to our economy. However, other fungi have the potential to be very expensive for humans. These fungi may cause illness in humans, pets and livestock, and crops. While researchers are finding more ways to efficiently grow and mass-produce with the first group of fungi, they are also finding ways to slow and prevent growth of the latter group (Borkovich, 2004).

II. Phylum Ascomycota

Ascomycota is a division of Kingdom Fungi. The ascomycota make up the largest clade within Kingdom Fungi representing the most species. Organisms that fall into this category contain a paired nucleus for a part of their life cycle; therefore, all ascomycota are within the division of dikarya. The defining characteristic for the phylum Ascomycota

is the production of ascospores within asci, the sexual spores in a specialized sac (James et al., 2006).

III. *Neurospora crassa*

A number of model organisms have been studied and continue to be studied because of their ability to represent a particular event or occurrence. These organisms are selected based on a variety of reasons. In microbial research, a few of these reasons include ease of growth, inexpensive maintenance, and quick reproduction periods.

Neurospora was first characterized as red bread mold in 1843. In the 1920s, work was done to differentiate species of *Neurospora*. Early studies were soon carried out looking at the genetics of the ascospores produced during reproduction. The recent discovery of genetic crossing over in *Drosophila* could be applied and further studied in the *Neurospora*. By 1940, *Neurospora* had become a model system for both plants and animals due to the similarities in reproduction and genetic inheritance (Perkins, 1992).

Molecular studies by Beadle and Tatum lead to the theory that is now one-polypeptide, one-enzyme in 1941 (Beadle, 1945; Perkins, 1992). More studies have been done over the years with the genome being completely sequenced since 2003 (Gallagan et al., 2004). The genome is 41 Mb and is arranged in seven different linkage groups. Linkage groups are organization for genetic material similar to chromosomes. *N. crassa* is still used today as a model organism because of its complexity and ease of use (Borkovich et al., 2004).

IV. Gene clusters in fungi

There is evidence for clusters of genes within the eukaryotic group fungi. Many of these systems are reminiscent of prokaryotic gene operons. There are many theories that attempt to explain the presence of clusters in eukaryotes. One theory is horizontal gene transfer from prokaryotes. However, there are cases where one or more of the genes within a fungal cluster are not able to be found in the related prokaryotic gene cluster. A gene cluster is a tightly linked group of genes that act together to perform a function such as the metabolism of a substance. Some of these clusters are termed dispensable metabolic pathways because they are not needed under most conditions, but add a benefit to the organism under certain environmental conditions, like sub-prime carbon availability.

There are four known dispensable pathways in fungi that provide a mechanism for utilizing less-preferred nutrients; they include ethanol, nitrogen, proline, and quinic acid. An organism may contain more than one gene cluster for nutrient utilization. Almost all of these systems are controlled by two different mechanisms of regulation. The first level of regulation is based on a specific activator gene that typically contains a zinc cluster motif, and the second involves either a broad acting regulatory gene or carbon catabolite repression (Keller and Hohn, 1997). Explaining the specific mechanisms used to regulate these clusters gives insight to eukaryotic gene regulation of simple clustered systems that are still being discovered in higher eukaryotes.

V. Qa gene cluster

The quinic acid gene cluster is composed of seven genes that span a 17.3 kb region on linkage group VII. Some of the significant points of this cluster of genes are that they are all regulated by the carbon source that is present, and that this regulation occurs, in part, because of regulatory genes that are adjacent to the group of structural genes. In this system, structural genes are those that encode proteins that are involved in metabolizing quinic acid. Regulatory genes, on the other hand, alter gene expression levels for the system.

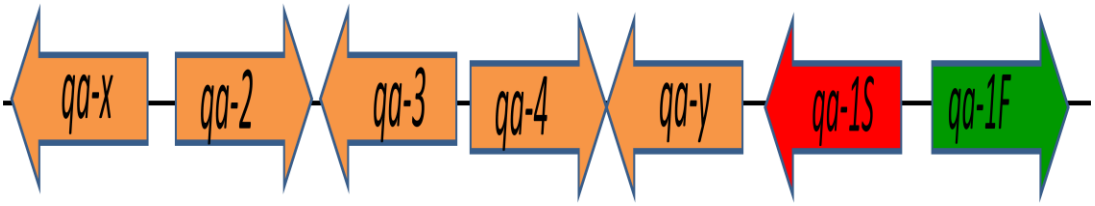
Five of the seven genes in this cluster are structural, while the other two are regulatory. Three of the five structural genes *qa-2*, *qa-3*, and *qa-4*, are involved in the metabolism of quinic acid. The gene *qa-x* has no known function at this time. The fifth structural gene is quinic acid permease encoded by *qa-y*. The regulator genes, *qa-1S* and *qa-1F*, encode a repressor and activator protein respectively (Geever et al., 1989).

All of the genes are expressed in three bidirectional pairs, with the exception of *qa-y*, which is expressed on its own. Each pair has its own promoter that will allow for increased expression in the presence of an activator. There is a region within the promoter that has the same 16 bp conserved sequence at the 5' end of each of the genes. This 16 bp region of the promoter is where the activator protein, QA-1F, will bind after translation, in the presence of quinic acid (Giles, 1991).

VI. *Qa* genes and the function of their products

Quinic acid is an aromatic ring that is a metabolite found in many different species of plants. Quinic acid does not enter into glycolysis directly, like glucose and is

Figure 1: The map of the qa gene cluster. Structural genes are found on the left hand side of the cluster, while regulatory genes are on the right hand side. The gene *qa-y* separates the regulatory genes from the rest of the cluster.

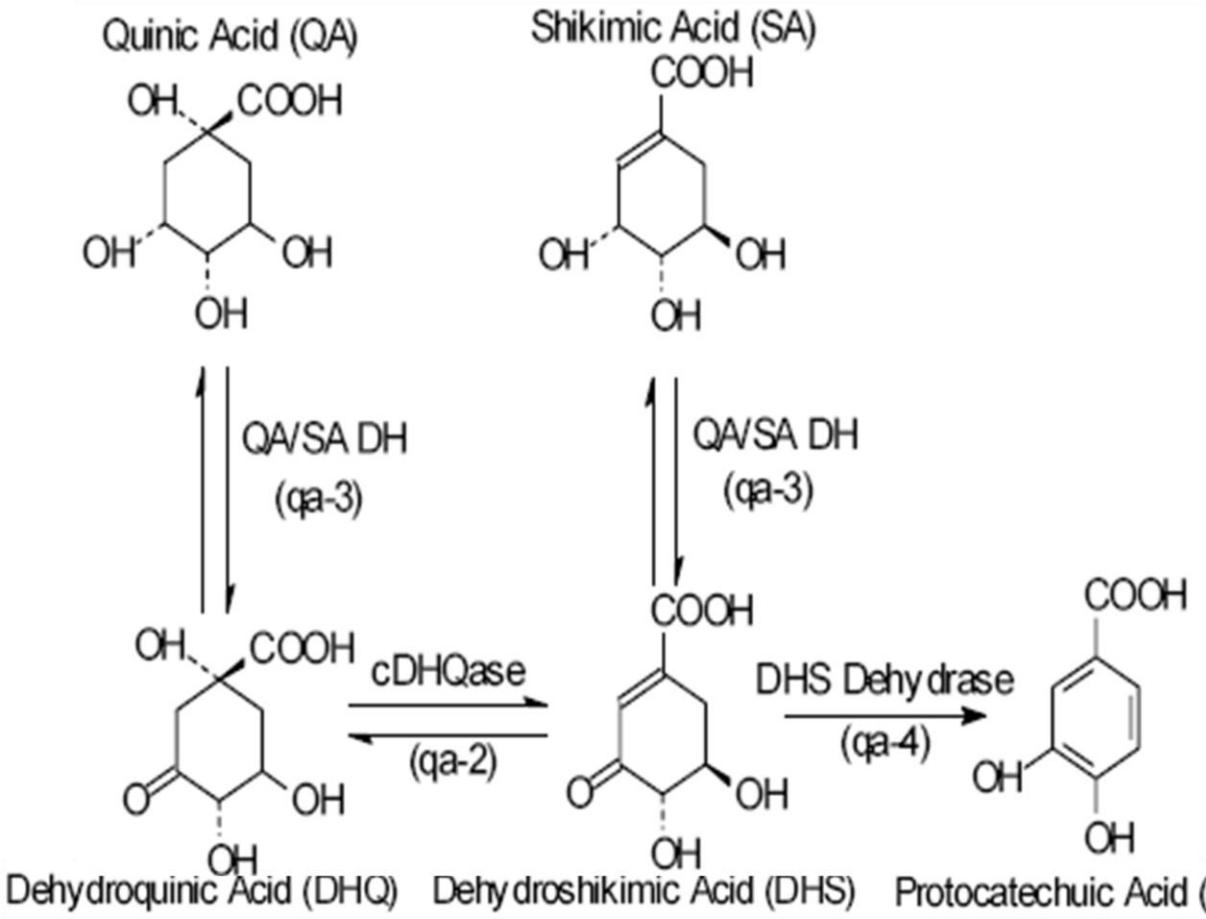

qa-x
qa-2
qa-3
qa-4
qa-y
qa-1S
qa-1F

therefore not a preferred carbon source. Although quinic acid is not preferred, a number of organisms have metabolic pathways to utilize it. Some of these systems involve metabolizing quinic acid into necessary compounds such as tryptophan and nicotinamide (Pero et al., 2009). Other systems, like the *qa* cluster in *N. crassa*, are able to metabolize quinic acid as the sole carbon source (Geever et al., 1987).

The three gene products responsible for the conversion of quinic acid to protocatechuic acid are dehydro- quinase, *qa*-2, quinic acid dehydrogenase, *qa-3*, and DHS dehydrogenase, *qa-4*. When quinic acid is brought into the cell via the permease, it will be converted into dehydroquinate (DHQ) by the *qa-3* gene product, as shown in Figure 2. The next step in the pathway involves the *qa-2* gene product converting DHQ to dehydroshikimate (DHS). The DHS dehydrogenase then converts DHQ into protocatechuic acid (Giles, 1991). The protocatechuic acid is further metabolized through pathways found in a variety of microbial species. (Giles et al., 1985; Chaleff, 1974). Protocatechuic acid eventually is converted to succinate and acetyl-CoA where it is able to enter into the citric acid cycle to provide energy to the organism (Kuswandi and Roberts, 1992; Griffin, 1994).

Mutant strains in *N.* crassa gave rise to the assignment of genes involved in the metabolism of quinic acid to the enzymes that carry out that metabolism. The *qa* gene cluster, when induced by quinic acid, produces gene products that carry out the metabolism of quinic acid. Researchers found that if a mutation was found in any of the three metabolically relevant genes, quinic acid was not metabolized properly. Strains of *Neurospora* that contained mutations within *qa-3* not only had a problem metabolizing quinic acid, but also, shikimate (Chaleff, 1974). There are two links between the quinic

Figure 2: The metabolic pathway that quinic acid to the more common metabolite, protocatechuic acid in *N. crassa*. Abbreviations: QA/SA DH - quinic/shikimic acid dehydrogenase; cDHQase - catabolic dehydroquinase; DHS dehydrase - dehydroshikimate dehydrase (Giles et al., 1985).


Quinic Acid (QA)
Shikimic Acid (SA)
QA/SA DH
(qa-3)
QA/SA DH
(qa-3)
cDHQase
(qa-2)
DHS Dehydrase
(qa-4)
Dehydroquinic Acid (DHQ)
Dehydroshikimic Acid (DHS)
Protocatechuic Acid (PCA)

acid catabolic pathway and the anabolic aromatic (AROM) pathway. One of the interchangeable steps, in *Aspergillus*, is due to isozymes produced by the *qa*-3 homolog, *qutB*, and another gene from the AROM pathway (Hawkins, 1993). Previously, the two enzymes were thought to be the same protein acting in both the AROM and *qa* systems for *Neurospora*; however, they may be two isozymes that are dependent on each other. The other link to the AROM pathway is between the *qa-2* and *arom-9* genes.

The other genes in the quinic acid cluster were found by Northern blot analysis. When looking at transcript levels, there were increased levels of *qa-x* and *qa-y* transcripts. Since they were induced by quinic acid, like the metabolic genes, they were thought to be involved in its utilization as well. Mutants with a deletion of the *qa-x* gene show only one unique phenotype, a brown pigment, produced when quinic acid is present as the only carbon source (Giles et al., 1991; Case, 1992).

The *qa-y* gene is believed to encode a quinic acid permease based on two reasons. Case (1992) described the first reason after producing transformants with *qa-y* deletions. Induction levels for *qa-2* and *qa-3* in the Δ*qa-y* strain were both less than 10% of the wild-type strain when grown on quinic acid as the sole carbon source. The Δ*qa-y* strain was not viable when grown with quinic acid as a sole carbon source because induction levels were not able to reach the necessary level. The reason that the activator, quinic acid, was not up-regulating the system was likely to be due to lack of quinic acid transportation. The other work suggesting that *qa-y* encodes a permease is based on a few studies that have looked into similarities between known sugar transporters, like *qutD* in *A. nidulans.* These studies have documented high levels of homology between sugar

transporters and *qa-y*. In these other systems transportation of a carbon source must occur in order to induce the appropriate genes (Arnett et al., 2009; Geever et al., 1989).

VII. Gene Regulation

Many different metabolic pathways are closely regulated. Currently, not all of the mechanisms of regulation are fully understood. A number of studies have looked into regulation of gene systems similar, in some ways, to the quinic acid gene cluster. This regulation can be compared to an "on/off" switch where genes are up regulated and turned on only at times when the gene product is needed. There is a system in some species of *Aspergillus* where protease genes are turned on by regulator genes located elsewhere in the genome (Punt et al., 2008). Although this is an "on/off" type of regulation, it is not regulated in the same fashion as the *qa* cluster because of the location differences. The quinic acid utilization (*qut*) system, found in different species of *Aspergillus,* is a regulatory system more similar to the *qa* gene cluster in *N. crassa*. The regulatory similarity comes from the regulator genes being clustered with the *qut* structural genes.

Although the *qut* and *qa* systems are very similar, containing many of the same genes, neither system has been studied extensively. The galactose (GAL) operon in *Saccharomyces cerevisiae*, is another system that is closely regulated. The GAL system has been studied in more detail than the *qut* system in *Aspergillus* species or the *qa* genes in *N. crassa,* and provides more insight about system regulation. The complexity of these systems is only beginning to be detected and understood (Wightman et al., 2008). These systems seem to be widespread and occur within *N. crassa* as well. Exploring the

complex and varied mechanisms of fungal gene regulation can provide insight on eukaryotic gene regulation.

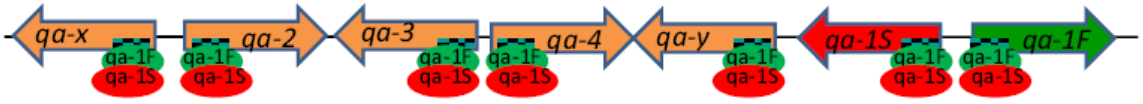
VIII. Regulation of the *qa* cluster

Regulation of the *qa* gene cluster is mediated by two different mechanisms. The first mechanism of regulation is in the presence of quinic acid. The activator gene, *qa-1F*, is activated when quinic acid is detected. This activation causes an up-regulation of all of the *qa* genes, including itself and the repressor, *qa-1S.* The second state is the carbon catabolite repression method. In this method, if a preferred carbon source, glucose, is available the *qa* gene expression will be reduced to basal levels.

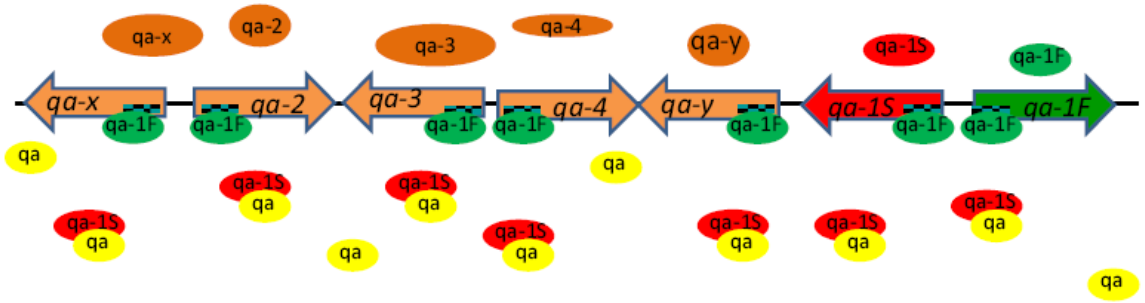
Under standard conditions, where glucose is the carbon source and quinic acid is absent, the current hypothesis leads us to believe that the repressor protein (QA-1S) will be bound to the activator (QA-1F) preventing induction of the cluster. At this point, it is not known exactly where the repressor protein is binding. One theory is that the repressor binds to the activator gene, *qa-1F* promoter region. In this scenario, while *qa-1F* is in this bound state, it is unable to be transcribed or translated. Without the QA-1F protein, the qa gene binding regions would remain empty. This theory does not have much support though because many studies looking for the DNA binding domain in the repressor have not been successful. The current theory is that the 816 amino acid, 89 kD, QA-1F protein is present, and possibly bound to the qa gene binding region, but the activation is inhibited by the binding of the repressor protein, as shown in Figure 3 (Huiet, 1984; Giles et al., 1985; Giles et al., 1987; Giles et al., 1991; Case et al., 1992).

Figure 3: Regulation of the qa gene cluster is carried out by two regulatory genes, *qa-1S* and *qa-1F*. Part A shows the system without quinic acid present and little transcript is produced. In part B, quinic acid is present and transcription of the *qa* genes is up-regulated due to the binding of the QA-1F protein to the activator binding site.

A) Quinic acid absent



B) Quinic acid present



Both theories are the same when quinic acid is introduced to the system. Quinic acid attaches to the 918 amino acid, 101 kD repressor protein, QA-1S, at the inducer-binding domain (Geever et al., 1989; Giles et al., 1985). This causes the repressor to release the activator. Once QA-1F is in its unbound state, it is able to activate transcription of the *qa* genes. This is the "on" state for the *qa* genes. It will continue to express the *qa* genes at a higher rate until the quinic acid is metabolized and no longer detectable. The QA-1S will be released and bind to the activator returning the system to its "off" position (Giles et al. 1991).

IX. Carbon catabolite repression

There are a number of systems that use catabolite repression as a mechanism of gene regulation. Most of this regulation is mediated by the elimination of the alternative carbon source, or by direct repression of the genes involved with its metabolism. One of the best studied systems in fungi is the galactose genes (GAL) in *Saccharomyces cerevisiae.* The GAL system consists of nine genes known to be involved in the utilization of galactose as a carbon source. Out of the nine genes currently known to be involved in the GAL system, three are regulatory and the other six are structural genes encoding enzyme that are involved in the metabolism of galactose. These genes are located on four different linkage groups, and are therefore not clustered together. The mechanism for carbon catabolite repression in *S. cerevisiae* has been studied extensively. It is known that carbon catabolite repression affects the GAL system, but the mechanisms and pathways by which the affects are carried out are not fully understood.

The current model for catabolite repression in yeast involves two different pathways (reviewed by by Westergaard et al., 2006). The first pathway deals with the detection of glucose. There are two proteins, Snf3p and Rgt2p, that bind to glucose at low or high concentrations respectively. Detection of glucose triggers expression of the hexose transporter encoding genes, HXT, thus producing new hexose transporter molecules. The other pathway is involved in a number of regulatory pathways, including the GAL system. When glucose is present, Snf1 is inactive; this leaves the DNA-binding protein Mig1 bound to a variety of genes within the nucleus. When the levels of glucose are no longer detected, the repression is lifted by activating the Snf1, which will then phosphorylate Mig1. The Mig1p releases the DNA and moves out into the cytosol.

The GAL system in *S. cerevisiae* is repressed specifically by the Mig1 complex. Mig1, in the presence of glucose, will bind to the promoter region of the activator gene, GAL4. This will prevent induction of the other *GAL* genes, but it also will bind to the promoter region of the other *GAL* genes increasing the effects of repression by preventing transcription of most all *GAL* genes. As the glucose in the system becomes less available, the Snf1 complex becomes activated, and, as in the general model, activates the Mig1 complex The activated Mig1 complex then exits the nucleus thus releasing the *GAL* genes for transcription. Glucose also has a great affect on limiting inducer by three different mechanisms: repressing *GAL2*, galactose permease encoding gene; inactivating the present permeases; and competitive antagonism at the permease receptor. Another effect of glucose directly acting on a gene is the regulatory gene, *GAL3*. Without glucose, the GAL3 protein acts on the GAL4/Gal80 complex leading to expression of the GAL genes by GAL4. When glucose is there, however, the GAL4/Gal80 complex remains

intact and prevents GAL gene expression through signal transduction (reviewed in Gancedo 1998).

Many other research groups have shown that transporters are repressed or altered when preferred carbon sources are available. When the additional nutrients are no longer needed, or when glucose is available, the permease transcript levels drop, or in some way are rendered inactive. This is more evidence that carbon source can affect the availability of extracellular nutrients by inducer exclusion in both bacteria and fungi (Görke and Stülke, 2008; Bailey and Arst, 1975; Peter et al., 2006).

X. Carbon catabolite repression in *N. crassa*

The catabolite repression offers a second line of regulation to the quinic acid gene cluster. When glucose, or another preferred carbon source is present, the expression of the *qa* genes is repressed. Although carbon catabolite repression has been known to play a role in the regulation of the *qa* gene cluster, the mechanisms for the repression still are not fully understood. Giles recorded in 1991 that, when glucose is present, there is an approximate 10% reduction in the transcription of the *qa* gene products, as compared to quinic acid as the sole carbon source. Data that is more current has suggested that the level of reduction is greater.

Recent research has shown that the catabolite repression is caused, in part, by the limitation of the system's activator, quinic acid. This research was carried out using a deletion mutant, ΔS, that lacked the *qa-1S* repressor gene (Case et al., 1992) and therefore was unable to repress the qa gene cluster using the primary mechanism. This meant that any repression of the system was due to the effects of carbon catabolite

repression. When RNA was isolated from tissue grown in either glucose supplemented or quinic acid supplemented media, there is approximately a 10-fold decrease of the *qa-y* transcript in the glucose sample. All of the genes, except *qa-2* and *qa*-4, are decreased in the presence of dextrose, but nowhere near the level of transcriptional change found in *qa*-y. Since *qa-y* encodes quinic acid permease, the molecule responsible for allowing quinic acid into the cell, it seems that glucose inhibits the production of quinic acid permease thereby limiting the activators entry and up regulation of the system (Arnett et al., 2009).

Another study, by Logan et al., looked at 11,000 genes from *N. crassa* to find genes that have a QA-1F binding site, and are responsive to carbon source shift from sucrose to quinic acid. Out of the 11,000 genes studied, 895 genes were reactive to quinic acid. The findings revealed main functional protein categories; these included carbon utilization, protein modification, amino acid metabolism, protein degradation, and ribosome biogenesis. The last three of these categories were largely found to be down regulated and termed "off". Protein modification genes, encoding kinases and phosphatases, on the other hand, were up-regulated. These proteins are involved in cascade pathways and may indicate a larger reaction to gene regulation by quinic acid than just the genes with a QA-1F binding site. Although this is not typical carbon catabolite regulation, it does seem to be a global method of gene control that may play into the general carbon catabolite pathways (Logan, 2007).

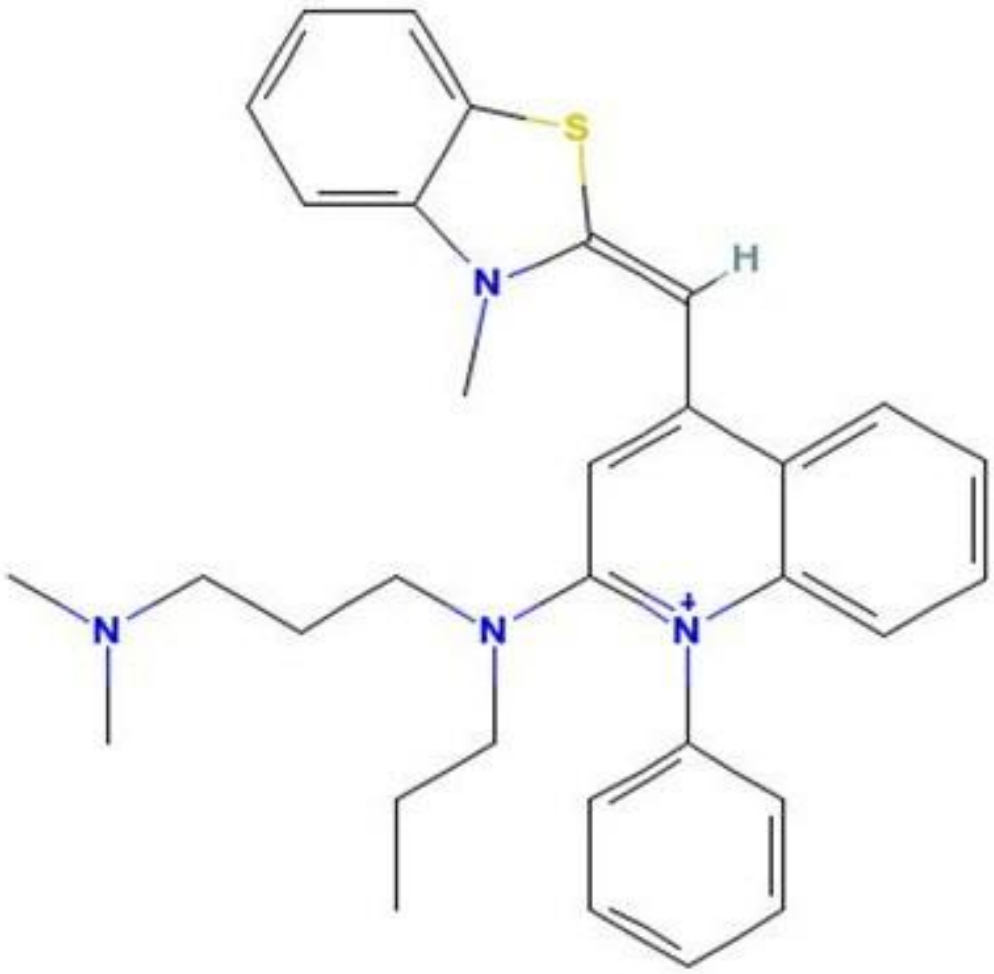
XI. Kinetics of gene expression

Eukaryotic gene expression is highly regulated. This high level of regulation makes gene transcription more specific to the needs of the system than what is observed in prokaryotes. This is the reason rates of induction are not as high in eukaryotes (Bengtsson et al., 2005). The amount of change between an induced and un-induced gene depends on the gene being studied and the environmental conditions being exposed to the gene. Some mechanisms of gene regulation differ while other aspects are more conserved. It would be ideal to find and fully understand the conserved methods for gene expression and the kinetics behind the system.

Northern blotting has previously been carried out to detect amounts of transcription along with calculating the amount of time the genes can be up or down regulated. These results show the general time-frame that changes occur in. Blot analysis reveals a four-fold increase in the amounts of *qa* gene transcripts found when the quinic acid is the only carbon source available. These results were calculated based on computer programs estimating the amount of pixilation in a band on the agarose gel (Arnett, 2009).

These experiments will use the newer technology of quantitative reverse transcription polymerase chain reaction (qRT-PCR) to calculate the changes in transcript levels based on the amount of florescence given off from the SYBR green dye. SYBR green is a non-symmetrical cyanine dye that contains a monomethine bridge that links an N-alkylated benzothiazolium or benzoxazolium ring system to a pyridinium or quinolinium ring system and carries a substituent with a heteroatom, as shown in Figure 4. The dye binds to the minor groove of dsDNA after the extension phase of amplification during qRT-PCR. After binding, there is a conformational change that

Figure 4: Structure of SYBR green dye (PubChem Compound, 1996).


S
N
H
N
N
N
+

causes the bound SYBR green to emit a wavelength of 524 nm wavelength with an excitation signal of 485 nm is transmitted. This allows for more sensitive levels of detection. The benefits of this technology are increased sensitivity due to higher affinity for DNA, and more data piece collection throughout the reaction, not just at the end as in Northern Blots using ethidium bromide (Zipper et al., 2004).

The regulation of these systems is extremely complex. In some ways it is so highly organized, it appears to be completely unorganized. Frequently one change in transcription of one gene will lead to a cascade of effects. This complicates tracking pathways. One answer to this tracking problem is to gather as much data as possible and form a mathematical model for the system (Harris et al., 2002). In the past ten years, thanks to techniques allowing for transcriptional analysis and bioinformatic databases becoming readily available, a number of different approaches for mathematical modeling have been developed.

A mathematical model has been developed for the quinic acid gene cluster in *N. crassa* (Battogtokh, 2002). This model was developed using previous data. Thus far, the model has stood up to the trends of the system as seen by the analysis of Northern blots. The data collected from this research using qRT-PCR may provide new insight to either support the current model, or provide more data to improve the model. Mathematical modeling of basic systems, like the *qa* gene cluster of *N. crassa*, may provide the beginnings for other, more complex regulatory systems.

Specific Aims

This research will quantitate the kinetics of gene induction within the quinic acid gene cluster in *Neurospora crassa.* Five of the seven genes within the *qa* cluster, *qa-2,*

qa-3, *qa-4*, *qa-y*, and *qa-1F*, will be used as representatives of the bidirectionally expressed system. Relative induction rates will be calculated using quantitative reverse transcription polymerase chain reaction (qRT-PCR). It is thought that transcription of the genes within the *qa* system will reach the highest levels of transcription within three hours post-induction with quinic acid. After the gene reaches peak induction, the transcript levels will drop off toward basal levels. Additionally, genes with higher levels of activator binding site conservation will be detected at increased levels, like *qa-2*, before other transcripts due to affinity.

Materials and Methods

I. Tissue Growth:

Neurospora crassa wild-type strain, 74A (FGSC NO. 2489), was obtained from laboratory stock collection, and grown on Horowitz complete media (22 mM potassium tartrate, 47 mM sodium nitrate, 1.5 mM dipotassium phosphate, 2 mM magnesium sulfate, 1.7 mM sodium chloride, 0.9 mM calcium chloride, 1.6% (v/v) glycerol, 0.025% (wt/v) casein, 0.5% (wt/v) yeast extract, 0.5% (wt/v) malt extract) with 1.5% agar at 30°C for two days. The samples were then transferred to room temperature under florescent lights for an additional 8-12 days (Horowitz, 1947).

II. Media Shift:

Conidia were collected after an approximate two-week incubation period and suspended in 25 mL liquid Vogel media (11.6 mM sodium citrate, 28.7 mM dipotassium hydrogen phosphate, 25.0 mM ammonium nitrate, 1.8mM calcium chloride, 0.5% (v/v) trace element solution [237.9 mM citric acid, 173.9 mM zinc sulfate, 26.7 mM ferric ammonium sulfate, 10.0 mM copper sulfate, 14.8 mM manganese sulfate, 8.1 mM boric acid, 2.1 mM sodium molybdate, 1.0% chloroform], 0.5% (v/v) biotin solution [409 µM biotin, 50% ethanol]) supplemented with 2% dextrose as a carbon source (Vogel 1956). Media was filtered through sterile cheesecloth so that the conidia were collected in an Erlenmeyer flask. The solution was divided into equal volumes. These portions were added to 50 ml Vogel media and supplemented with 2% dextrose. Samples then incubated over night in a 30°C incubator shaking at 150 RPM. The samples underwent

vacuum filtration to remove the tissue from the media. The pads were washed in sterile water, and vacuum filtered again. The pads were then shifted into Vogel media with either 2% dextrose, or 0.3% quinic acid. These flasks were incubated for up to three hours in the 30°C incubator shaking at 150 RPM. After the allotted time passed, the samples were vacuum filtered until dry again, and pads were frozen at -70°C for storage.

III. RNA Isolation:

Total RNA was isolated from both the dextrose and quinic acid grown tissues at all time points. Liquid nitrogen, baked sand, and ceramic mortar and pistol set were used to homogenize the tissue. After the tissue was ground into a fine powder, it was divided into eppendorf tubes and 1 mL RiboZol^{TM} (Ameresco, Solon, Ohio) was added to each tube. The protocol was followed as described by the manufacturer. Briefly, after adding the 1 mL RiboZol^{TM}, the samples were incubated at room temperature for 10 minutes. 500µL of chloroform was added next. The samples were vortexed and incubated at room temperature for an additional 3 minutes. Tubes were centrifuged at 13500 RPM. The supernatant, containing the RNA, was removed. The RNA was then precipitated with 100% isopropanol, washed with 75% ethanol, and left to air dry for 6-8 minutes. The isolated RNA was re-suspended in RNA storage buffer (Ameresco) and stored at either -20°C for short-term, or -70°C for long-term storage. The purity and concentration of the RNA was calculated for each sample using a spectrophotometer at 260nm and 280nm wavelengths.

IV. Primer Selection:

Primers were selected for the histone-3 (*his*-3), actin, 18S rRNA, and β-tubulin genes, as controls, and *qa*-2, *qa-3*, *qa-4*, *qa-y*, and *qa-1F* genes using previously sequenced DNA. The *his*-3 was chosen because its transcription remained constant regardless of carbon source based on Northern Blot data (Case, 1992; Battogtokh, et al., 2002; Arnett, Ph. D., 2005). The other control genes were selected because they were more commonly used control genes for microarray or qPCR (Nicot et al., 2005; Yan and Ziou, 2006; Zhang and Hu, 2007). All primers used in this study for RT-PCR and PCR are shown in Table 1.

V. Reverse Transcriptase Polymerase Chain Reaction:

RNA was used as the template sequence for OneStep RT-PCR (Qiagen), for reverse transcriptase PCR (RT-PCR), and HotStart Taq Plus Master Mix kit (Qiagen), for PCR as a control for DNA amplification. One microgram of RNA was added to each reaction for the template. Reactions for each sample contained a total volume of 50 µL with a final concentrations for the reactions being 1X Qiagen OneStep RT-PCR Buffer, 400 µM each dNTP, 0.6 µM each primer. A thermocycler was programmed using manufacturer recommendations as follows for RT-PCR: 30 minutes at 50°C for reverse transcriptase activity; 15 minutes at 95°C for the initial denaturing and inactivation of reverse transcriptase; 30 cycles of the following denaturing, annealing, and extension as 45 seconds at 94°C, 45 seconds at 56°C, and 1 minute at 72°C; and a final extension at 72°C. The PCR program was identical except for the reverse transcriptase step, which was removed. The PCR confirmed that the product originated from RNA transcript, not

from DNA contamination. Without the reverse transcriptase activity, RNA cannot be converted to cDNA and therefore cannot be amplified in a standard polymerase chain reaction. Gel electrophoresis was carried out to check for amplification and proper size on a 1.5% agarose gel.

VI. Quantitative Reverse Transcriptase Polymerase Chain Reaction:

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was carried out using the QuantiFast SYBR Green kit (Qiagen) with the qI5 thermocycler (BioRad). Each reaction contained 1X QuantiFast SYBR Green RT-PCR Master Mix, 1 µM each of two corresponding primers, 0.25 µL QuantiFast RT Mix, and water to bring reaction to 20 µL. RNA, within the range of 9.9×10^{1} ng to 9.9×10^{-5} ng, was added to the reaction. Reactions were run in 96-well plates (BioRad) with clear adhesive sealant (BioRad). Adhering to recommendations set by the manufacturer, the thermocycler was programmed as follows for qRT-PCR: 10 minutes at 50°C for reverse transcriptase activity; 5 minutes at 95°C for the initial denaturing and inactivation of reverse transcriptase; 35 cycles of the following denaturing (95°C for 10 seconds) and combined annealing and extension phases (60°C for 30 seconds). Following each extension phase, a reading of the fluorescence in each well was taken and recorded by the camera within the qI5. The qI5 software then generated a graph based on the readings. A melt curve analysis began after all 35 cycles were carried out with a temperature range from 55°C up to 95°C with 0.5°C increment temperature changes.

Table 1: Primers used throughout experiment for RT-PCR, PCR, and qRT-PCR. Predicted bands sizes are under 200 bp for all primers as required for qRT-PCR.

Gene	Name	Primer
Histone-3	H3F2	5’ – AGA TTC GTC GCT ACC AGA AG – 3’
Histone-3	H3R2	5’ – CGG AGG TCG GAC TTG AAG – 3’
Actin		5’ – GCC GAG CGC GAA ATC GTT – 3’
Actin		5’ – GCT CTG GGC AGC GGT CTG A – 3’
β-tubulin		5’ – CGT CTT CGG CCA GTC CGG – 3’
β-tubulin		5’ – CCG GTA CCA CCA CCG AGG G – 3’
18s rRNA		5’ – TGG CCG GAA GGT CCG GGT – 3’
18s rRNA		5’ – AGT AGC GAC GGG CGG TGT GT – 3’
qa-2	qa-2 F	5’ – GAC GCG CTT CTG GGG ACA GG – 3’
qa-2	qa-2 R	5’ – ACG GCC CCA AAC CGC AAA TGA – 3’
qa-3	qa-3 F	5’ – CGT CCA GCC CCG ACA GCA AG – 3’
qa-3	qa-3 R	5’ – CGG GTG TGC TGG TGA GGT GA – 3’
qa-4	qa-4 F	5’ – CGC CGG CTG CTC TGG ATT GT– 3’
qa-4	qa-4 R	5’ – TGG TCT CGC GCG TGG ATG TC– 3’
qa-y	qa-y F1	5’ – GGT ATC AAT GCC ATC AAC TAT TAC– 3’
qa-y	qa-y R1	5’ – GCC ACA GAA GCC AGA TAA TG – 3’
qa-1F	qa-1Fforward	5’ – TGC CGG GGC AAA GAC ATC CG – 3’
qa-1F	qa-1Freverse	5’ – CCG GGC CTG GTA GAG TGC G – 3’

VI. Data Analysis

Data points were analyzed using ddC_T method in the iQ5 software. Averages of each sample in triplicate were compared to detect increases or decreases in gene expression for a number of variables. Variables include gene studied, amount of time during incubation, and different carbon sources.

Results

I. Isolation

Neurospora crassa was grown on complete media for approximately two weeks before the conidia were harvested in Vogel's minimal media. Media was supplemented with 2% dextrose, grown overnight, and shifted to 0.3% quinic acid, as described above, in Methods. RNA was isolated using RiboZol and stored in RNA storage buffer at -70°C. The purity and concentration of all RNA samples was calculated using a spectrophotometer with absorbance readings of 260 nm and 280 nm wavelengths. RNA with purity levels below 260:280 ratio of 1.5 were not used. Average 260:280 ratio ranged from 1.7 to 1.9. Primers were tested on each batch of RNA using RT-PCR to ensure that both RNA and primers were functional. Gel electrophoresis was then carried out to ensure one band products of the proper size. All batches of RNA were also checked for DNA contamination through PCR. Without the reverse transcription to cDNA, provided by RT-PCR, the polymerase is unable to replicate the RNA template. Gel electrophoresis was carried out after PCR was performed on the RNA template; any band produced from RNA samples without reverse transcription indicates contaminated RNA. Samples of pure RNA were then used as template for qRT-PCR analysis.

II. Internal Controls

Looking at other experiments involving mRNA quantification, in fungi, there were a number of genes commonly used as a reference genes in different studies. In many cases however, the expression levels were being compared under conditions involving temperature change or light application. When changing the environmental condition, and

not nutrient contents, the housekeeping genes are more likely to remain at relatively constant levels. Previous research on the *qa* gene cluster had chosen the histone-3 gene, *his-3*, as a control. Based on the Northern blot data *his-3* was approximately equal (Case, 1992; Battogtokh, et al., 2002; Arnett, Ph. D., 2005), however, initial results with qRT-PCR showed there was an approximate 60% drop in transcription of *his-3* when grown in quinic acid as a sole carbon source (Figure 5). These results lead to the decision to test more genes to find the most constant gene expression levels.

Tissue was grown for the three-hour incubation time to maximize the effects of nutrient limitation in both dextrose and quinic acid. RNA was isolated and qRT-PCR was carried out using the newly designed primers for genes encoding 18s rRNA, β-tubulin, actin, and histone-3. The results are shown in Figure 6 and indicate that *18s rRNA* remain the most constant in a sub-prime carbon source. There is an approximate 35% drop in transcription when tissue is grown in quinic acid, but this is the most constant, and seems to represent the starving cells most appropriately.

Before analyzing the data further, the melt curve data for each of the wells was checked to ensure that there was only one product produced per well. The expected size and the percentage of CG content in the product affect the melting temperature. Using the equation $\mathbf{t_m = 64.9 + 0.41\ (\%GC) - (500/length\ of\ DNA)}$ produced by The Biology Project at the University of Arizona, the melt temperature was calculated. This information, along with the collected experimental melting temperatures, is listed in Table 2.

Figure 5: Results of qRT-PCR using the reference gene, *histone-3*, which had been previously used as an internal control for RNA work in this lab. There was approximately 40% transcript level when tissue was grown using quinic acid.

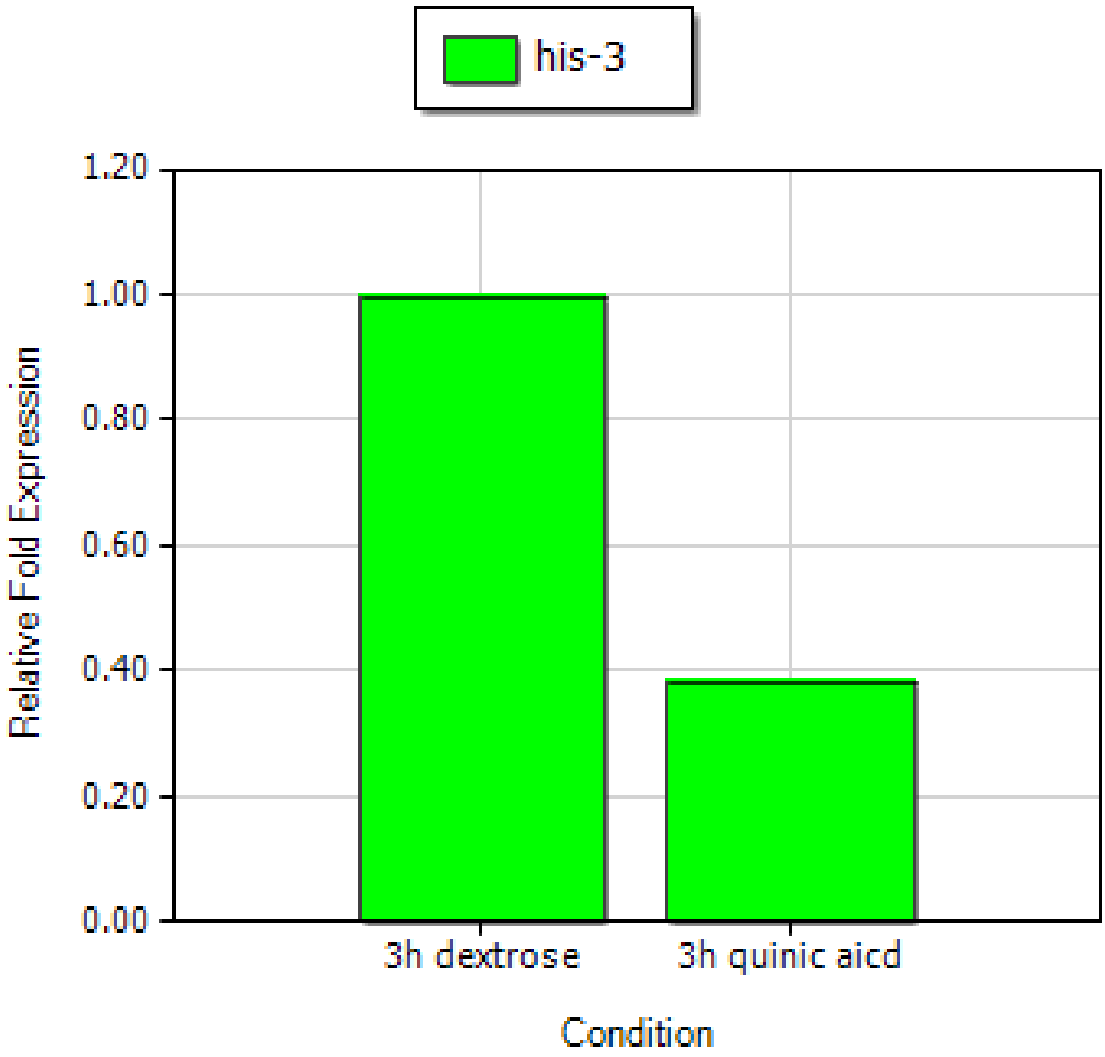

his-3
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Relative Fold Expression
3h dextrose
3h quinic aicd
Condition

Figure 6: Results of qPCR to determine the best reference gene in *N. crassa* when under the experimental stress of a less preferred carbon source.

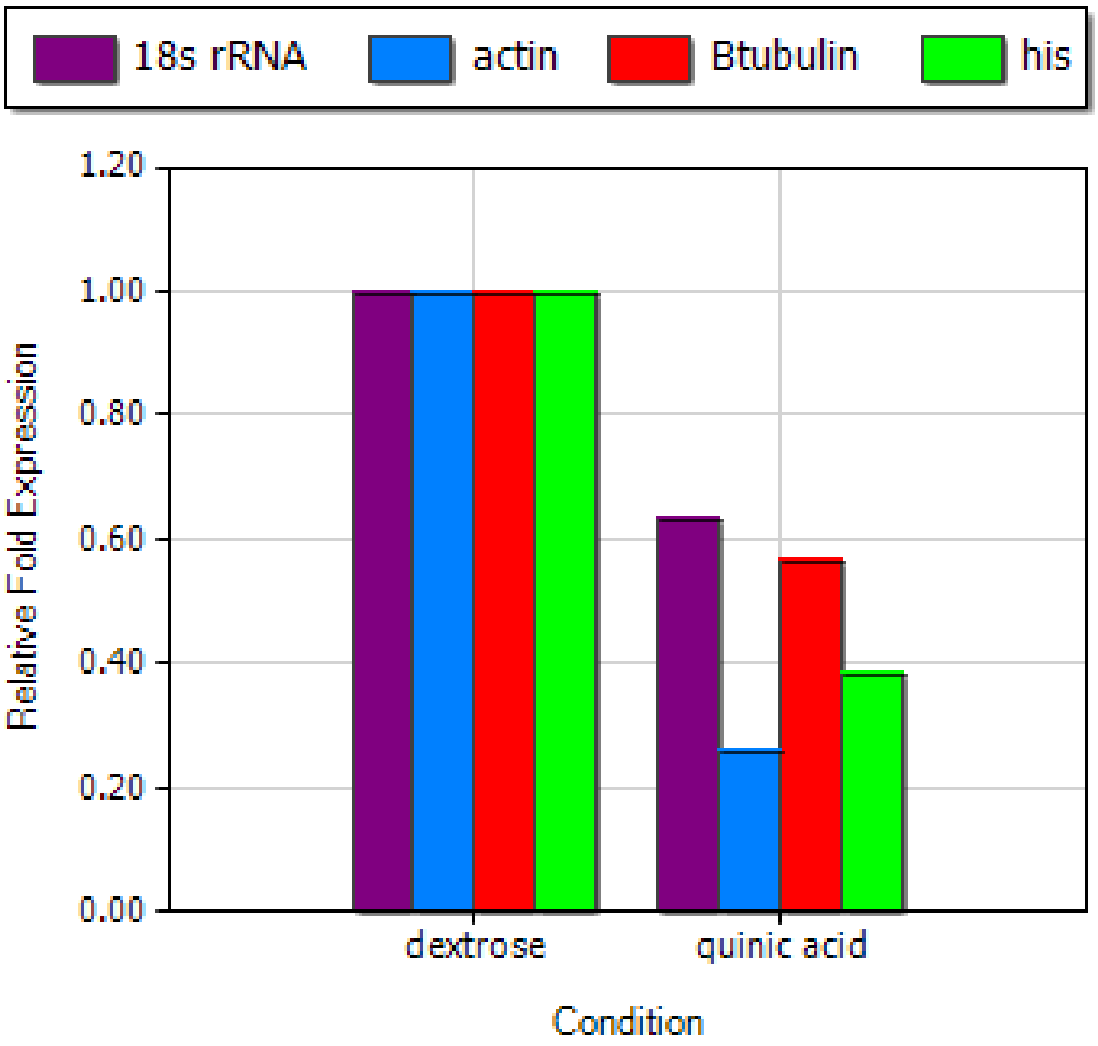

18s rRNA
actin
Btubulin
his
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Relative Fold Expression
dextrose
quinic acid
Condition

Table 2: Information for the gene products produced in qRT-PCR.

Gene	Product Size (BP)	%GC	Th. Melt Temp (°C)	Exp. Melt Temp (°C)
his-3	100	58	83.7	83.5
actin	90	58.9	83.5	83.5
β-tubulin	162	63.6	87.9	87.3
18s rRNA				83
qa-2	130	54.6	83.4	83.5
qa-3	47	66	81.3	multiple
qa-4	113	60.2	85.2	84.5
qa-y	136	52.9	82.9	82.5
qa-1F	98	59.2	84.1	83.5

III. Expression pattern for the *qa-2* gene

Previously, the expression of the *qa* gene cluster has been studied. To analyze the levels of each of the genes within the system *N. crassa* was grown overnight in dextrose supplemented minimal media. The mycelia were vacuum filtered, washed, and re-suspended in minimal media with either 2% dextrose or 0.3% quinic acid and allowed to incubate for up to three hours. The desired times for incubation were 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, or 3 hours. After incubation, the samples were vacuum filtered again. Total RNA was isolated from this tissue and used for qRT-PCR analysis.

Expression of the *qa-2* gene product was measured using primers designed specifically for qRT-PCR, and is shown in Figure 7. For this gene, peak levels of transcription occur at the 1 hour time point. The amount of m*qa-2* at 1 hour post-induction in quinic acid grown tissue compared to dextrose grown tissue is over 400 times the amount. The transcription levels decrease steadily after the 1 hour time point and would be expected to return to basal levels if more time points were taken.

IV. Expression pattern for the *qa-3* gene

Transcription levels were detected for *qa*-3 using total RNA that had been isolated as described above. Results for the *qa-3* gene transcription were unable to be determined at this time due to multiple melting points for the PCR product. The data output for the melt curve analysis is shown in Table 3. Most of the reactions, approximately 70% of the wells tested, had two or more products. When using SYBR green it is not possible to differentiate between products within the same

Figure 7: Results of qRT-PCR showing expression of the *qa-2* gene over the time period of 15 minutes post inoculation to 3 hours post inoculation.

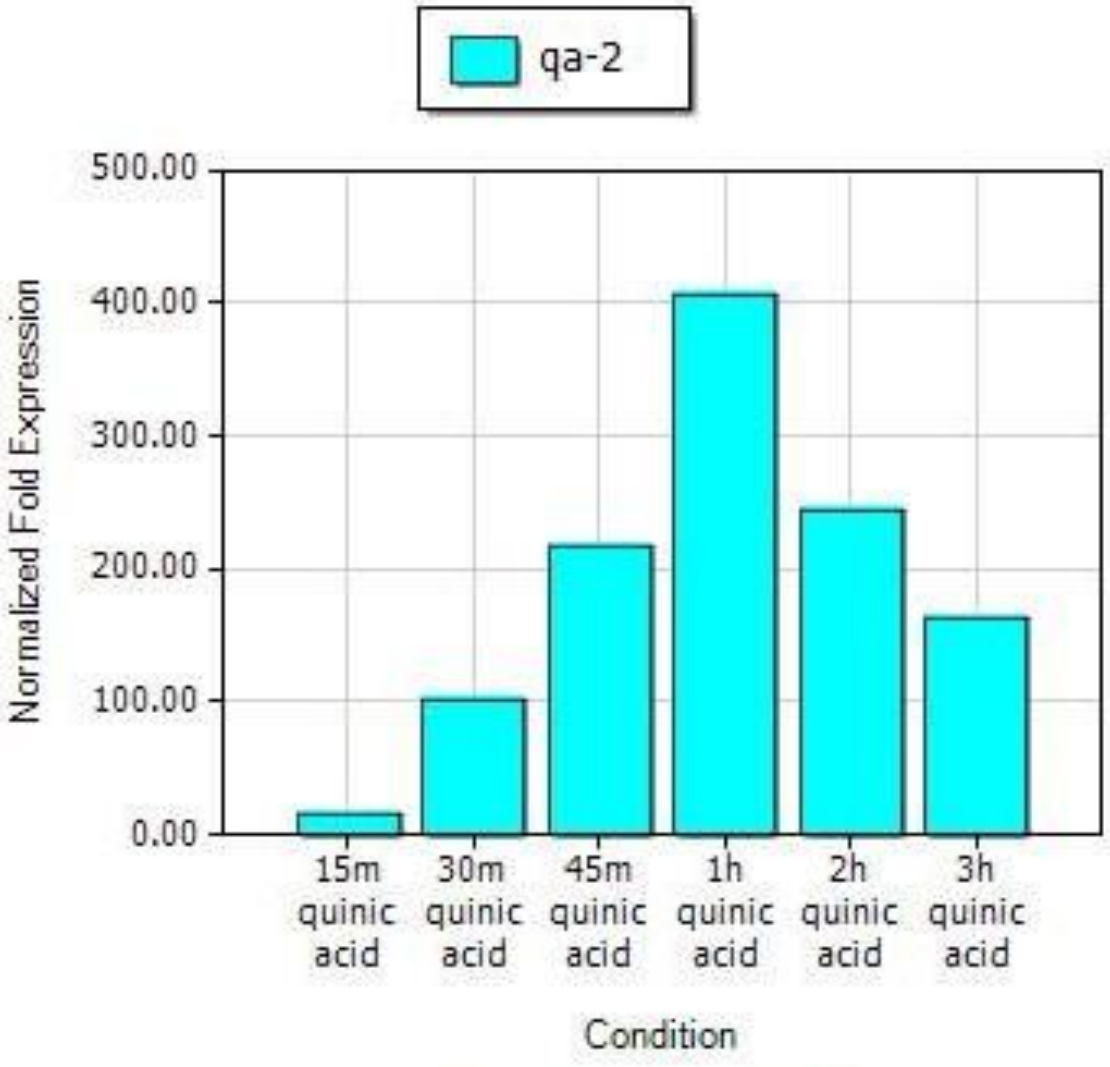

qa-2
500.00
400.00
300.00
200.00
100.00
0.00
Normalized Fold Expression
15m quinic acid
30m quinic acid
45m quinic acid
1h quinic acid
2h quinic acid
3h quinic acid
Condition

Table 3: Displays the multiple melt curve temperatures from *qa-3* qRT-PCR runs which indicate that the primers bind nonspecifically to template cDNA in reaction therefore producing multiple products.

Well Number	Melt Temp (°C)		Well Number	Melt Temp (°C)
A01	86.50		D01	81.00
A02	87.50			84.50
A03	87.50		D02	81.50
A07	86.50			84.50
A08	81.00		D03	81.50
	86.50		D07	81.50
A09	80.50			85.00
	86.00		D08	81.50
B01	81.00			85.00
	85.50		D09	81.50
B02	80.00			84.50
	83.00		E01	81.50
	85.50		E02	81.50
B03	85.00		E03	81.50
B07	80.50		E07	81.00
	84.50			85.00
	86.50		E08	81.00
B08	81.00			85.50
	86.00		E09	80.50
B09	82.50			85.50
	86.00		F01	81.00
C01	80.00		F02	81.00
	85.00			84.50
C02	80.00		F03	81.00
	85.00			85.50
C03	80.50		F07	81.00
	85.00			85.00
C07	80.50		F08	81.00
	85.50			85.00
C08	83.00		F09	82.00
C09	80.50			86.00
	86.00			

sample and therefore this set of primers is not able to be used for *qa-3* transcript detection.

V. Expression pattern for the *qa-4* gene

The *qa*-4 gene transcription levels were measured from the same total RNA isolated and used in the aforementioned gene studies. The *qa-4* gene product is produced at slightly higher levels when induced as compared to dextrose conditions. The graphic output, Figure 8, shows a peak for this gene after 45 minutes of induction. The largest difference in transcript levels appears to be just under 14 times the non-induced state.

VI. Expression pattern for the *qa-y* gene

The gene encoding a permease, *qa-y*, is increases transcription less than 15 minutes after induction with quinic acid and continues to increase until approximately 1 hour post-induction using fresh aliquots of isolated RNA. The normalized fold induction, shown in Figure 9, reveals there is an increase in transcript level up to 12 times the dextrose conditions. At the 3 hour time point the induction is still at 2/3 the peak level, and therefore still remains relatively high as compared to the equivalent dextrose time point sample.

Figure 8: Results of qRT-PCR measuring expression of the *qa-4* gene over the time period of 15 minutes post inoculation to 3 hours post inoculation.

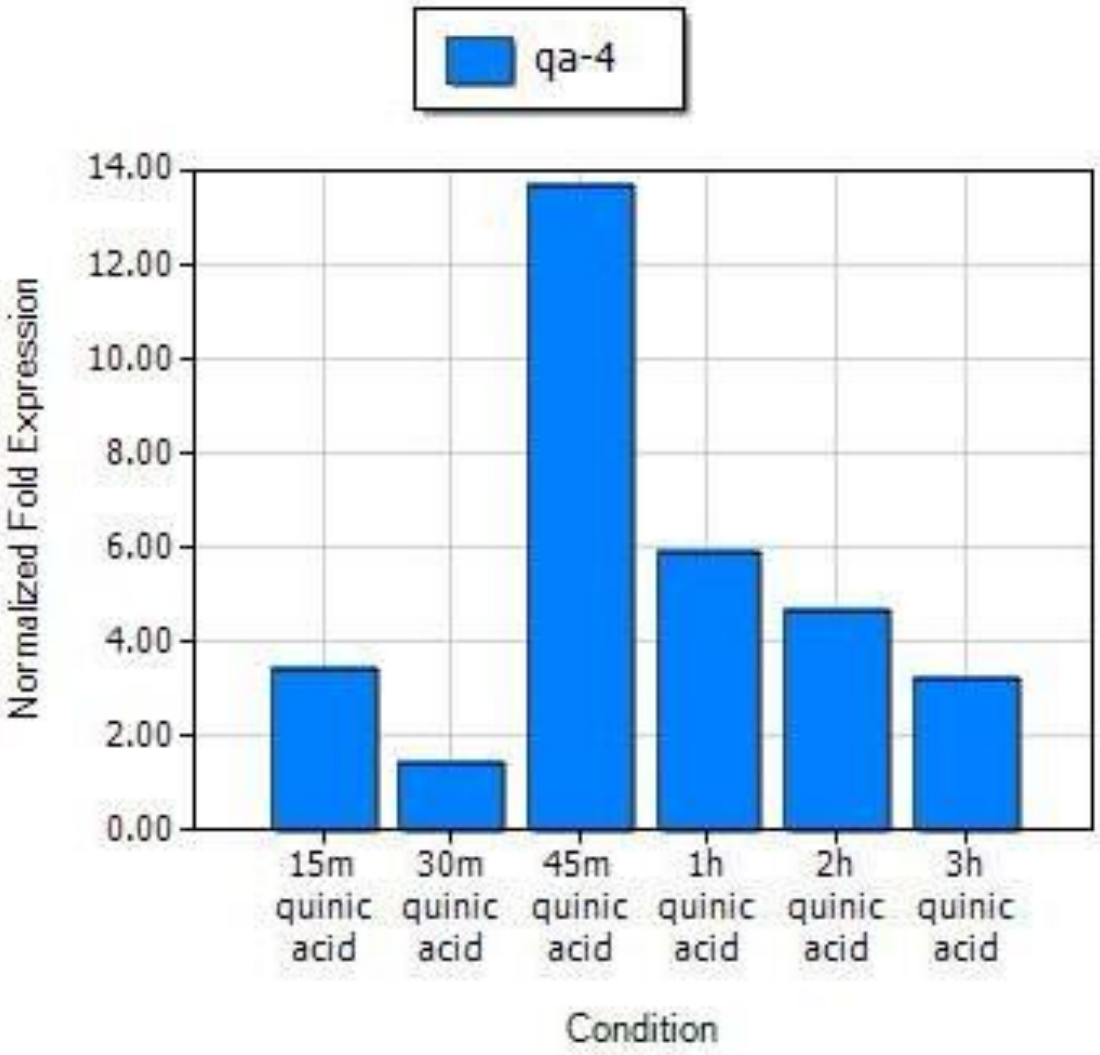
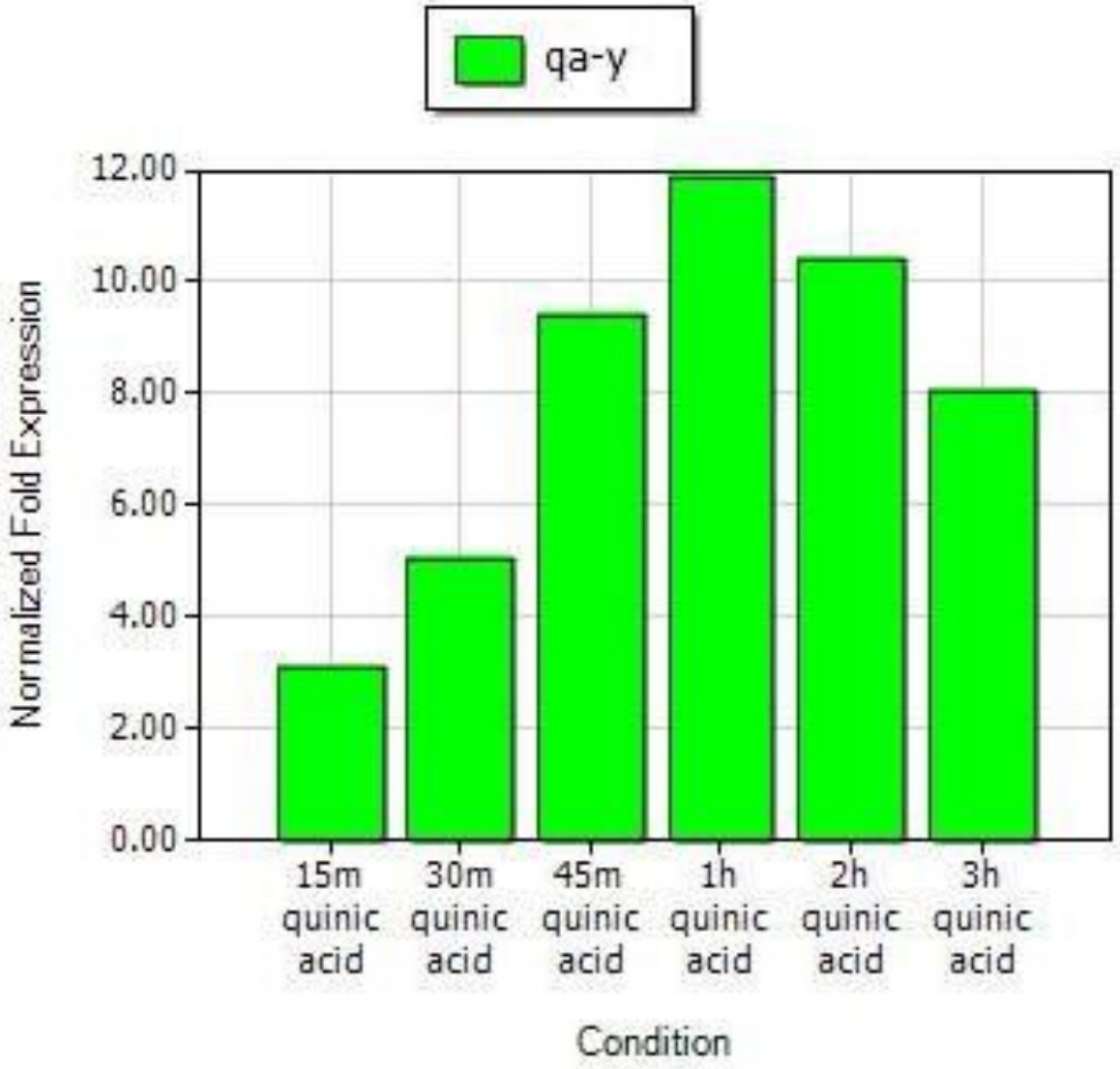

qa-4
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
Normalized Fold Expression
15m quinic acid
30m quinic acid
45m quinic acid
1h quinic acid
2h quinic acid
3h quinic acid
Condition

Figure 9: Results of qRT-PCR measuring expression of the *qa-y* gene over the time period of 15 minutes post induction to 3 hours post induction.


qa-y
12.00
10.00
8.00
6.00
4.00
2.00
0.00
Normalized Fold Expression
15m quinic acid
30m quinic acid
45m quinic acid
1h quinic acid
2h quinic acid
3h quinic acid
Condition

VII. Expression pattern for the *qa-1F* gene

The results for *qa-1F* appear to be the least consistent of the genes tested thus far. The measurements were taken from the total RNA that had been isolated as described above. There is not a gradual, bell-shaped change in transcription levels (Figure 10). The amount of fold induction goes up and down between measurements. At the 1 hour time point there is a large increase in production of transcript, although this may be exaggerated by relatively low amounts of transcript measured at the 45 minute and 2 hour time points. The peak level of transcription reaches 90 times the dextrose condition.

VIII. Combining data

The previous data was all compiled and a new graph, shown in Figure 11, that displays all of the time points, all of the experimental genes, for the quinic acid induced genes. This data has been normalized to minimize differences between the different files, the control gene 18s rRNA, and dextrose conditions. This allows the overall picture to be interpreted by using one scale for all of the genes. This graph shows that *qa-4* and *qa-y* have very similar amounts of up-regulation, and not as strongly up-regulated. The *qa-2* gene has the highest up-regulation. All of the genes are up-regulated after only 15 minutes of induction with quinic acid.

Figure 10: Results of qRT-PCR measuring the expression of *qa-1F gene* over the time period of 15 minutes post inoculation to 3 hours post inoculation.

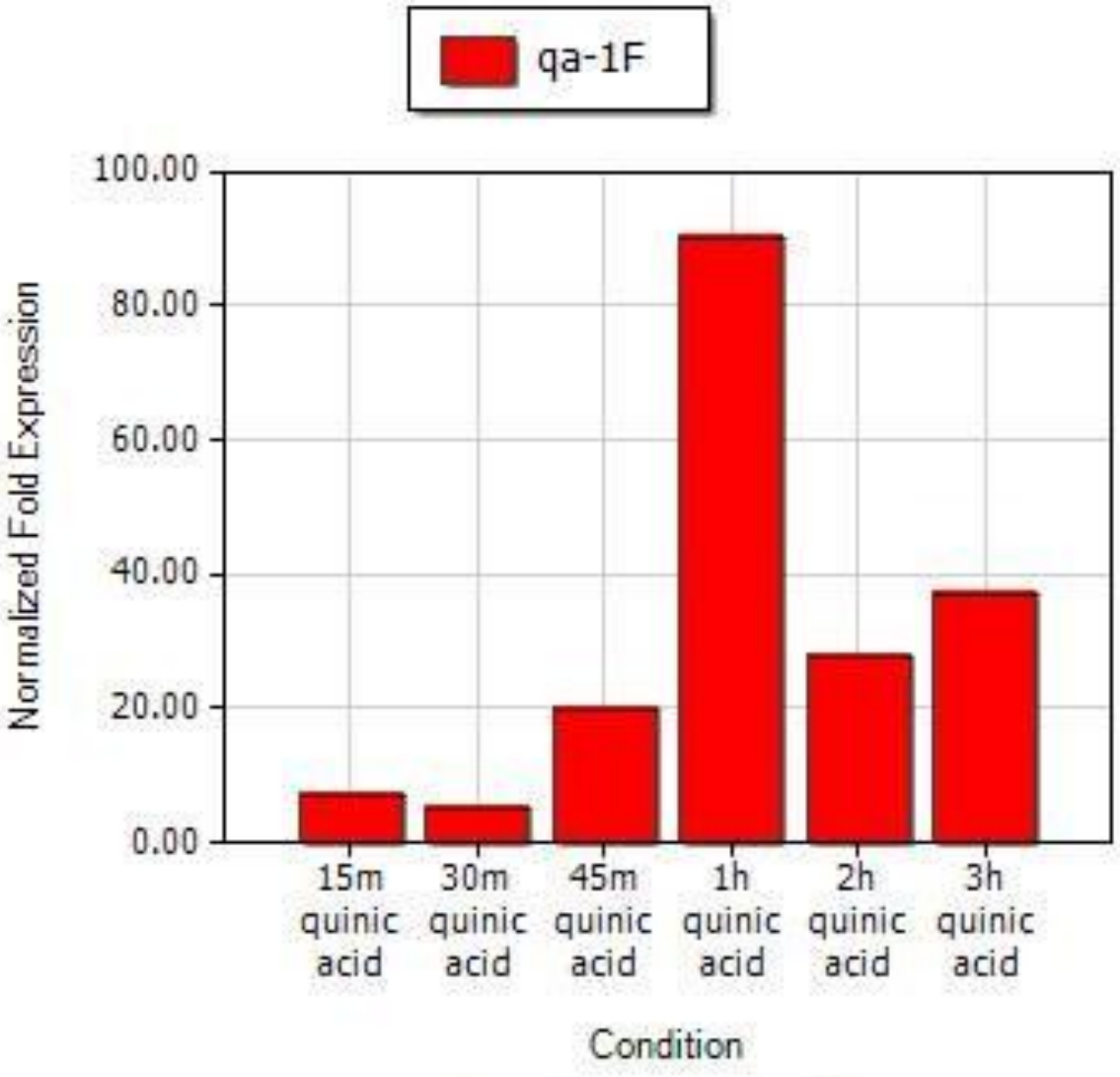
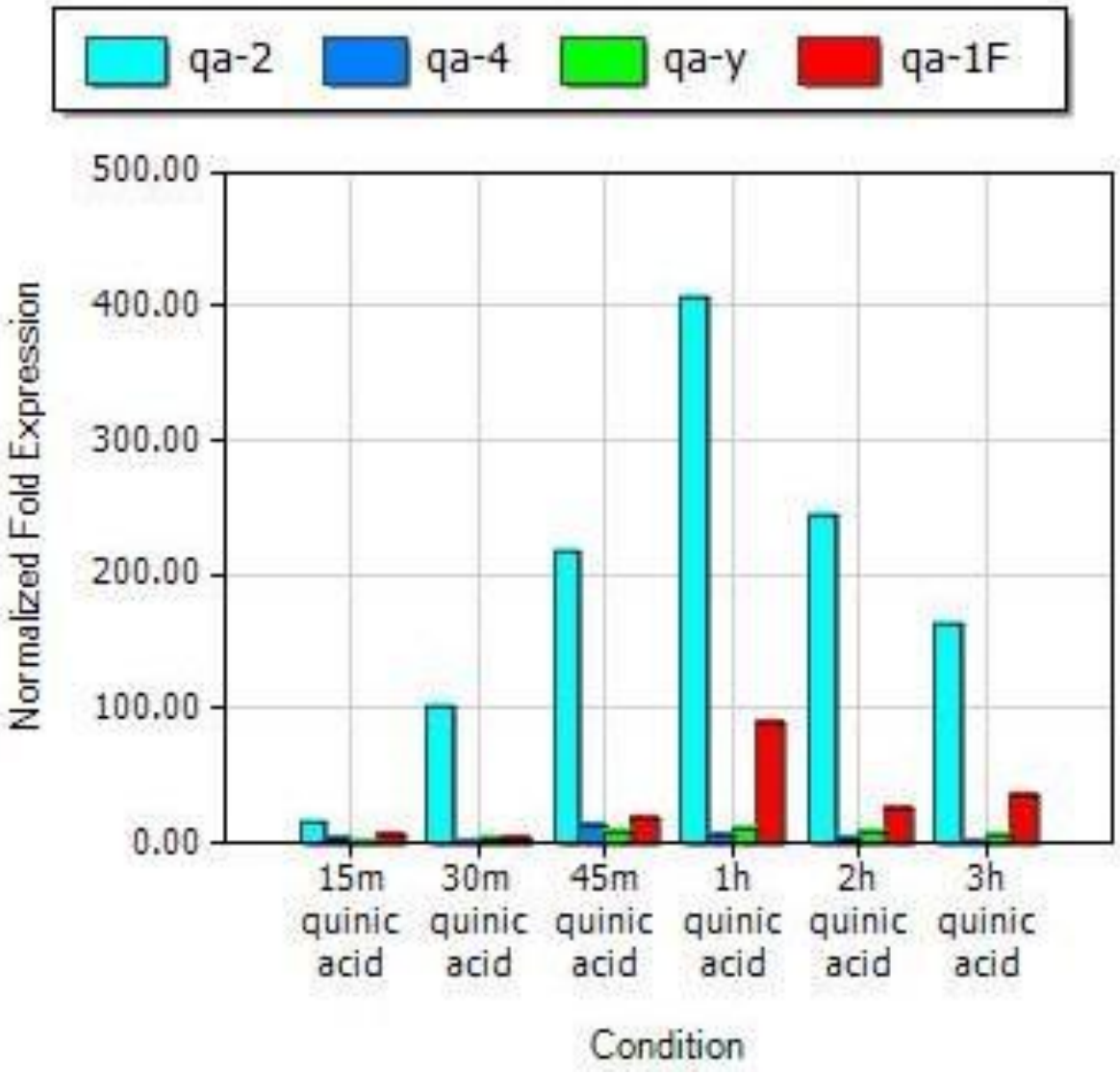

qa-1F
100.00
80.00
60.00
40.00
20.00
0.00
Normalized Fold Expression
15m quinic acid
30m quinic acid
45m quinic acid
1h quinic acid
2h quinic acid
3h quinic acid
Condition

Figure 11: Expression pattern for all *qa* genes over the time period of 15 minutes post inoculation to 3 hours post inoculation.


qa-2
qa-4
qa-y
qa-1F
500.00
400.00
300.00
200.00
100.00
0.00
Normalized Fold Expression
15m quinic acid
30m quinic acid
45m quinic acid
1h quinic acid
2h quinic acid
3h quinic acid
Condition

Discussion

Control genes

Little work has been previously carried out on *Neurospora crassa* using sensitive mRNA detection techniques like microarray or qRT-PCR. Both of these mechanisms are able to detect very minute changes in transcription levels. This level of sensitivity makes finding a gene that remains constitutively expressed, at the exact same levels, under two or more conditions challenging. For this research, the experimental conditions involved a control of a preferred carbon source, dextrose, compared to a poor carbon source, quinic acid. Although it is known that *N. crassa* is able to metabolize quinic acid, the organism is not thriving under these conditions. When microbial species are exposed to stress, unnecessary gene transcription is depressed. Many housekeeping genes are not needed when the extra stress is added to the organism.

Research in this area is only beginning to be documented. There have been two studies which have looked at the regulation of genes in *Neurospora crassa*. One of the two, looks only at the differences in gene transcript levels for genes containing the QA-1F binding domain. While the findings are interesting, and provide the basis for future studies, the remaining 10,000 genes were not discussed further. It would be interesting to see if there were better control genes picked out in this experiment (Logan et al., 2007). The other study, by Aign et al., looked at the effects of poor carbon source on gene transcription, including the effects of acetate as a carbon source (Aign et al., 2003). Again, the controls were not defined and therefore the data was not helpful to this project for internal reference gene selection.

This research looks at four of the most commonly used reference genes in fungi for q-PCR. Thus far, the literature has not shown a comparison between typical reference genes for fungal species. Many other groups, including mammalian and bacterial, have such studies published (Vandesompele et al., 2002; Desroche et al., 2005). This research may be able to be applied to other groups studying related fungal species such as other *Neurospora*, or many species of *Aspergillus*. Studies that show levels of RNA quantification in a variety of environmental conditions are very helpful to researchers beginning new projects or trying to increase the accuracy of the current project. This research has shown that out of the three genes most commonly used as controls for fungi, *18s rRNA*, *β-tubulin*, and *actin*, that the *18s rRNA* seems to be most constant, even in the stressed state of starvation.

_Qa_ gene cluster

Within the *qa* gene cluster thirteen activator binding sites have been identified through DNase footprinting (Baum et al., 1987). Each of these binding sites was found, *in vitro*, to have a different level of binding affinity with the activator protein. Some genes are associated with more than one binding site, however, the overall effect of these multiple sites and their strength is not known *in vivo*. The overall affinity for each of the genes can be interpreted from the individual binding affinities. The *qa-2* gene is associated with three of the 16 bp activator binding sites. One of these binding sites has the highest affinity out of all of the binding sites for the cluster as seen in Table 4. The data is supported the qRT-PCR data as shown above. The *qa-2* gene transcript is up-regulated to a higher degree than the other genes that have been tested within the cluster. The three other genes, *qa-4*, *qa-y*, and *qa-1F*, have an increase in transcription within

Table 4: Activator binding sites for all *qa* genes are listed along with their relative location and the relative binding affinity (adapted from Baum et al., 1987).

Gene	Binding Strength*
qa-2-qa-x	0.25
qa-2	<0.1
qa-2	1
qa-4	0.25
qa-4	<<0.1
qa-3	0.5
qa-3	0.15
qa-3	<0.1
qa-y	0.25
qa-y	0.4
qa-y	<<0.1
qa-1S	0.1
qa-1F-qa-1S	0.8

* Activator binding strengths are based on relative amounts of activator required to completely bind the DNA binding sites. The numerical value was collected by comparing the amount of activator for the strongest site to all 13 sites.

two-fold of each other. Looking at the affinities for *qa-4* and *qa-y, in vitro,* they are very similar with the strongest relative affinity for both genes around 0.25 compared to the *qa-2* binding site affinity. The findings in this study show similar transcription levels for *qa-4* and *qa-y*. Again, this was expected from the known binding affinities and supports that the *in vitro* findings represent the *in vivo* reactions.

This research may support the *qa*-regulation hypothesis that includes the activator and repressor proteins being previously bound to the activator binding sites based on the rate at which the system is up-regulated. This data shows that there is a change in the level, over two-fold, for all of the gene transcripts tested within the *qa* gene cluster by the 15 minute incubation period with quinic acid. It is difficult to imagine that the activator is nearby, but not bound to, the binding site, and would be released from the repressor, and aid in the up-regulation of the genes in such a short time frame.

Previously, this study had been carried out using Northern Blots. The analysis of these blots lead to the mathematical modeling of this system through a series of equations (Battogtokh, et al., 2002). It appeared that the transcription of the *qa* genes was less dramatic of an up-regulation, and that it occurred over a longer period of time. Earlier research on the proteomic expression of *qa-2* (Giles et al., 1985) showed approximately 45% levels of DHQase in the dextrose conditions compared to quinic acid grown tissue. There are a few explanations for the smaller fold increase from the in vitro enzyme assays as compared to the qRT-PCR findings. One reason is that the protein activity is more fragile, and changes are harder to detect than the RNA; another could be the degradation of protein before expression levels are recorded properly. Whatever the

reasons may be for the increased levels of up-regulation, it seems that the response to quinic acid may be much larger than previously expected.

One of the genes, *qa*-3, was previously able to be quantified, but in this study the detection was not accurate. As of now, a primer pair was not able to be designed that would not bind to and amplify other genes within the *N. crassa* genome. The likely reason for the challenging design is due to the nature of the gene sequence. The protein product for *qa-3* is only 321 residues long. Also, as mentioned above, *qa-3* encodes a dehydrogenase. Dehydrogenases are abundant, with most sharing a very similar active domain. The similar functional domains therefore have retained sequence homology. Out of the nucleotide sequence, approximately 822 bp are involved in the conservation of the active domains out of the total 965 bp sequence. The first 93 nucleotides and the last 24 nucleotides do not encode the domain, however the end is too small for sequence amplification, and the beginning too similar to other genes. Conserved sequences among different enzymes are not able to be used for unique primer sequences. Out of the remaining sequence, there is not a fragment length appropriate for PCR.

Future work

This research could have been improved by increasing the number of samples that were analyzed. This would most likely give more data points and help in lowering the overall standard deviation between samples. More samples would also help to determine false readings due to the sensitivity of the SYBRgreen binding dye.

Research in this area could be expanded to more time points throughout the experiment to attempt to find exact times for initial regulatory changes. This would include more studies at the beginning, to see up-regulation, and longer length, possibly

2.5 and 4 hour samples, to see the return to basal transcription. This strategy, however, may not lead to exact, reproducible results because quantitating small time ranges accurately becomes less reliable than looking for levels at spaced time points for general trends. Isolation of RNA would need to be frozen at a very specific time point, and currently there would most likely be too many errors in tissue preparation before isolation to be this exact.

Looking at results from research that is currently being conducted on proteomics of *N. crassa* may provide other genes involved with the metabolism of less-preferred carbon sources. Analyzing the level of transcriptional control for the related systems might provide more insight to the overall regulation. Other genes for the system should be looked into, along with genes that are known to be involved in carbon catabolite repression.

The findings here support the hypotheses that transcription of the *qa* genes reached peak levels by the three-hour time-point post-induction incubation with quinic acid, and that after each gene reaches peak induction the transcript levels decrease toward a level of enzyme maintenance. Based on our findings, the *in vivo* expression levels are related to the data produced *in vitro* for affinity strength of the activator binding sites. The strength of the sites are, greatest to least, *qa*-2 > *qa-1F* > *qa-y* > *qa-4*, and overall levels of transcription follow this same pattern.

References

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