Expression of Genes in *Neurospora crassa* Outside of the Quinic Acid Gene Cluster During Quinic Acid Metabolism

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John William Savopoulos

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Signature: _____

Mr. John William Savopoulos, Student Date

Approvals: _____

Dr. David K. Asch, Thesis Advisor Date

_____

Dr. Jonathan J. Caguiat, Committee Member Date

_____

Dr. Xiangjia "Jack" Min, Committee Member Date

_____

Dr. Salvatore A. Sanders, Dean of Graduate Studies Date

Abstract

The quinic acid (*qa*) gene cluster in *Neurospora crassa (N. crassa)* consists of two regulatory genes (*qa-1F* and *qa-1S*) as well as five structural genes (*qa-2, qa-3, qa-4, qa-y,* and *qa-x*) that are transcribed in the presence of quinic acid with suppressed levels of glucose (Greever R.F., et al. 1988). The corresponding activation and repression of this cluster enables the survival of the organism in less than hospitable environments. A research study hypothesized that some 50 genes were under the control of the *qa-1F* gene and another *qa* transcription factor. Furthermore, it was shown at the proteomic level in the work of Kayla Brown, Katie Allen, and Dana Tirabassi that glycogen phosphorylase (NCU07027), peptidyl-prolyl cis-trans isomerase (NCU04140), and NCU08332 *Hex-1*: Woronin Body Coding Protein all had heightened expression at the proteomic level in the presence of quinic acid. Therefore, the scope of this work aimed to analyze these three genes to determine their levels of transcription in the presence of quinic acid (utilizing qRT-PCR), and to determine if they are related to the quinic acid gene cluster.

The results indicate that heightened glycogen phosphorylase expression seen in prior works is not related to quinic acid metabolism and is not a result of starvation. However, it is predicted that the cell may be holding on to this protein for other purposes not related to the scope of this work. Peptidyl prolyl cis-trans isomerase appeared to not be related to the quinic acid gene cluster, but rather showed heightened expression as a result of starvation. Last, the NCU08332 *Hex-1* Woronin body major protein gene appeared to have a direct relation to quinic acid metabolism and the quinic acid gene cluster. Furthermore, bioinformatics analyses presented also supported this assumptions.

Acknowledgements

Although I am the only author of this work, it goes without saying that I had a huge amount of help not only scholastically but also personally from a great group of people. First and foremost, I want to thank Dr. David Asch for all of his exceptional advice from my freshman year of undergraduate work all the way through my graduate studies. Working in his lab has been an absolute privilege and a distinct honor that I will cherish for the rest of my life and has allow me to excel academically and reach my goals. Furthermore, I would also like to thank Dr. Jonathan Caguiat. Dr. Cagiuat has been a tremendous help in writing and analyzing all of the components of my thesis. If I was ever in doubt of a certain lab technique that I was utilizing, I knew I could always stop in Dr. Caguiat's lab to get some pointers. Also, I would like to thank Dr. Xiangjia "Jack" Min. Dr. Min has taught me everything I know regarding bioinformatics. I am by no means a "computer guy", but Dr. Min made the subject very comprehendible which has allowed me to conduct this research. Beyond that, Dr. Min also has given me excellent feedback every time I have needed to revise my manuscript. Beyond the members of my committee, I would also like to thank Dr. Mark Womble and Dr. Deborah Benyo. It has been an honor to work with Dr. Womble and Dr. Benyo as well as to get to know both of these individuals throughout my graduate coursework. In addition, both Dr. Benyo and Dr. Womble have given me a lot of helpful tips in the logistical parts of writing my thesis. I also want to thank Joshua Engle and Mr. Julio "Ed" Budde for all of their help with qRT-PCR. Without their help none of this research could have been possible. Last, I want to give a special thanks to the undergraduates that have worked with me in the lab. I

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Chapter 1: Introduction

I. Fungi

With 90,000 species identified and some 1.5 million species estimated to be in existence, Eumycota or Fungi represent a vast category of Eukaryotes that have been and remain a significant group in scientific discovery. As of 2005, the International Society of Protistologists included fungi in the supergroup *Opisthokonta;* a classification containing all metazoans. Typically though, the kingdom Fungi is classified into six phyla: Ascomycota, Basidiomycota, Chytridiomycota, Glomeromycota, Microsporidia, and Zygomycota (Willey J.M., et al. 2008). Each of these classifications can be further simplified with each member having its own significant traits, but with each ultimately containing the relative principles that constitute them in the kingdom Fungi. Furthermore, all six phyla of the kingdom Fungi contain saprophytes or organisms that are capable of retrieving nutrients via osmosis (osmotrophy) through the release of degradative enzymes.

II. Neurospora crassa: A Member of the Phylum Ascomycota

Neurospora crassa has been a model organism in genetic and biochemical studies for many generations. From its first reference in bakeries throughout Paris to the Nobel Prize winning " one gene- one enzyme" hypothesis of Beadle and Tatum (Beadle G.W., Tatum E.L. 1941), experimentations with *N. crassa* have led to various fundamental genetic principals such as mitochondrial protein import, DNA methylation, circadian rhythms, post-transcriptional gene silencing, and DNA repair (Deacon J., 2006). With its greater than 40 megabase (Mb) genome, *N. crassa* is more complex than other common model organisms, such as *Saccharomyces cerevisiae,* making it ideal for certain analyses.

N crassa's genome encodes for roughly 10,000 protein coding genes which is approximately 25% less than *Drosophila melanogaster* (Galagan J.E., et al. 2003). Its ease of genetic manipulation via transformation with foreign DNA, rapid growth rates, availability of well-characterized mutants, and relative stability make *N. crassa* a preferred organism in many genomic research laboratories (Deacon J., 2006). As of 2003, James Galagan and his associates completed the sequencing of the entire 40 Mb genome. Galagan and his associates noted many important genomic features in their studies **(Table 1)**, namely that, as of 2003, roughly 41% of the protein coding sequences had no similarity to known sequences in various proteomic databases (Galagan J.E., et al. 2003). This shows that although this organism has been studied for the past three quarters of a century and genomic and proteomic technologies have drastically advanced since initial investigation, there is still a wealth of information that is unknown about the genetic features of this Fungi.

Feature	Value
General	
Size (bp) (assembly 5)	38,639,769
Chromosomes	7
G + C content (%)	50
Protein-coding genes	10,082
Protein-coding genes >100 amino acids	9,200
tRNA genes	424
5S rRNA genes	74
Per cent coding	44
Per cent intronic	6
Average gene size (bp)	1,673 (481 amino acids)
Average intergenic distance (bp)	1,953
Predicted protein-coding sequences	
Identified by similarity to known sequences	1,336 (13%)
Conserved hypothetical proteins	4,606 (46%)
Predicted proteins (no similarity to known sequences)	4,140 (41%)

Table 1: *Neurospora crassa* Genome Features (Galagan J.E., et al. 2003)

Ascomycetes and Basidiomycetes are often referred to as "higher Fungi" due to their considerably more complex genome when compared to other Fungi (Alexopoulos

C.J. 1962). *Neurospora crassa,* a well-studied organism belonging to Ascomycetes, is often referred to as a "sac Fungi" due to the sac like reproductive structure known as an ascus (Willey J.M., et al. 2008). *Neurospora crassa (N. crassa)* is classified as a heterothallic species meaning that the organism has sexes that exist in different individuals (Russo E.A., Pandit N.N. 1992). However, *N. crassa* is not only capable of reproducing sexually, but can reproduce asexually as well **(Figure 1)**. Asexual reproduction typically involves the production of conidia where sexual reproduction involves ascus formation. During asexual reproduction, initially a conidium germinates on a suitable form and eventually develops into a mat of hyphae known as mycelium (Willey J.M., et al. 2008). After several days of growth on a suitable media, such as Vogels plus Sucrose minimal media, macroconidia (variable in nuclei) and microconidia (mono-nucleated) form. With sexual reproduction, formation of the protoperithecia marks the initiation of the sexual cycle. These female structures contact cells of other mating types which

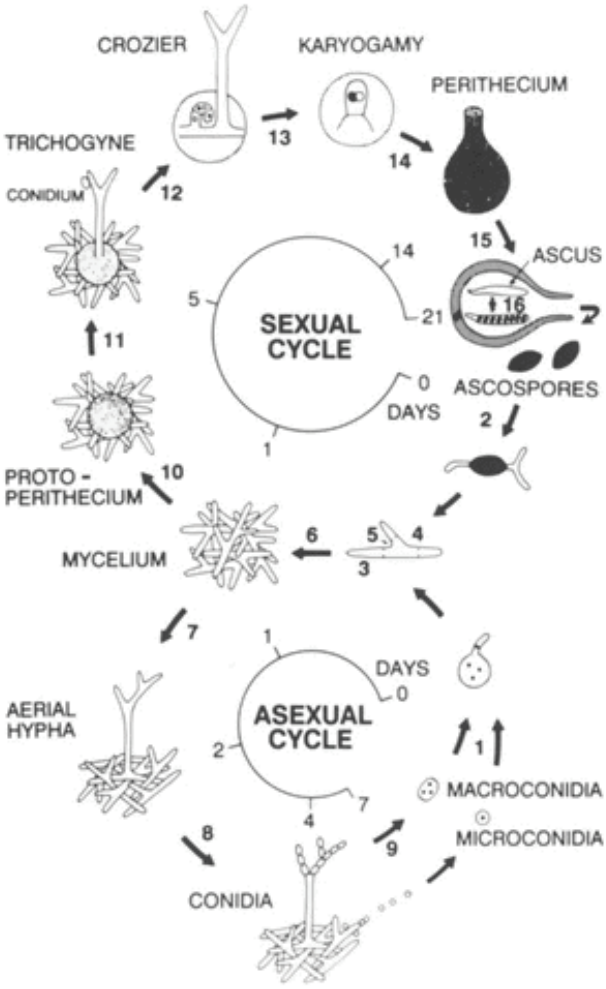

Figure 1: Reproductive Cycles of *Neurospora crassa* (Russo E.A., Pandit N.N. 1992)

initiates fertilization. Protoperithecia are noted as female due to the fact that they provide mitochondria and cytoplasm to the ascospores following the sexual cycle. Later on in the sexual cycle the protoperithecia appears much larger and is then deemed the perithecium. This structure contains roughly 200-400 asci having eight ascospores per ascus. After approximately fourteen days post fertilization, the perithecia ejects ascospores in groups of eight which occurs for several days (Russo E.A., Pandit N.N. 1992).

N. crassa possesses the widest array of genomic defense mechanisms among eukaryotic organisms (Deacon J., 2006). Recently, it has been discovered that repeated induced point mutations are prevalent in *Neurospora.* Repeated induced point mutations typically occur in *N. crassa* during sexual reproduction in haploid nuclei, between fertilization and meiotic DNA replication (the haploid dikaryotic phase). This phenomenon detects and mutates each copy of the reproduced gene via mutations from G: C to A: T pairs. This event often leads to DNA methylation which causes gene silencing (Deacon J., 2006). This function has been noted in other fungal species as well, and in *N. crassa* has been assumed to cause a lack of genome evolution ever since the acquisition of the repeated induced point mutation function at some time in its evolutionary lineage (Deacon J., 2006).

III. Quinic Acid Metabolism: a Function of the Quinic Acid (qa) Gene cluster in N. crassa

The quinic acid (*qa*) gene cluster in *N. crassa* consists of two regulatory genes (*qa-1F* and *qa-1S*) as well as five structural genes (*qa-2, qa-3, qa-4, qa-y,* and *qa-x*) that

are transcribed in the presence of quinic acid **(Figure 2)** with low levels of glucose (Greever R.F., et al. 1988). The corresponding activation and repression of this cluster then enables the survival of the organism in less than hospitable environments. In the presence of quinic acid, a cyclohexanecarboxylic acid found in cinchona bark and coffee beans, the *qa-1F* gene in *N. crassa* acts as an activator, resulting in the expression of the *qa* gene cluster to aid the organism's altered metabolic pathway. This pathway is then repressed by the *qa-1S* gene in the absence of quinic acid **(Figure 3).** While the absolute function of the *qa-x* gene in the cluster is unknown, the other structural genes have known functions; the *qa-y* encodes for a protein that is a carrier protein, aiding the movement of quinic acid into the cell (Logan D.A., et al. 2007) while the *qa-2* gene encodes for the catabolite dehydroquinase (5-dehdroquinate hydrolase); *qa-3* encodes for quinate dehydrogenase (quinate NAD oxioreductase) and the *qa-4* encoding for 5-dehydroshikimate dehydratase (Case M.E., Giles N.H. 1976). This then aids in the initial step of converting quinate to protocatechuic acid under conditions when quinic acid acts as the sole carbon source for metabolic processes in *N. crassa* **(Figure 4)** (Anton I.A., et al. 1987). Furthermore, protocatechuic

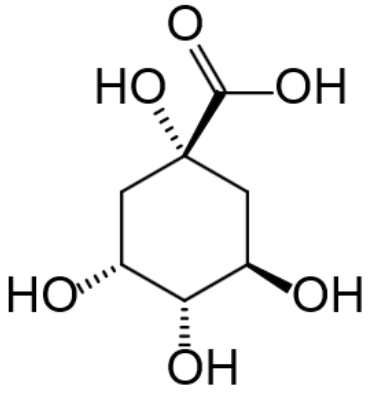

Figure 2: Quinic Acid: A 192.167 g/mol cyclohexanecarboxylic acid

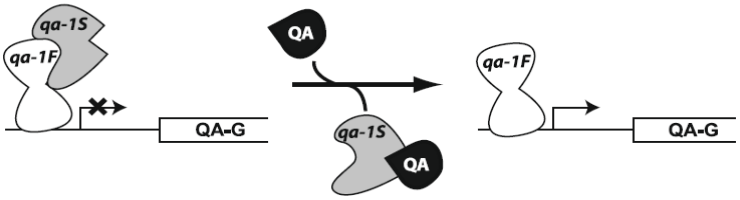

Figure 3: Activation of the quinic acid (qa) genes (QA-G). In the absence of qa, the qa-1S protein binds to the qa-1F protein preventing transcription of the qa genes. In the presence of qa, qa bind to the qa-1S protein dissociating it from the qa-1F protein allowing for transcription of the qa genes. (Figure adapted from Arnett D.R., et al 2009)

acid is oxidized by oxygenase enzymatic activity prior to ortho **(Figure 5)** or meta **(Figure 6)** cleavage of the protocatechuic acid substrate.

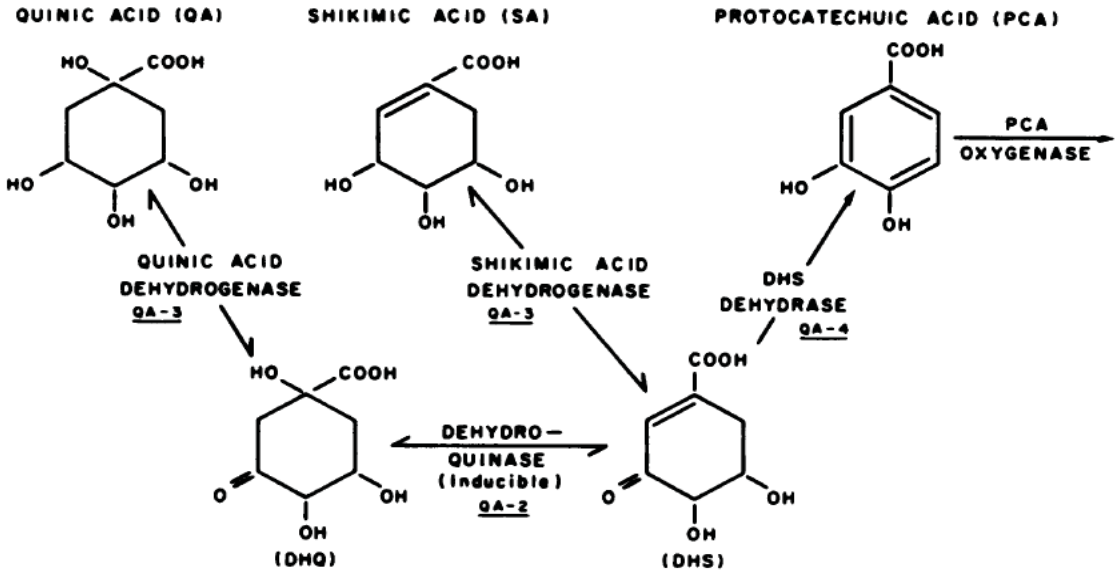

Figure 4: Conversion of Quinic Acid to Protocateuchuic Acid via Enzyme Products of the *qa* Gene cluster in *N. crassa.* Each gene is underscored below the corresponding enzyme it is associated with. (Figure adapted from Giles G.H., et al. 1985)

A research study at the University of Georgia hypothesized (via microarray analysis) that some 50 genes outside of the quinic acid gene cluster were under the control of the *qa-1F* gene and another *qa* transcription factor. Furthermore, both transcription factors are members in the fungal binuclear Zn(II)2Cys6 cluster family (Tang X., et al. 2011). Prior to this, other experimentations indicated that *qa-1F* plays a positive role in regulation where the *qa-1S* acts as the suppressor. Studies by Layne Huiet also provide evidence supporting the idea that although the *qa-1S* gene is induced by quinic acid, it also is under subsequent control of the *qa-1F* activator gene (Huiet L. 1983). The *qa-1F* gene encodes a 2.9 kb mRNA and the *qa-1S* gene encodes both a 4.1 kb and a 3.4 kb mRNA. Through the use of *qa-1F* and *qa-1S* mutants, it was supposed

that since *qa-1F* was transcribed at miniscule amounts in the *qa-1S* mutants that the *qa-1S* gene product may regulate the *qa* structural genes by regulating the transcription of the *qa-1F* in concordance with quinic acid acting as a suppressor (Huiet L. 1983). Subsequent studies of this phenomenon pointed out that mutants in the *qa-1F* gene are also involved in the regulation of the synthesis of *qa-1F* mRNA (Patel V.B., Giles N.H. 1985). It was shown that while being induced, the *qa-1S*$^-$ mutant, as proposed by V.B Patel and his colleagues, produced lower levels of *qa-1F* mRNA in a relatively similar amount to that of *qa-1F*$^-$ mutant (induced conditions) and to the same degree as *qa-1F*$^+$ mutant under non-induced conditions. This evidence along with other prior studies exemplified the hypothesis that *qa-1F* is an inducer of the seven genes of the quinic acid gene cluster, and the *qa-1S* gene is indeed a

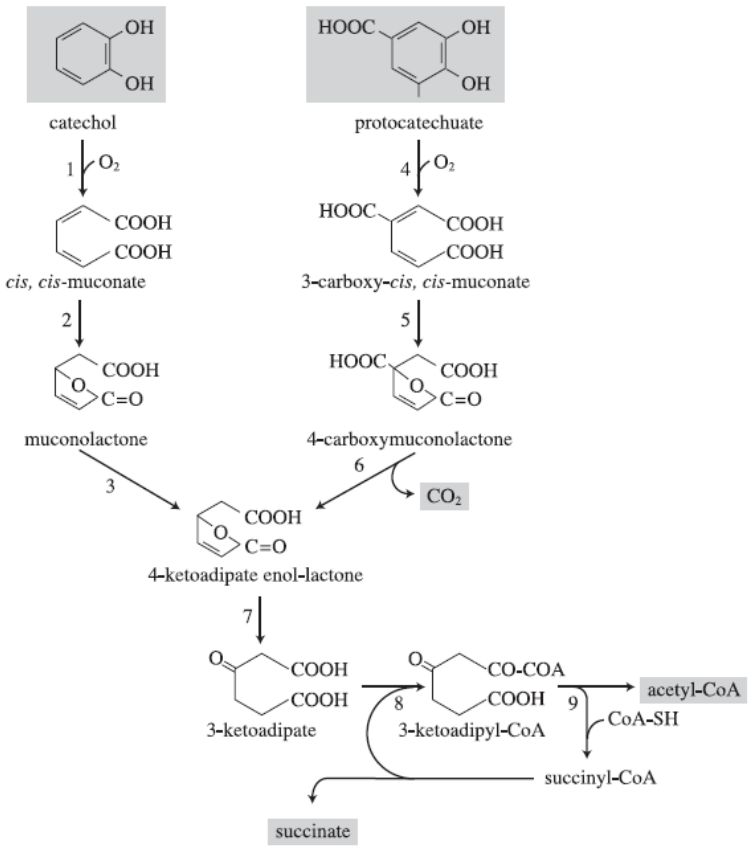

Figure 5: Ortho cleavage of protocatechuate resulting in acetyl-CoA and succinate (Kim B.H., Gadd G.M. 2008)

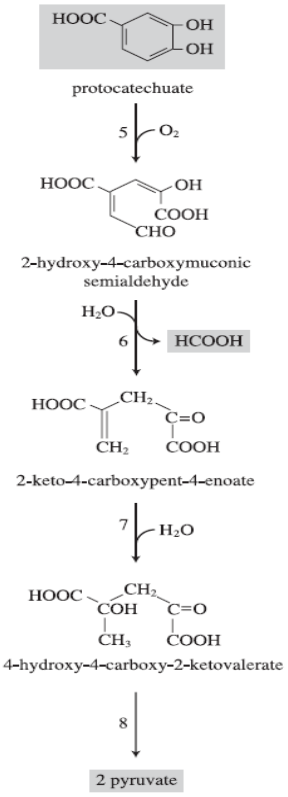

Figure 6: Meta cleavage of protocatechuate (Kim B.H., Gadd G.M. 2008)

suppressor of the cluster, hindering the functionality of the *qa* cluster in the absence of quinic acid.

Among the structural genes in the *qa* gene cluster, *qa-2* encodes for 5-dehydroquinate hydrolyase, a catabolic dehydroquinase (Stroman P., et al. 1978). Generally as with most of the *qa* structural genes, *qa-2* is typically activated via a functional activator protein encoded by the *qa-1F* activator gene, but this is not always the case. A study by Robert Geever and his associates at the University of Georgia, determined the presence of 12 mutations, via Southern blots, in the *qa-2* gene that allow for the fractional expression of 5-dehydroquinate hydrolase without the presence of the activator protein encoded by the *qa-1F* gene. It was also noted in this study that there is a positive correlation between activity of the catabolic dehydroquinase enzyme produced and the amount of mRNA transcription activity of the *qa-2* gene (Geever R.F., et al. 1983). This simply means that with an increase in enzyme activity (5-dehydroquinate hydrolyase) comes a proportional increase in *qa-2* mRNA transcription. One of the mutations of specific interest showed roughly 45% of *qa-2* expression, with regard to the wild-type gene. This amount in the mutant was considered high by Geever and his colleagues so further tests were done via the use of cloning and sequencing. The 158-33 mutation began by fusing specific unique DNA sequences from *N. crassa* 378 base pairs up-stream (-378) from the normal functioning initiation site. It was not determined that this mutation causes change in RNA pol II recognitions sites since this is not often seen in other eukaryotic organisms. This mutation also was not comparable to analogous prokaryotic genetic systems. This then led to the hypothesis that the 158-33 mutation of the *qa-2* gene requires an alternate method of action to initiate an increase in the

transcriptional activity of *qa-2*. Geever and his associates described this phenomenon as simply being an upstream activation sequence that increases the basal transcriptional levels of *qa-2* from a distance away. It is important to note then, that similar upstream activators present in the 158-33 mutant are present in other organisms. Geever noted that within normal *his3, cyc1,* and *gal10* in yeast (*Saccharomyces cerevisiae*) and the herpesvirus *tk* gene, similar upstream regulating factors exist backing the claim regarding the 158-33 mutant's ability to increase transcription of the *qa-2* gene. Furthermore, subsequent studies investigated the relation between the *qa-x-qa-2* intergenic (non-coding) regions of other species of *Neurospora.* DNA of the intergenic region of the two genes of interest were digested with *Bg/*II, *Hind*III, and *Bam*HI in several heterothallic and homothallic species of *Neurospora. N. tetrasperma, N. intermedia, N. sitophila,* and *N. galapagosensis* (heterothallic) revealed highly conserved restriction fragment size after enzymatic digestion via three enzymes (*Bg/*II, *Hind*III, and *Bam*HI). After digestion of the sequence in the homothallic species, a high degree of polymorphism was shown as compared to *N. crassa.* Regardless of this result, further discovery showed that the *qa-x-qa-2* intergenic region was located on the 2.4kb *Hind*III fragment in the homothallic species (*N. Africana, N. dodgei,* and *N. lineolate*) leading to a further result showing that the *qa* gene order of *N. crassa* is conserved among the three homothallic species tested (Asch D.K., et. al. 1991).

The *qa-3* structural gene in *N. crassa* encodes for NAD oxidoreductase, a quinate dehydrogenase. This is the first enzyme available in the quinic acid catabolic pathway in *N. crassa* (Case M.E., et al. 1978). The *qa-3* gene is comprised of 322 codons. Of these, 27 are considered to be rare. They include nine GGG codons encoding for glycine (Gly) ,

eight AGG/AGA codons coding for arginine (Arg) and six codons that encode for proline (pro) (Li-Bing L., et al. 2006). Outside of the species *Neurospora,* researchers have experimented with implementation of the *qa-3* gene in *E. coli.* Quinic acid is a chemical product that not only can act as a carbon source for *N. crassa,* but also has an important role in chemical industry. Quinate 5-dehydrogenase is product of the *qa-3* gene and must be imported into *E. coli* in order to create a quinic acid engineering bacterium (Li-Bing L., et al. 2006). Although it has been tested and shown that the *qa-3* gene cannot be expressed in *E. coli,* insight into the rare codons present in the gene provide insight as to how this may be accomplished. Although the *qa-3* gene has not been able to be expressed in *E. coli,* cloning and expression of the *qa-2* gene has been accomplished in *E. coli* species. (Stroman P., et al. 1978).

3-dehydroshikimate dehydratase catalyzes the third step of the quinic acid catabolic pathway in *N. crassa.* This enzyme is encoded for by the *qa-4* structural gene in the *qa* gene cluster. Purifications of this enzyme have shown that it is a monomer roughly 37 kilo Daltons (kDal) in size. Within the *qa-4* gene it also has been determined that there are no introns within the *qa-4* coding region (Rutledge B.J. 1984). A study at the University of Georgia further analyzed the base composition of the *qa-4* gene in order to determine percentages of bases. Within the coding region of *qa-4*, it was shown that 20.7% base composition consisted of Adenine, 28.7% consisted of Cytosine, 27.1% consisted of Guanine, and 23.6% consisted of Thymine. These figures are considered fairly consistent. 100 nucleotides upstream toward the 5' end of the sequence (-100 to +1), the results were quite different. Adenine accounted for 19.0% of the total base composition while Cytosine counted for 38.0%, Guanine 11.0%, and Thymine 32.0%.

Guanine is present in rather low amounts (11.0%) while Cytosine is quite high (38.0%). Similar results were also seen 50 nucleotides upstream from the ATG start codon (-50 to +1). At this position on the sequence, Adenine accounted for 20.0% of the total base composition while Cytosine accounted for 42.0% (similar to the -100 to +1 region), Thymine 24.0%, and Guanine 14.0% (similar to the -100 to +1 region). Downstream from the TAA stop codon, base composition was again very different from the prior three regions. 100 nucleotides downstream from the TAA codon, Adenine and Cytosine constituted 34.0% base composition (17.0% per base respectively), while Guanine accounted for only 15.0% and Thymine constituting 51.0% of the total base composition. 50 nucleotides downstream from the TAA stop codon, similar results were presented. Thymine showed the highest composition at 51.0% (compared to 46.0% 100 nucleotides downstream), Cytosine constituted 16.0% of the total base composition (compared to 17.0% 100 nucleotides downstream), and Guanine and Adenine made up roughly 38.0% of the total composition (Rutledge B.J. 1984). In short then, upstream of the start codon, cytosine was the most prevalent and downstream of the stop codon, thymine was the most prevalent nitrogenous base.

IV. Peptidyl-prolyl cis-trans isomerase (NCU04140)

Previous cluster and microarray analyses of genes expressed during quinic acid metabolism in *N. crassa* have shown that there are greater than 100 gene products linked to quinic acid metabolism (Logan D.A., et al. 2007). Although it was not shown in the microarray analysis, Katie Allen proposed that one possible protein product produced is peptidyl-prolyl cis-trans isomerase (PPIase) (Master's thesis). This enzyme produced by

the peptidyl-prolyl cis-tans isomerase gene (*fkr-2)*, catalyzes a reversible conformational change from cis to trans in peptide bonds that are followed by a proline amino acid residue (i.e. xx-Pro) (Hanes S.D. 2015). In some cases, isomerization of the prolyl bond, either *in vivo* or *in vitro*, is the rate-limiting step in folding of the protein (Pinto D., et al. 2008). Rather than utilizing energy from Adenosine triphosphate (ATP) to catalyze the cis to trans transformation, energy is gained from conformational changes in protein substrates, thus this may be a possibility for this process being the rate-limiting step in protein folding (Hanes S.D. 2015). Initially discovered in 1984 by Gunter Fischer and his associates in pig kidney tissue, PPIase are typically divided into three highly conserved groups (cylophilins, FKBPs (FK506 binding proteins), and parvulins) that are found in the cellular compartments of all organisms: Archaea, Eukaryotes, and Prokaryotes. PPIases are important in signal transduction, apoptosis, rDNA silencing, cell-cycle regulation, protein assembly, control of transcription, and protein trafficking (Pinto D., et al. 2008).Cyclophilins and FKBP's also have roles in tumorigenesis via prolyl cis/trans isomerase signaling pathways (Theuerkorn M., et al., 2011). It also has been proposed that PPIases promote folding of ribosomal proteins that are formed before folding to their active shape (nascent proteins) (Tremmel D., Tropschul M., 2007).

```
   1 ggcttcaacc cacacaagta agctctttga gaggtttatt acctctggat acctctacct
  61 agaggtaggt ttcaagaggt cgaggtcaag caacctcgag gtcggaaaag aacgctgttg
 121 gttccatttt ccagcagtta gtttgcttat acacaaacgt gccgacccgc gctgtgctga
 181 ttctggcatt cattcagtga tctccctgta catattccct gattcccgtt cccagcttgg
 241 aaggctcact gcaggatcgc tttgcctcct gccctccttg ctcgttctgc ccctctcttt
 301 cgtccccttc cactcaacag gttcaccaat ctaattacgc catgaacggg ataaagcttg
 361 cgccccaccg cctcagggtc gtcgctcctc gtctttccct cctccgcccc tcgttcctct
 421 tttctgtgcc caagattctg aactctctcc cgtacaccac tcctcaaaca aacccaaaac
 481 accacttcac caccacatcc agcaagatga ctattcccca gcttgacggt cttcagatcg
 541 aggtccagca ggagggccag ggcacccgtg agacccgtcg cggcgacaac gtcgacgtcc
 601 actacaaggg tgtcctcacc agcggtaaga agttcgacgc tagctacgac cgtggcgagc
 661 ctctcaactt caccgttggc cagggccagg tcatcaaggg ctgggatgag ggtctcctcg
 721 gcatgaagat tggcgagaag cgcaagctca ctatcgcccc tcacctcgcc tacggcaacc
 781 gcgccgtcgg tggcatcatc cccgccaact ccaccctcat cttcgagacc gagctcgtcg
 841 gtatcaaggg tgtccagaag ggcgagtaaa tggatcactt acaaatgcat ctatagctct
 901 gggatacagg cggattaacg ggtcaatgga agatcatttt cggcgttatg tctcttaccg
 961 gtgctgactc gccctgagcg cttctcgagt cgcgattggg gctggacaga tactatttga
1021 gggtcatatt tagggaggaa acgttcgcat aaaacagacc gcgttggctg cctacctact
1081 tgctttgcaa tcctccgagt gcccctagtg atgagtacag gccgcccatg ccgtattggt
1141 tcacacattg acttgcgttt cggttgtttg gttaagagtt tgttagttcg ggaagtgtac
1201 taatcctact actagcgttc ttcacaggct cgtcctcgtg agtactccta actggaccat
1261 gcagcggcac tatacagttc cctaactgtc tataccttct gtcggcatac actagtccga
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1561 cgtccctacg cacatccatc gcctcacggt tggcaaccac acacccgcat tcactctcca
1621 gccctctgaa caccttttct ggcccttcgc tgtttccccc ggtcatccag tccgcaatcg
1681 ccgcgcccgc gatgtcatct gtaaggatat ggatattgaa ccgcctgctg gcgcgcaatg
1741 cgtccagtgt ccgggacaaa tggttgatgt tgaagctgac aatgggcgag ggggaaaggc
1801 cgaggcaggt gagagaggac atggtcatgc cgcgcatggt cggctta
```

Figure 7: mRNA from the 1847bp *fkr-2* gene. The coding sequence (CDS) which codes for the 120 amino acid protein Peptidyl-prolyl cis-trans isomerase fkr-2 (NCBI Accession P20080) (Figure 8) is highlighted in brown.

The Neurospora Sequencing Project at Whitehead Institute and MIT's Center for Genomic Research showed that FKBP11, FKBP13, FKBP22, and FKBP50 were present in *N. crassa's* genome. FKBP22's acts in the luminal space of the endoplasmic reticulum where is has PPIase function as well as chaperone activity due to its direct interaction with the major HSP70 chaperone BiP, forming a folding helper complex that has great chaperone activity preventing aggregation of unfolded rhodanese (Pinto D., et al. 2008). This homodimeric protein also has shown involvement in the development of microconidophores (Tremmel D., et al., 2007). Although initially isolated from cytosol, FKBP13 has a second location in the mitochondrial matrix of *N. crassa* (Pinto D., et al. 2008). Both the FKBP13 and FKBP22 proteins are translated from RNA that is

```
 1 mtipqldglq ievqqegqgt retrrgdnvd vhykgvltsg kkfdasydrg eplnftvgqg
61 qvikgwdegl lgmkigekrk ltiaphlayg nravggiipa nstlifetel vgikgvqkge
```

Figure 8: The 120 amino acid protein Peptidyl-prolyl cis-trans isomerase fkr-2 (NCBI Accession P20080)

transcribed from the *fkr-2* (FK506 resistant-2) gene **(Figure 7)** (NCBI Accession Number XM_956203.3). This 1847 bp DNA sequence, found on the right arm linkage group V on the fifth chromosome (Chromosome V) in *N. crassa,* contains two (2) exons and codes for the 120 amino acid cytosolic protein **(Figure 8)** FK506-binding protein 1A (also referred to as FKBP-1A or Peptidyl-prolyl cis-trans isomerase fkr-2 (NCBI Accession P20080)). This protein was initially described in Katie Allen's analysis (Master's thesis) of the proteome during *N. crassa's* growth on quinic acid. Although this protein was produced, the degree to which the coding gene was expressed in the presence quinic acid as the sole or partial carbon source during quinic acid metabolism by *N. crassa* is still unknown. Likewise, little is known of the exact role of the protein product in qa metabolism if it has a function in this process at all.

FK506-binding protein or peptidyl-prolyl cis-trans isomerase derived from *N. crassa* has high similarities in sequence to the same gene (FKBP) in *Homo sapiens* (Barthelmess I.B., Tropschug M., 1993). FK506 is an antibiotic derived from the soil-borne bacteria *Streptomyces tsukabaensis* that is frequently used after organ transplants or tissue grafts due to its decreased nephrotoxic and neurotoxic properties when compared to other immunosuppressant drugs such as cyclosporine (Barthelmess I.B., Tropschug M., 1993; McDiarmid S.V., et al., 1993). In nanomolar concentrations, FK506 can inhibit T-cell initiation, thus its importance when there is a need for immunosuppression. As the name suggests, FK506 binds directly to the intracellular protein FK506-binding protein. In *N.*

crassa, FK506 is toxic when bound to the major receptor FKBP13 (encoded for by *fkr-2*) (Pinto D., et al. 2008). Furthermore, in the 1993 study by Ilse Barthelmess and Maximilian Tropschug, it was proven via induced mutation that when FK506-binding protein was missing from *fkr-2*, cytotoxic effects lacked, but when present, FK506 was toxic to *N. crassa* showing that FK506 binding protein facilitated the cytotoxic effect of this antibiotic (Barthelmess I.B., Tropschug M., 1993).

V. Glycogen Phosphorylase in N. crassa (NCU07027)

The highly conserved enzyme glycogen phosphorylase catalyzes the rate limiting step in the breakdown of glycogen to glycogen and glycogen-6-phosphate via glucogenolysis **(Figure 9)**. Glycogen phosphorylase is a dimer of identical 842 amino acid residues that undergoes one of two conformational changes which deems the protein active or inactive. The active or R conformation is characterized by the conformation of the enzyme that has an accessible, high affinity, catalytic phosphate binding site where the inactive or T state has an active site that is rather secluded due to the protein's shape inhibiting phosphate binding (Voet D., et al., 2008). After the breakdown to glucose-1-phosphate and glycogen, glucose-1-phosphate must be further catalyzed to glucose-6-phosphate in order to be further metabolized via glycolysis. Glycogen phosphorylase has been analyzed in many organisms from dog thyroid gland to maize endosperm, but it was

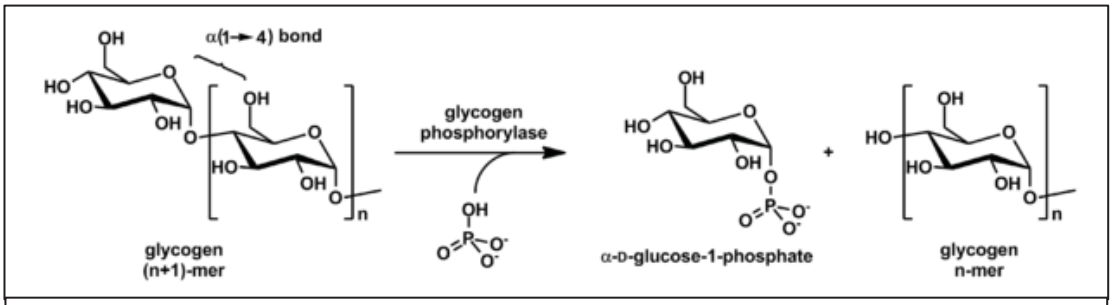

Figure 9: Glucogenolysis by Glycogen phosphorylase into glycogen and glucose-1-phosphate (Voet D., Voet J.G., 2011)

not until 1969 when experimentation by David Shepherd and Irwin Segel analyzed this protein in *N. crassa*. They noted in their work that glycogen phosphorylase was remarkably similar to liver and muscle phosphorylases, and was significantly more active than phosphorylase in *E. coli.* In *N. crassa,* glycogen phosphorylase is an 817 amino acid protein **(Figure 10)** (NCBI accession number XP_962166) that is translated from the RNA that is transcribed from the 3010 bp glycogen phosphorylase gene (NCBI accession number XM_957073 (NCU07027)) **(Figure 11).** This protein coding gene contains four

```
  1 masnttqrvp lrerrpsvga plvdiqggva pagvsrpkhk rtltgfgpge iknveasipe
 61 pqrkawlahq tsgfkdkdgf etevvrhvet tlarsmyncd eqaaysacsl afrdrlilew
121 nrtqqrqtfa dskrvyylsl eflmgraldn amlnigqkdv akaglaelgf riedvieqeh
181 daalgngglg rlaacfldsl aslnysawgy glryrygifk qeiidgyqve vpdywldfnp
241 wefprhdvtv diqfyghvtk rtddngktia tweggeivka vaydvpipgy atpstnnlrl
301 wsskaasgef dfqkfnsgdy ensvadqqra etisavlypn dnldrgkelr lkqqyfwvaa
361 slydivrrfk ksrrpwkefp dqvaiqlndt hptlavvelq rilvdlegld weeawnivtn
421 tfgytnhtvl pealekwsvp lfqhllprhl qliydinlff lqsverkfpk dremlarvsi
481 ieesqpkmvr mahlaivgsh kvngvaelhs dlikttifkd fvevfgpdkf tnvtngitpr
541 rwlhqanprl selissktgs qnflkdltel akiehykddk afrkewaeik yankvrlakh
601 ikkttgvdvn psalfdvqvk riheykrqqm nifgvihryl tlkslspeer kkfqprvsif
661 ggkaapgywm akqiihlina vgavvnndkd igdllkvifl edynvskaem iipasdlseh
721 istagteasg tsnmkfvlng gliigtcdga nieitreige qniflfgnla edvedirhnh
781 tygsytvdpd lvkvfeaiek gtfgepndfm gmisavrdhg dfylvsddfh syietqelvd
841 kayrdqegwi tksiesvarm gffssdrcin eyaegiwnie plavkdq
```

Figure 10: The 817 Amino Acid Protein, Glycogen Phosphorylase from *N. crassa* (NCBI accession number XP_962166)

exons and is located on the chromosome IV in *N. crassa.* Although there is a wealth of knowledge available on this gene and gene product, there is very limited understanding of its exact role and expressional level in quinic acid metabolism in *N. crassa* or in the cells response to poor carbon sources.

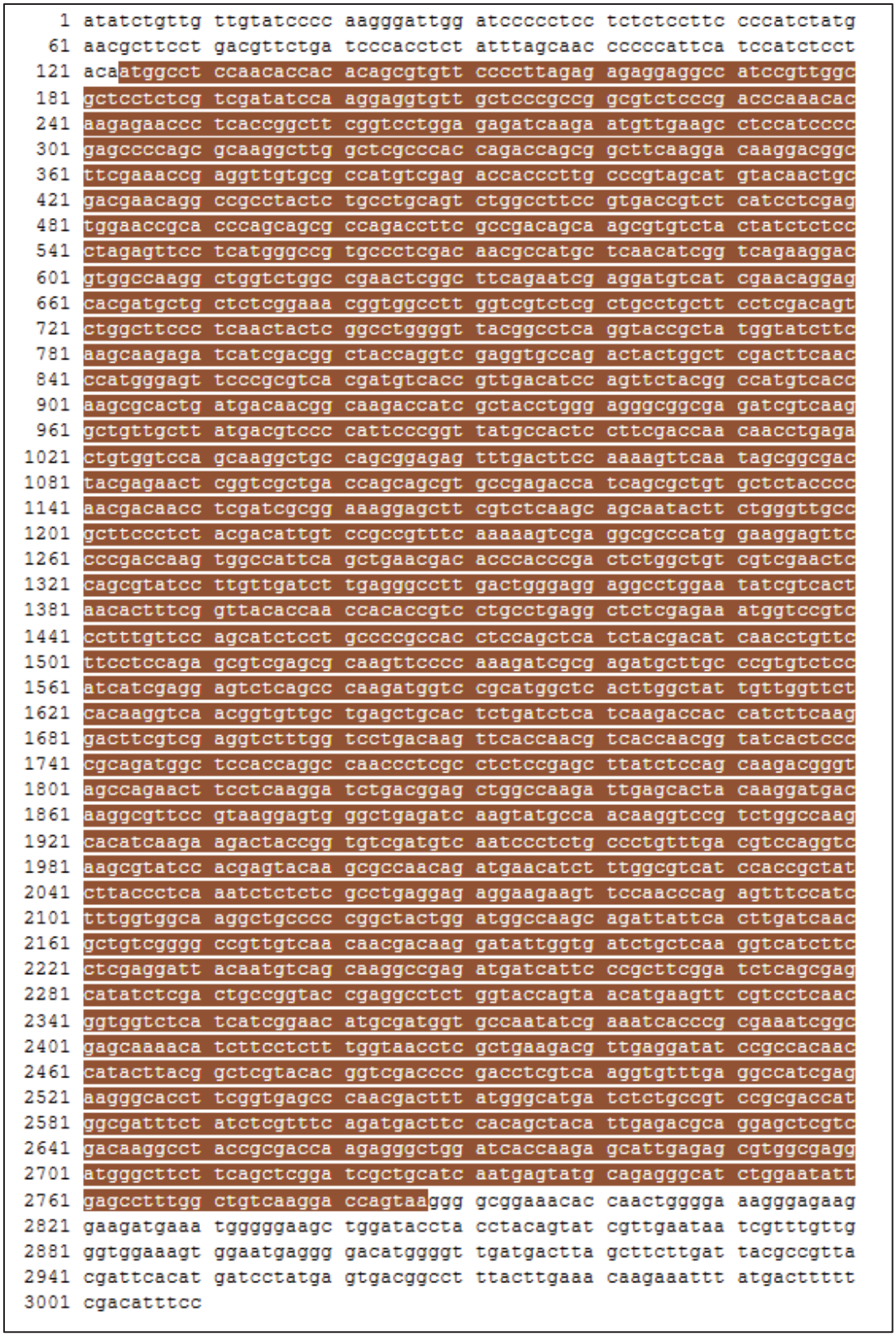

Figure 11: The 3010 bp gene: glycogen phosphorylase (NCBI accession number XM_957073). The CDS for the protein glycogen phosphorylase (Figure 9) is highlighted in brown.

VI. Hex-1: Woronin Body Coding Protein (NCU08332)

Woronin bodies are a peroxisome-derived microbody that is found near the septae that divide hyphal compartments in Ascomycota. Microbodies or peroxisomes are types of organelles that have similar origins of biogenesis but are extremely diverse in nature (Managadze D., et al. 2010). Woronin bodies were first discovered by Russian botanist Mikhail Stepanovich Woronin, and are described as hexagonal shaped organelles with an electron-opaque matrix that are found in the mycelial hyphae of a variety of fungi belonging to Ascomycota, including *Fusarium, Aspergillus* and *Neurospora.* In fungi, Woronin bodies are typically found near the septal pore of the hyphae with the major function of these organelles to prevent the escape of cytoplasm in the occurrence of hyphae damage. Each of these dense bodies are formed by a variety of proteins with the majority of which being a single protein (*hex-1*) that form a hexagonal crystal giving Woronin bodies their shape (Figure 13). The *hex-1* protein is located in the Woronin body core and contains a peroxisome targeting signal that suggests that Woronin bodies are in fact related to peroxisomes (Yuan p., et al. 2003). It was noted that the *Hex-1* Woronin Body protein gene coded for the protein that gives this structure its hexagonal matrix (Managadze D., et al. 2010). Furthermore, it was demonstrated through tandem mass spectrometry that this was the major protein among the variety that make up this organelle. This protein in *N. crassa* is coded for by the *Hex-1* Woronin Body protein gene (NCU08332) (Figure 12). Studies have shown that through the deletion of the *hex-1* gene in *N. crassa,* Woronin bodies were

eliminated from the cytoplasm. This mutation showed that cytoplasm would leak trough the pores followed by cell lysis, further enhancing the hypothesis that the protein, *hex-1*, is crucial in septal pore sealing and the function of the Woronin body (Yuan P., et al. 2003). In *N. crassa, Hex-1* is a 1565 base pair gene (Figure 12) containing two exons and coding for a 176 amino acid protein (Figure 13).

A study at Yale University aimed to determine if nutrition had an effect on perithecial development in *N. crassa* by growing the fungi on Synthetic Crossing Medium and natural Carrot Agar. Following this, microarray data comparing cDNA was analyzed. It was shown that *Hex-1* (NCU08332) had similar expression levels in both treatment conditions (Wang Z., et al. 2012). However, in the thesis work of Dana Tirabassi, it was shown that when *N. crassa* was grown in the presence of quinic acid, there was a heightened translational product of the NCU08332 gene via mass spectrometry of a spot on a protein gel. From this, it seemed as though this gene showed heightened expression at the proteomic level due to the presence of quinic acid. Furthermore, it was seen in Michael Werry's thesis that this gene also seemed to respond to nitrate as a poor nitrogen source rather than ammonia.

```
   1 agcaaatccc tccagtggtc acaacttttc tgaaccatcc ttcgaaaagc ctcatcatca
  61 ccactcctct tctcatcatc aacaatcctc cgacagcgcc agcgctatcg acattgctga
 121 gcatcaatac cggtccaagt tccagcccgc ctaccacaac gaaggtcacg tagtcggttc
 181 caccgtcgac gtcgctagct accgtcccag ccacaaggag tccacccacg tcgacgacac
 241 tactgtcgac ttccctgcac tccctgcacc ccctagatcc acttcttaca aggagaagat
 301 cgttgtcgac gagactaccg tcgacttccc cacctaccgt ccggctcata agaaggcacc
 361 cagcctctac agcgagtcta ctgtcgacat ccaagaaaac gaactttctc tagtcaaagt
 421 ttccaacaac aacaacacat cttccaaaat gggctactac gacgacgacg ctcacggcca
 481 cgttgaggct gatgccgcgc cccgtgccac caccggcact ggcaccggct ctgcttccca
 541 gactgtcacc atcccctgcc accacatccg cctcggcgat atcctcatcc tccagggccg
 601 cccttgccag gtcatccgca tctccacctc cgctgccact ggccagcacc gttacctcgg
 661 tgtcgacctc ttcaccaagc agctccatga ggagtccagc ttcgtctcca accctgcccc
 721 cagcgtcgtc gtccagacca tgctcggccc cgtcttcaag cagtaccgtg tcctcgacat
 781 gcaggatggc tccatcgtcg ccatgaccga gactggcgat gtcaagcaga acctccccgt
 841 cattgaccag agctctctct ggaaccgtct ccagaaggct ttcgagtctg gccgcggctc
 901 cgtccgtgtc ctcgtcgtct ccgaccacgg tcgcgagatg gccgtcgaca tgaaggtcgt
 961 ccacggttcc cgcctctaaa tcgagggttc cgtcatccgt tcaataatat gactttcccc
1021 cttcctggaa tcggacacac gacacacacg gttgggttaa taagggctgc ttggatgtta
1081 tgatatggtc tctctgatgc tgggtctgat tttttgcgcg cgtctagaag atgggttgga
1141 taatttgtac tggtagtatg ctcccctccc atccatgtat atgaaaattg taatggaagg
1201 agtcgtcagt aatacaacct taaaaaactc gcttcgtctt gttccaattg attgtgtgtg
1261 aacagcattt gcatgaaagt gactgcattc ttctggtgct aaaggtcagt tcacatcttg
1321 ttgacagctt gatctcgctt gaggtgtcga acaatcatag tgcatcagac tagcgacgtc
1381 ctcgacttct gattggtagg attcgaagat catcgtcctg accaatcgtg tcttgtctct
1441 ttttctgtaa gtcgaactgt cgatgtacta gtatacatag tcaacaagag tcaatacacg
1501 ccaaccaatg agagagccaa tccacgtcaa cgaataggag agccaaaagt ctcgatggtg
1561 agtgg
```

Figure 12: *Hex-1* Woronin Body Protein Gene (NCU08332). A 1565 base pair gene. The coding region is highlighted in brown

```
   1 mgyydddahg hveadaapra ttgtgtgsas qtvtipchhi rlgdililqg rpcqvirist
  61 saatgqhryl gvdlftkqlh eessfvsnpa psvvvqtmlg pvfkqyrvld mqdgsivamt
 121 etgdvkqnlp vidqsslwnr lqkafesgrg svrvlvvsdh gremavdmkv vhgsrl
```

Figure 13: *Hex-1* Woronin Body Protein (XP_963707). A 176 amino acid protein.

VII. Specific Aims

Proteomic data suggests that both Glycogen Phosphorylase, Peptidyl-Prolyl cis-trans Isomerase, and *Hex-1*: Woronin Body Coding Protein are expressed in relation to quinic acid exposure and metabolism in *Neurospora crassa.* In prior works, products from each gene were seen in the proteome of *Neurospora crassa* in the presence of quinic acid. However, these genes were not specifically defined as some of the 100 that were previously described to be associated with the qa gene cluster through microarray analyses. Thus, the aim of this work is to determine the level of transcription of these three genes in the presence of quinic acid as compared to glucose and to determine if there is indeed a relation between these three genes and the quinic acid gene cluster itself.

VIII. Hypothesis

qRT-PCR experiments will most likely show elevated expression levels of Glycogen Phosphorylase (NCU07027), Peptidyl-prolyl Cis-trans Isomerase (NCU04140), and the *Hex-1*: Woronin Body Coding Protein gene (NCU08332) during quinic acid metabolism as the central dogma of biology suggests.

Chapter 2: Materials and Methods

I. Samples and Growth

For analysis, *Neurospora crassa* wild type sample 74A (FGSC 2489) as well as FGSC 11034 (*qa-1F* knockout mutant) were obtained either from laboratory stock samples or from the Fungal Genetic Stock Center at Kansas State University. Both 74A and FGSC 11034 samples were prepared in the same fashion. Samples were first streaked onto Vogel-FIGS plates as described in Table 4. Plates were then placed into a Blue M STABIL-THERM© dry type gravity convection bacteriological incubator at 30° C for approximately 24 hours in order to isolate individual colonies. Colonies were then isolated with a MEIJI TECHNO EMT series microscope under 1X magnification. Individual colonies were then used to inoculate Vogels-Sucrose slants according to Table 5. Slants then were placed at 30° C for approximately 48 hours. Post incubation, each slant was then placed under fluorescent light at room temperature for an additional 72 hours for continued growth. Samples were then utilized to inoculate Vogels-Sucrose media (50 mL) in a 250 mL Erlenmeyer flask according to Table 6. Post inoculation, samples were incubated at 30° C for 24 hours. After incubation, conidia samples were grown for at least 1 week under florescent light at room temperature before utilization.

Table 2: 50x Vogels Minimal Media (1L)

Component	**Amount**
Na_3 citrate - 5.5 H_2O	150g
KH_2PO_4 - anhydrous	250g
NH_4NO_3 - anhydrous	100g
$MgSO_4$ - 7 H_2O	10g
$CaCl_2$ - 2 H_2O	5g
Trace Element Solution (as follows) Citric acid - 1 H_2O : 5g $ZnSO_4$ - 7 H_2O : 5g $Fe(NH_4)2(SO_4)2$ - 6 H_2O : 1g $CuSO_4$ - 5 H_2O : 0.25g $MnSO_4$ - 1 H_2O : 0.05g H_3BO_3 - anhydrous : 0.05g Na_2MoO_4 - 2 H_2O : 0.05g Deionized H_2O : 998 ml Chloroform : 2 ml	5ml
Biotin Solution (as follows) Biotin : 5mg 50% Ethanol : 50mL	2.5ml
Deionized H_2O	992.5 mL

Table 3: 20X FIGs Sugar Solution (100ml)

Component	**Amount**
Sorbose	400 g
Fructose	10 g
Glucose	10 g
Deionized H_2O	100 ml

Table 4: Vogels-FIGS Plates (200mL). Note that the solution was autoclaved prior adding FIGS solution. Furthermore, the 200ml solution made approximately 8 plates for inoculation.

Component	Amount
50X Vogels Minimal Media (Table 2)	4mL
Bacteriological Grade Agar	3g
Deionized H_2O	186mL
FIGS solution (Table 3)	10mL

Table 5: Vogels Sucrose Agar Slants (100mL) Note: After autoclaving solution, the solution is poured into test tubes for inoculation.

Component	Amount
Vogels Minimal Media (Table 2)	2mL
Sucrose	2g
Bacteriological Grade Agar	1.5g
Deionized H_2O	98mL

Table 6: Vogels Sucrose Flasks (50mL) Note: Solution was placed into a 250ml Erlenmeyer flask prior to autoclaving. Media was then allowed to cool prior to inoculation.

Component	Amount
Vogels Minimal Media (Table 2)	1mL
Sucrose	1g
Bacteriological Grade Agar	0.75g
Deionized H_2O	49mL

II. Media Shifts for Induction of Genes

In order to initiate expression of genes of interest, several media growth shifts took place to develop various conditions for experimentation. Before each shift, both 74A and FGSC 11034 samples were transferred from solid media (Vogels sucrose flasks described previously) to 50mL of liquid Vogels sucrose media as described in Table 7. The samples were then placed into a GeneMate Incubated Shaker at 30°C oscillating at 150 revolutions per minute. This occurred for 24 hours. Following this growth period, the samples were filtered off using simple vacuum filtration with Whatman filter paper followed by a brief vigorous wash in 50mL of autoclaved deionized water to remove any excess sucrose not removed by the initial filtration. The samples were then again poured over a Buchner funnel and filtered off. Following this step the samples were transferred into three different liquid media types: Vogels 2% sucrose (non-induced), Vogels 0.3% quinic acid (induced), and Vogels (starved) as follows:

Non-induced:

Non-induced samples for both 74A and FGSC 11034 acted as the control. Following filtration from the 24 hour growth period, the samples were re-introduced to Vogels sucrose liquid media as described in Table 7. The samples were then placed into an incubated shaker at 30°C oscillating at 150 revolutions per minute for 3 hours. Following this 3 hour growth period, the samples were filtered off using vacuum filtration and stored at -80° C.

Table 7: Vogels Sucrose Liquid Media (50mL)

Component	Amount
Vogels Minimal Media (Table 2)	1mL
Sucrose	1 gram (2% by volume)
Deionized H_2O	49mL

Induced:

After filtration from the 24 hour growth period, the samples were placed into Vogel's QA liquid media as described in Table 8. The samples were then placed into an incubated shaker at 30°C oscillating at 150 revolutions per minute for 3 hours. Following this period, the induced samples were filtered over a Buchner funnel. Samples were then stored at -80°C.

Table 8: Vogels QA Liquid Media (50mL)

Component	Amount
Vogels minimal media (Table 2)	1mL
Quinic Acid	0.15g (0.3% by volume)
Deionized H_2O	49mL

Starved:

Starved media lacked both glucose and quinic acid as carbon sources. After filtration from the 24 hour growth, the samples were placed into Vogels liquid media as described in Table 9. The samples were then placed into an incubated shaker at 30°C oscillating at 150 revolutions per minute for 3 hours. Following this period, the starved samples were filtered over a Buchner funnel. Samples were then stored at -80°C.

Table 9: Vogels Liquid Media (50mL)

Component	Amount
Vogels Minimal Media (Table 2)	1mL
Deionized H_2O	49mL

III. RNA Extraction

After developing various conditions for analysis, RNA was extracted from non-induced, induced, and starved samples from both 74A and FGSC 11034 strains following

the same protocol. To start, each sample was manually ground with a mortar and pestle with the aid of liquid nitrogen (N_2) to lyophilize the sample. After lyophilization occurred, the sample was evenly distributed into four (4) Eppendorf Safe-Lock 1.5 mL micro-centrifuge tubes. Following distribution, 700µL of AMRESCO's Ribozol™ RNA Extraction Reagent was added. This single stage phenol solution allows for isolation of total RNA while subsequently inhibiting RNase degradation allowing for relatively decent yields of RNA. Following the addition of Ribozol™, each sample was manually shaken by vigorously inverting and reverting the micro-centrifuge tubes for a period of approximately 30 seconds. This was then followed by a 15 second agitation of the sample using a Scientific Industries Vortex-Genie 2 laboratory vortex mixer set to its most vigorous mixing speed. The samples were then centrifuged for 10 minutes in a BioExpress SpinMate 24 micro-centrifuge at 13,300 revolutions per minute (RPM). Post centrifugation, the samples were incubated at room temperature (approximately 23°C) for 5 minutes. Following incubation, 200µL of chloroform was added to each tube to remove proteins from the solution. The tubes were then mixed for 15 seconds using a laboratory vortex mixer as described previously. The samples were then incubated at room temperature for 3 minutes. Following incubation, the samples were centrifuged at 13,300 (RPM) as described previously for 15 minutes. Following centrifugation, three phases were present in each tube: a lower phenol chloroform phase containing DNA and proteins, a middle interphase containing proteins, and an upper clear aqueous phase containing RNA. The upper phase was then removed from each sample with a micropipette and carefully transferred to 4 new 1.5 mL micro-centrifuge tubes. In addition to the aqueous phase containing RNA, 500µL of isopropanol were added to each

of the 4 micro-centrifuge tubes. Since all nucleic acids are somewhat insoluble in alcohol, the isopropanol allowed for further purification of the RNA in the sample. The sample was then frozen at -20°C overnight.

On the second day of extraction, the samples were thawed out at room temperature after removal from the -20°C freezer. The samples were then centrifuged at 13,300 RPM for 10 minutes. After removal from the centrifuge, a whitish pellet of RNA was visible at the bottom of each tube. The supernatant was then removed and discarded. In order to further isolate the RNA, 1,000µL of 75% ethanol was added to each tube. At this point, the pellet of RNA was broken up intentionally with the tip of the micropipette by gently drawing up and ejecting the pellet through the micropipette tip. The samples were centrifuged again at 13,300 RPM for 5 minutes. After centrifugation, the ethanol was removed from each of the 4 tubes and disposed of properly. A pellet of RNA was then again visible and allowed to air dry briefly in the micro-centrifuge tube. Lastly, the pellet in each of the tubes was re-suspended in 200µL of nuclease free water and stored at -80°C.

IV. RNA Quality Analysis and Quantitation

Following RNA extraction from each tissue sample of interest, quality and quantity of RNA was tested using a Thermo Scientific NanoDrop© 2000c spectrophotometer. After setting general parameters of the program to initiate the analysis, 5µL of nuclease free water were placed onto the optical probe of the NanoDrop© 2000c spectrophotometer for approximately five minutes to ensure degradation of any residual sample that may have been improperly cleaned off by the

previous user. The probe was then wiped with a KIMTEC Science Brand Kimwipe© delicate task wiping cloth. Following thorough cleaning of the optical probe, 2µL of nuclease free water were placed on the optical probe of the spectrophotometer. Since the RNA was suspended in nuclease free water as a buffer, the system was "blanked" using nuclease free water to ensure the software recognized that nuclease free water was the buffer solution. After "blanking" the system, the probe was again wiped with a Kimwipe© to ensure removal of the 2µL of nuclease free water. Then, 3µL of each sample was loaded onto the optical probe with removal of each sample via a Kimwipe© between each sample. This allowed for measurement of the quantity of RNA in the sample (in nanograms per microliter (ng/µL)) as well as a 260/280 nanometer light frequency (nm) ratio to indicate the quality of the sample. From these results, RNA samples were selected from each condition (induced, non-induced, and starved) from each sample (74A and FGSC 11034) based on the highest concentration and best 260/280 nm value. A 260/280 nm value of roughly 2.0 is considered to be "pure" RNA, so values ranging from 1.8-2.1 and concentrations higher than 400 ng/µL were favorably selected.

In order to determine the integrity of the extracted RNA from the samples of interest, 1% agarose gels were run. The agarose gel was made according to Table 10 before pouring into a BioExpress Model E-4109-1 electrophoresis tray. After cooling of the gel, the electrophoresis apparatus was filled with 1X TPE (Table 12). Then, 10µL of RNA from each sample was combined with 3µL of AMRESCO EZ Vision^{TM} loading dye in a fresh 1.5 mL Eppendorf tube. Each well was then loaded with 10µL of the corresponding RNA loading dye solution. The gel was then run at 60 volts and 74 Amps for 90 minutes using a Labnet International Inc. Endero MINI power supply. After the 90

minute time period, the slab gel was taken from the electrophoresis tray and set in a container for 20 minutes filled with approximately 200 mL of deionized water mixed with 50μL of ethidium bromide. The gel was then removed from the solution and imaged with a Fisher Scientific Transilluminator ultra violet light box.

Table 10: 1% Agarose Gel (75mL) **Note:** Contents were brought to a boil before pouring

Component	**Amount**
1X TPE (Table 12)	75mL
Agarose (powder)	0.75g

Table 11: 10X TPE Buffer Solution (500mL)

Component	**Amount**
Phosphoric Acid (85%)	7.75mL
0.5M EDTA	20mL
Deionized H_2O	472.25mL
TRIS base	54g

Table 12: 1X TPE Buffer Solution (1L)

Component	Amount
10X TPE (Table 11)	100mL
Deionized H_2O	900mL

V. Primer Selection

Primer pairs were determined using the National Center for Biotechnology Information's (NCBI) Primer Basic Local Alignment Search Tool (BLAST) software. Correct accession numbers as well as specific parameters were imputed prior to searching for a desired primer pair. Product lengths were limited to 200 base pairs and the length of the primers were limited to 20 base pairs. A combined Guanine and Cytosine percentage (GC %) of roughly 50% was selected as favorable. This selection process was done for three primer pairs of interest: NCU01635 Histone 3 as a reference gene (XM_950910.3), NCU07027 Glycogen Phosphorylase (XM_957073.3), NCU04140 Peptidyl-prolyl cis-trans isomerase (XM_956203.3), and NCU08332 *Hex-1*: Woronin Body Coding Protein (XM_958614.3). Following selection of the correct sequences, each primer was ordered from Integrated DNA Technologies (IDT). Lyophilized oligonucleotides were received from IDT. These oligonucleotides were centrifuged, then resuspended in nuclease free water according to the manufacturer's specifications to achieve a concentration of 100µM. The primers solutions were then stored at -20°C. This was conducted for Histone

3, Glycogen Phosphorylase, Peptidyl-prolyl cis-trans isomerase, *Hex-1*: Woronin Body Coding Protein, *qa-1F*, and *qa-y* primer pairs.

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

Following primer selection, RT-PCR was conducted with each primer pair in order to determine if the selected primers were functional and would work for qRT-PCR. RT-PCR takes mRNA targets and converts them to cDNA via reverse transcription followed by PCR amplification. This was conducted in one step utilizing a QIAGEN® One Step RT-PCR Kit. Several master mixes were made according to Table 13. Following making the master mix according to manufacturer specifications, less than or equal to 2µg of template RNA was added to each tube. Following aliquoting out RNA, each tube was placed into a BIO-RAD MJ Mini™ Personal Thermal Cycler in order for RT-PCR to take place. The thermocycler was pre-programed for the cycling conditions for one-step RT-PCR set by QIAGEN® (Table 14). Following RT-PCR, the cDNA was then analyzed on a 1.5% agarose gel (Table 15) to determine if the correct product was being amplified and to determine if the primers worked effectively. Gels were poured into a BioExpress Model E-4109-1 electrophoresis tray and allowed to solidify. Ten microliters of cDNA along with 3µL of EZ Vision Loading Dye™ was added to each well. Also, a 100 base pair DNA molecular weight marker ladder (AMRESCO® K180 100 BP Ladder) was utilized to determine if the length of the PCR product was indeed the correct amplicon. 2µL of the ladder, 10µL of nuclease free water, and 3µL of EZ Vision Loading Dye™ was added to one well. The gel was then run at 60 volts and 74 Amps for 90 minutes using a Labnet International Inc. Endero MINI power supply. After the 90

minute time period, the slab gel was taken from the electrophoresis tray and imaged with a Fisher Scientific Transilluminator ultra violet light box.

Table 13: Master Mix for RT-PCR (Note: After adding template RNA, the solution was gently mixed with a pipet to ensure a homogenous mixture)

Component	**Amount**
QIAGEN® One Step RT-PCR Buffer	5µL
dNTP Mix	1µL
Primer A	1.6µL (0.6µM final concentration)
Primer B	1.6µL (0.6µM final concentration)
QIAGEN® One Step RT-PCR Enzyme Mix	1µL
Template RNA (added last)	≤ 2µg per reaction
Nuclease Free H_2O	Bring to a final volume of 25µL

Table 14: Cycle Conditions

Step	Temperature	Time	Result
1	50°C	30 Minutes	Reverse transcription (cDNA)
2	95°C	15 Minutes	This initiates initial PCR activation by activating the QIAGEN® One Step RT-PCR Buffer and Enzyme Mix as well as denaturing the cDNA template.
3	90°C	30 Seconds	Denaturation
4	50°C	30 Seconds	Annealing
5	72°C	1 Minute	Extension
6	Steps 3-5 run 39 more times	78 Minutes	Amplification
7	72°C	7 Minutes	Extension
8	4°C	Forever	Holds sample at a stable condition

Table 15: Agarose Gel (1.5%) Note: Contents were brought to a boil before allowing to solidify

Component	Amount
Agarose (powder)	1.125g
1X TPE (Table 12)	75mL

VII. Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

QRT-PCR is utilized to simultaneously amplify and quantify a targeted DNA molecule. This process allows for the analysis and identification of specific amplified DNA fragments via their melting temperature. This in turn allows for an analysis of the

genes of interest and their change in expression depending on the carbon source present. Furthermore, it also allows for an analysis of the relation of these genes, via their expression, to the *qa* gene cluster along with efficiency tests of each primer. For this analysis each gene was analyzed under various conditions with RNA from noninduced, induced, and starved 74A samples as well as noninduced, induced, and starved FGSC 11034 Samples (Table 16, 17, and 18). BIO-RAD iTaq™ Universal SYBR® Green One-Step Kits were utilized for all reactions. First, a master mix was made according to manufacturer's specifications (Table 19). Next, 18µL of a specific master mix was aliquoted out into the desired well (s) of a 96 well qRT-PCR plate. Following this, 2µL (100ng/µL concentration) of the RNA of interest (i.e. condition) was aliquoted out to the desired well(s) in the 96 well qRT-PCR plate. Also, nuclease free water (2µL) was placed into their corresponding well(s) to act as non-template control samples. Each condition was run in triplicate in order to eliminate error or false result. Following aliquoting out corresponding master mixes, RNA, and/or nuclease free water to the correct well of the 96 well plate, the plate was sealed with a film type seal and placed into either a BIO-RAD iQ™ 5 Multicolor Real-Time PCR Detection System Thermocycler or a BIO-RAD CFX96™ Real-Time System C1000 Touch Thermal Cycler. After the logistics of the plate setup were programmed into the computer software, the thermal cycling protocol was imputed into the software according to manufacturer's specifications (Table 20). Following this, data was analyzed to determine the expression of each gene in the various condition presented. This was done a minimum of two times per gene per condition to elicit a consistent result.

Table 16: Conditions for Glycogen Phosphorylase

RNA	Conditions Analyzed
74A	Noninduced vs Induced
74A	Noninduced vs Starved
Qa-1F KO (FGSC 11034)	Noninduced vs Induced
Qa-1F KO (FGSC 11034)	Noninduced vs Starved

Table 17: Conditions for Peptidyl-Prolyl cis-trans Isomerase

RNA	Conditions Analyzed
74A	Noninduced vs Induced
74A	Noninduced vs Starved
Qa-1F KO (FGSC 11034)	Noninduced vs Induced
Qa-1F KO (FGSC 11034)	Noninduced vs Starved

Table 18: Conditions for *Hex-1*: Woronin Body Coding Protein

RNA	Conditions Analyzed
74A	Noninduced vs Induced
74A	Noninduced vs Starved
Qa-1F KO (FGSC 11034)	Noninduced vs Induced
Qa-1F KO (FGSC 11034)	Noninduced vs Starved

Table 19: iTaq™ Universal SYBR® Green Master Mix (450µL) Note: Following the addition of all components, agitation via vortex occurred

Component	Amount (µL)	Percent by Volume
SYBR	250µL	55.6%
Forward Primer (10µM)	50µL	11.1%
Reverse Primer (10µM)	50µL	11.1%
Nuclease Free H2O	93.75µL	20.8%
Reverse Transcriptase	6.25µL	1.4%

Table 20: Thermal Cycling Protocol (qRT-PCR)

Cycle	Step	Temperature	Duration	Times Repeated
1	1	50.0°C	10 Minutes	1
2	1	95.0°C	5 Minutes	1
3	1	95.0°C	10 Seconds	45
	2	60.0°C	30 Seconds	
4	1	95.0°C	1 Minute	1
5	1	55.0°C	1 Minute	1
6	1	55°C to 95°C	10 Seconds	81

Chapter 3: Data and Results

I. Introduction

With 90,000 species identified and some 1.5 million species estimated to be in existence, Eumycota or Fungi represent a vast category of Eukaryotes that have been and remain a significant group in scientific discovery. *Neurospora crassa* has been a model organism in genetic and biochemical studies for many generations. From its first reference in bakeries throughout Paris to the Nobel Prize winning " one gene- one enzyme" hypothesis of Beadle and Tatum (Beadle G.W., Tatum E.L. 1941), experimentations with *N. crassa* have led to various fundamental genetic principals such as mitochondrial protein import, DNA methylation, circadian rhythms, post-transcriptional gene silencing, and DNA repair (Deacon J., 2006). Furthermore, *N. crassa* has an ability to metabolize poor carbon sources in the absence of the preferred carbon source glucose. In the presence of quinic acid, *N. crassa* has the ability to metabolize this carbon source via the activation of the quinic acid gene cluster.

The quinic acid (*qa*) gene cluster in *N. crassa* consists of two regulatory genes (*qa-1F* and *qa-1S*) as well as five structural genes (*qa-2, qa-3, qa-4, qa-y,* and *qa-x*) that are transcribed in the presence of quinic acid with suppressed levels of glucose (Greever R.F., et al. 1988). *qa-1F* acts as an activator of the gene cluster where *qa-1S* acts as a repressor. In the presence of glucose, the protein product of *qa-1S* bind to the *qa-1F* protein blocking the activation domain of the *qa-1F* protein, inhibiting the transcription of these genes (Arnett D.R., et al. 2009). However, in the presence of quinic acid, quinic acid binds to the *qa-1S* protein dissociating it from the *qa-1F* activator, thus allowing for

transcription of the *qa* cluster. The corresponding activation and repression of this cluster then enables the survival of the organism in less than hospitable environments. While the absolute function of the *qa-x* gene in the cluster is unknown, the other structural genes have known functions; the *qa-y* encodes for a protein that is a carrier protein, aiding the movement of quinic acid into the cell (Logan D.A., et al. 2007) while the *qa-2* gene encodes for the catabolite dehydroquinase (5-dehdroquinate hydrolase); *qa-3* encodes for quinate dehydrogenase (quinate NAD oxioreductase) and the *qa-4* encoding for 5-dehydroshikimate dehydratase (Case M.E., Giles N.H. 1976). In the absence of *qa*, the *qa-1S* protein binds to the *qa-1F* protein preventing transcription of the *qa* genes. In the presence of *qa*, *qa* bind to the *qa-1S* protein dissociating it from the *qa-1F* protein allowing for transcription of the *qa* genes (Arnett D.R., et al. 2009).

A research study at the University of Georgia hypothesized (via microarray analysis) that some 50 genes were under the control of the *qa-1F* gene and another *qa* transcription factor (Logan D.A., et al. 2007). Furthermore, subsequent studies showed that *qa-1F* control was not sufficient to describe why these 50 genes showed expression profiles that related them to quinic acid metabolism (Tang X., et al. 2011). Additionally, although they were not described in previous work, it was shown through protein spot excision and sequencing by tandem mass spectrometry in the work of Kayla Brown, Katie Allen, and Dana Tirabassi (Master's thesis work) that at proteomic level, glycogen phosphorylase, peptidyl-prolyl cis-trans isomerase, and NCU08332 *Hex-1*: Woronin Body Coding Protein all had heightened expression in the presence of quinic acid. In these results, the scope of the work is to determine the level of transcription of these three genes during quinic acid metabolism as well as their relation to the quinic acid gene

cluster. Furthermore, bioinformatics analyses will be utilized to determine possible reasoning for the enhancement of transcription during metabolism of this poor carbon source.

II. Materials and Methods

II.A- Strains and Media:

A wild-type strain (74A) as well as a *qa-1F* knockout strain (FGSC 11034) both obtained from laboratory stock collections were utilized in this study. Strains were grown on Vogels-Sucrose (2%) slants for maintenance.

II.B- Media Shifts:

Strains 74A and FGSC 11034 samples were grown on solid media (Vogels sucrose flasks for 7-10 days) Conidia were harvested using Vogels- Sucrose (2%) media and transferred to 50mL of liquid Vogels sucrose (2%) media. The samples were then placed into an incubated shaker overnight at 30° C oscillating at 150 revolutions per minute. Following this growth period, the samples were filtered off using simple vacuum filtration followed by a brief vigorous wash in 50mL of autoclaved deionized water. The samples were then transferred into three different liquid media types: Vogels 2% sucrose (non-induced), Vogels 0.3% quinic acid (induced), and Vogels (starved) (each was grown for 3 hours). Subsequent samples were then utilized for extraction of RNA creating various conditions.

II.C- RNA Extraction and Analysis:

Mycelia were ground in liquid nitrogen and RNA was isolated using Ribozol™ according to the manufacturer's specifications. The RNA was then tested using a Thermo Scientific NanoDrop© 2000c spectrophotometer as well as analyses in 1% agarose gels to ensure quality.

II.D- qRT-PCR and Bioinformatic Analysis:

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) using a BIO-RAD iTaq™ Universal SYBR® Green One-Step Kit was conducted utilizing either a BIO-RAD iQTM 5 Multicolor Real-Time PCR Detection System Thermocycler or a BIO-RAD CFX96TM Real-Time System C1000 Touch Thermal Cycler. Primers were selected utilizing NCBI's Primer BLAST software. Subsequent bioinformatics studies utilizing NCBI's BLAST software as well as ClustalX2 alignment software were also conducted.

III. Data with Explanations

RNA Extraction:

Proteomic analysis had implicated several proteins (Glycogen Phosphorylase (NCU07027), Peptidyl-prolyl Cis-trans Isomerase (NCU04140), and the Hex-1: Woronin Body Coding Protein gene (NCU08332)) whose expression is induced during growth on

quinic acid. To examine this, RNA was extracted from tissue samples of wild-type 74A and the *qa-1F* knockout mutant (FGSC 11034) grown in either sucrose, or quinic acid as a carbon source. Also to determine if these proteins were produced in response to starvation, tissue from each strain was grown without a carbon source (Vogles only). After RNA extraction the quality and quantity of RNA was tested using a Thermo Scientific NanoDrop© 2000c spectrophotometer. 260/280 nanometer light wavelength frequency ratios were recorded as well as quantity of the RNA sample measured in ng/µL (See Appendix Table(s) 21-26).

Primer Selection:

Primer pairs were determined for each gene using the National Center for Biotechnology Information's (NCBI) Primer Basic Local Alignment Search Tool (BLAST) software. Special notice was taken when selecting the primers to ensure that the primer resulted in an amplified segment that corresponded to an exon or coding region of the sequence. Three of the six primer pairs (Table 27) that were utilized were previously tested in prior thesis works and were proven to show desired result. Histone-3 acted as a reference gene in all of the qRT-PCR analyses conducted in this study since it shows little variation with regard to changes in carbon energy sources since it is considered a "house-keeping" gene in the cell (George K., 2016). This means that it is essential for all of the cells routine basic functions to sustain life and is expressed at a relatively even level regardless of the conditions. *qa-y* and *qa-1F* were utilized in order to determine if the three hour induction (i.e. the shift from Vogels sucrose to Vogels quinic acid) did indeed elevate the expression of the quinic acid gene cluster and activate *N. crassa's* response to this poorer

carbon source. This ensured that each sample was indeed induced and metabolizing quinic acid. However peptidyl-prolyl cis-trans isomerase, glycogen phosphorylase and Woronin (*Hex-1*) primers were all selected for this specific study.

Gene	Product Length	Sequence	Direction	GC%
Histone-3	100 bp	5'-AGA TTC GTC GCT ACC AGA AG-3'	Forward	50.00%
		5'-CGG AGG TCG GAC TTG AAG-3'	Reverse	61.10%
qa-1F	98 bp	5'-TGC CGG GGC AAA GAC ATC CG-3'	Forward	65.00%
		5'-CCG GGC CTG GTA GAG TGC G-3'	Reverse	73.68%
qa-y	136 bp	5'-GGT ATC AAT GCC ATC AAC TAT TAC-3'	Forward	37.50%
		5'-GCC ACA GAA GCC AGA TAA TG-3'	Reverse	50.00%
Woronin (*Hex-1*)	184 bp	5'-CCA AAA TGG GCT ACT ACG ACG-3'	Forward	52.38%
		5'-GTG GAG ATG CGG ATG ACC TG-3'	Reverse	60.00%
Peptidyl-prolyl cis-tran isomerase	145 bp	5'-GCG GTA AGA AGT TCG ACG CT-3'	Forward	55.73%
		5'-AGG TGA GGG GCG ATA GTG AG-3'	Reverse	60.00%
Glycogen Phosphorylase	194 bp	5'-CCT CAA GGA TCT GAC GGA GC-3'	Forward	60.00%
		5'-GGC GCT TGT ACT CGT GGA TA-3'	Reverse	55.00%

Table 27: Primer pairs determined using the National Center for Biotechnology Information's (NCBI) Primer Basic Local Alignment Search Tool (BLAST) software. (utilized in this study)

From Table 27, it is important to note that although primer pairs that showed roughly 50% GC content were favored, qa-1F and qa-y were still utilized since prior experimentation proved both pairs to show desired result.

Following 3 hour media shifts to set up the three conditions (non-induced, induced and starved) for the genes of interest, several qRT-PCR analyses were conducted in order to determine if the RNA samples that were extracted from the 74A wild-type induced sample was indeed induced (i.e. The qa gene cluster was "turned on") in the three hour timeframe of the shift and in the presence of 0.3% quinic acid. In order to

determine this, first, the expression of the qa-1F gene was measured in a non-induced induced trial. *qa-1F* acts as the activator of the quinic acid gene cluster, thus the expected result would be that *qa-1f* would be significantly heightened in the induced RNA sample as compared to the non-induced. This was indeed the case as seen in Figure 14.

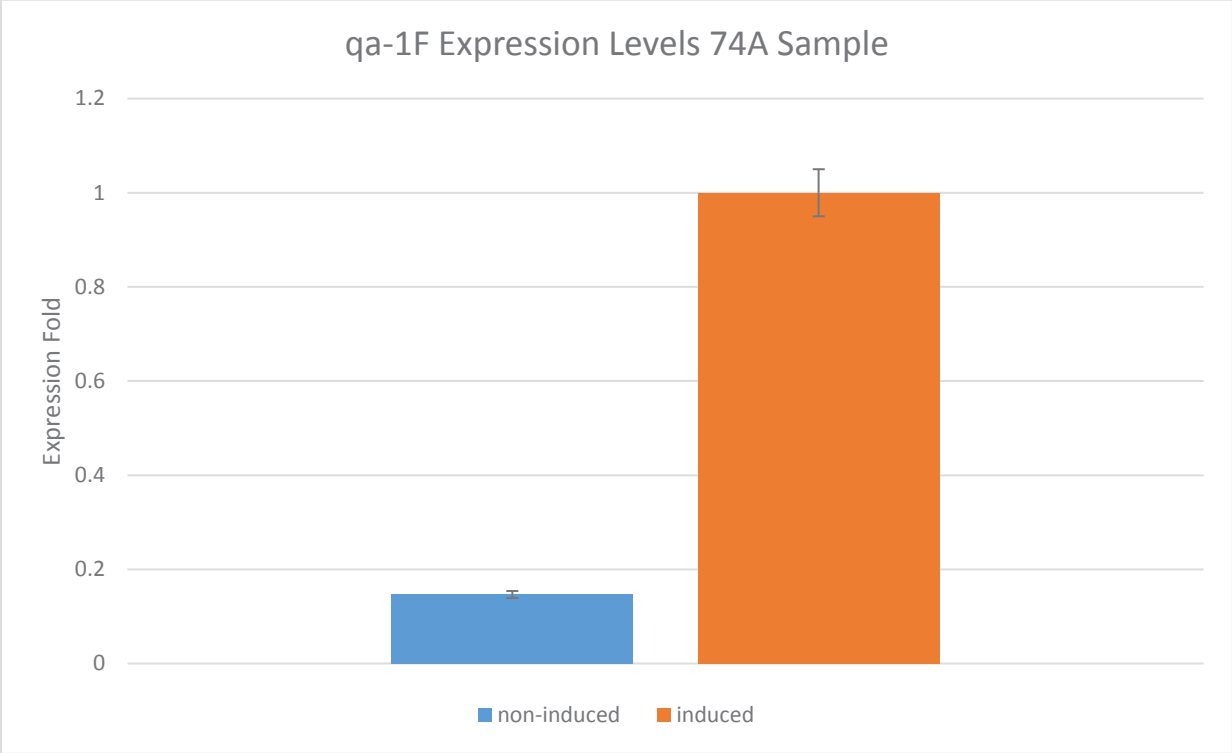

Figure 14: qa-1F Expression Levels in 74A Non-induced and Induced Samples. From the figure, it is clearly shown that in the induced sample (orange) the expression of the qa-1F gene is substantially greater than that of the qa-1F gene in the non-induced sample (blue). This was the expected and desired result.

A similar test was conducted with a 74A sample in a non-induced induced trial to determine the expression of the *qa-y* gene. If the samples that were produced during the media shift were in fact induced after the 3 hour media shift, the result would show a heightened amount of expression of the *qa-y* gene in the induced sample as compared to the non-induced sample similar to what was seen and expected in the *qa-1F* trial since the *qa-y* gene is a structural gene of the quinic acid gene cluster coding for a quinate permease protein. This was indeed the case as seen in Figure 15.

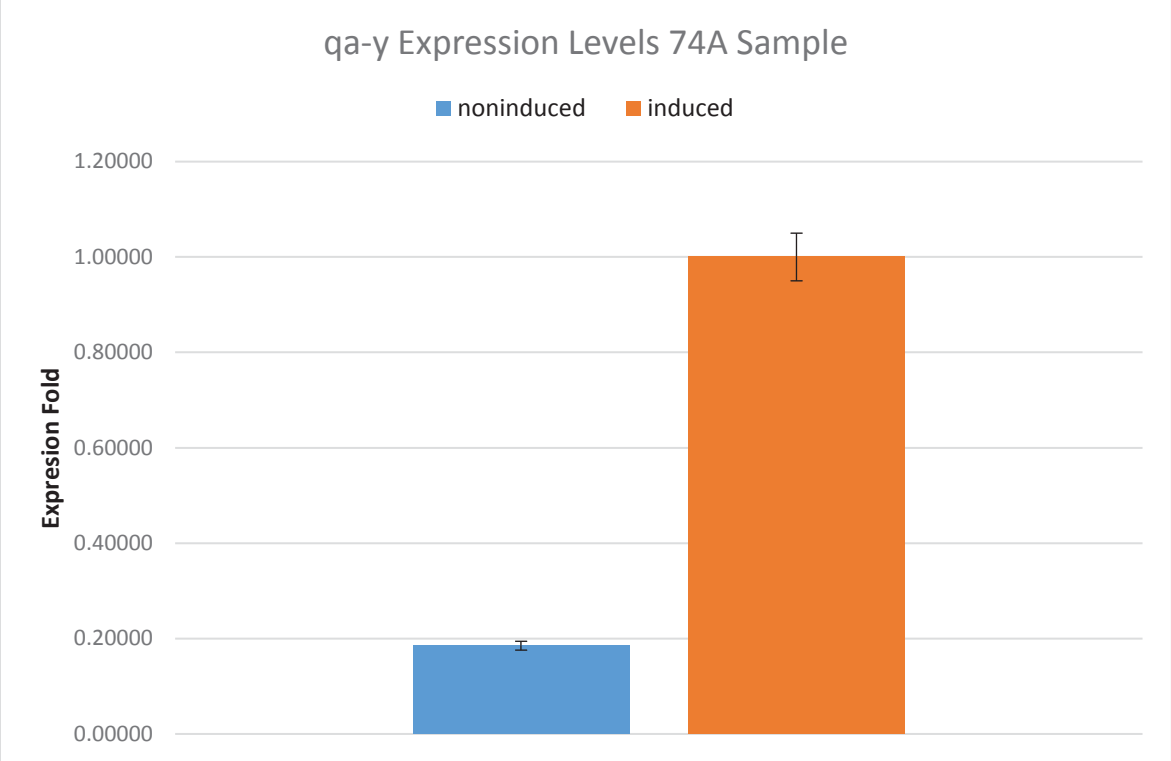

Figure 15: qa-y Expression Levels in 74A Non-induced and Induced Samples. From the figure, it is clearly shown that in the induced sample (orange) the expression of the qa-y gene is substantially greater than that of the qa-y gene in the non-induced sample (blue). This was the expected and desired result.

Glycogen Phosphorylase

Based upon previous data showing that the NCU07027 Glycogen Phosphorylase gene in *N. crassa* showed heightened expression through a proteomic analysis, first, a non-induced induced trial was conducted with qRT-PCR in order to determine the expression levels of this gene at the transcriptional level when quinic acid is the sole carbon source (Figure 16).

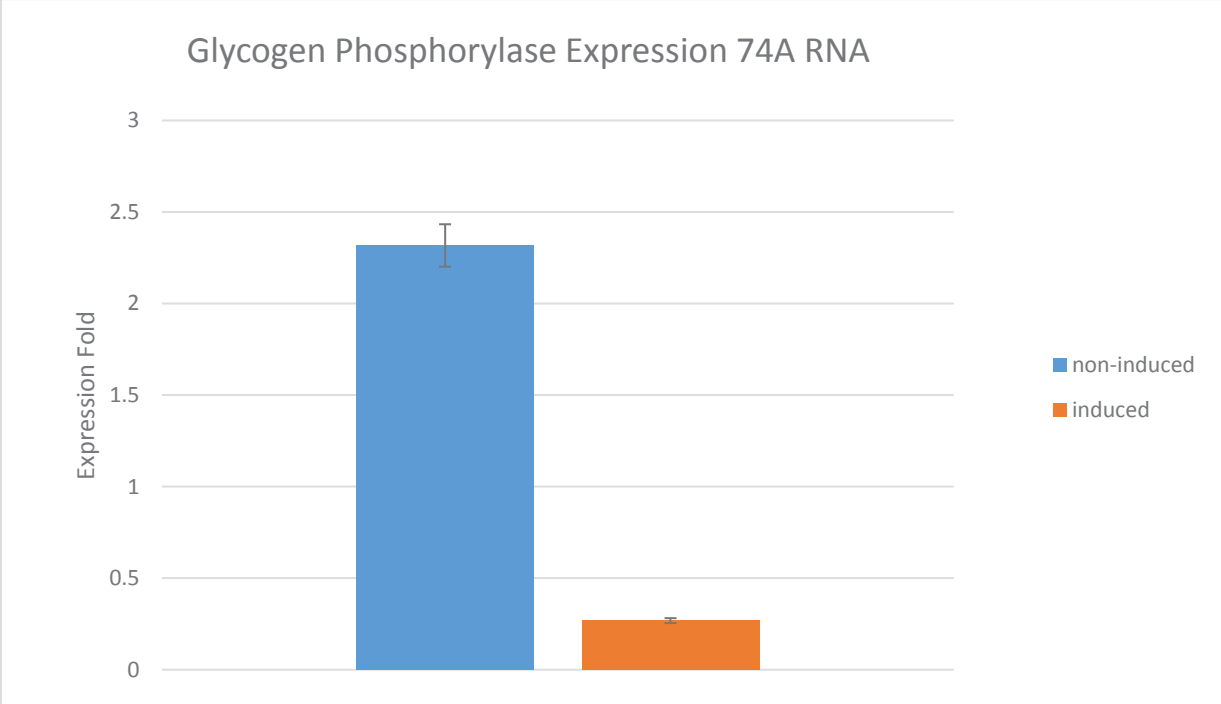

Figure 16: Glycogen Phosphorylase Expression Levels in 74A Non-induced and Induced Samples. From the figure, it is clearly shown that in the non-induced sample (blue) the expression of the Glycogen Phosphorylase gene is substantially greater than that of the Glycogen Phosphorylase gene in the induced sample (orange). This was not the expected result as prior studies would suggest.

From this test it was shown that Glycogen Phosphorylase was expressed at a much lower level in the induced sample as compared to the non-induced. This was not what was expected. Since previous data showed this gene to be translated at a higher level at the proteomic level during quinic acid metabolism, it was expected then that at the transcription level, the gene would be expressed higher as well when quinic acid was the sole carbon source. Since this was not found, the next step in the analysis was to determine if what was seen in previous studies was a result of starvation rather than quinic acid metabolism since quinic acid is a poor carbon source. The assumption then was that since quinic acid is a poor source of energy, that glycogen phosphorylase expression may be due to a starvation response rather than to quinic acid metabolism. However, in the qRT-PCR test of non-induced and starved RNA samples, the results did not show this. Instead, Glycogen Phosphorylase in the starved sample showed lower expression than in the non-induced sample (Figure 17). Glycogen Phosphorylase is important in all organisms in glucose metabolism, so the result that was achieved is consistent in that regard, but it is not consistent with the idea that the heighted level of expression of Glycogen Phosphorylase seen in prior works during quinic acid metabolism is a result of starvation.

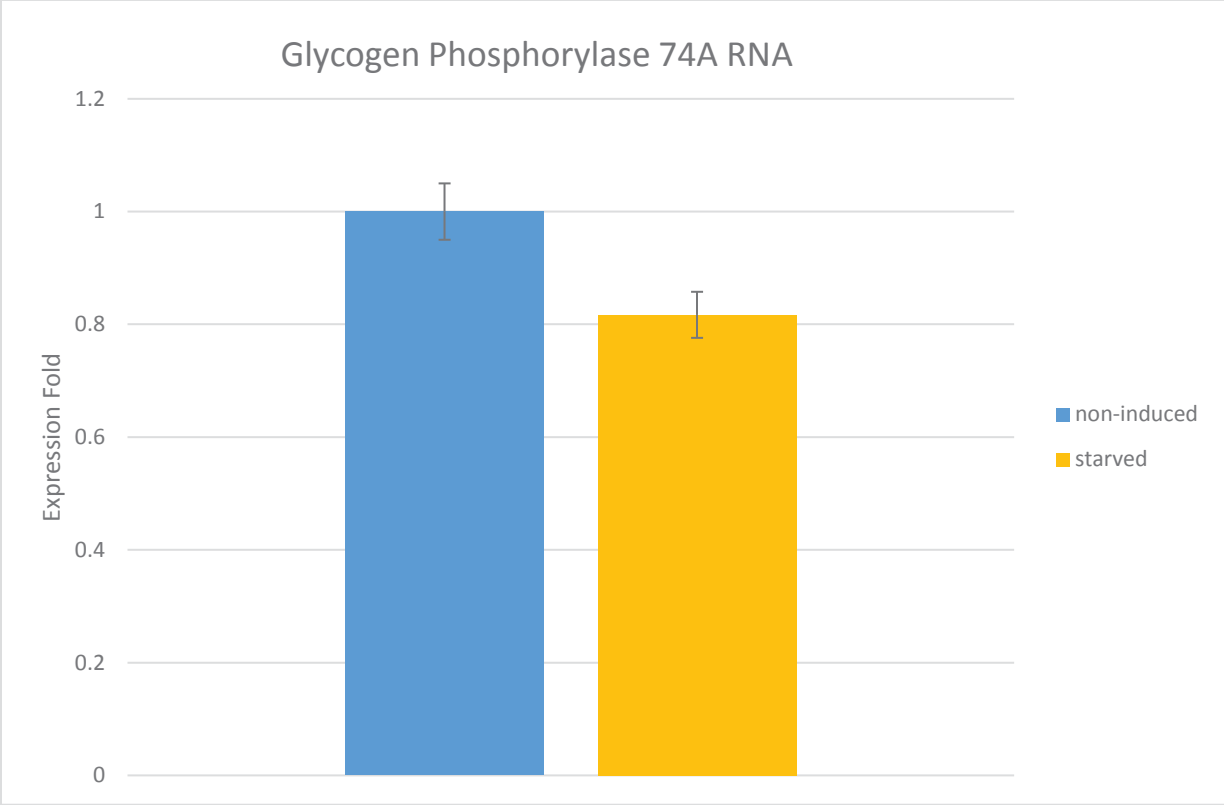

Figure 17: Glycogen Phosphorylase Expression Levels in 74A Non-induced and Starved Samples. From the figure, it is clearly shown that in the non-induced sample (blue) the expression of the Glycogen Phosphorylase gene is greater than that of the Glycogen Phosphorylase gene in the starved sample (yellow).

Based upon the qRT-PCR results that were acquired with the 74A samples, it can be hypothesized that the transcription of the Glycogen Phosphorylase gene is not related to quinic acid metabolism thus, in the *qa-1F* knockout samples, similar results to that of the 74A trials are expected. In the FGSC 11034 samples both the non-induced induced trial and the non-induced starved trial did in fact show similar results to the 74A trials as expected (Figure 18 and 19).

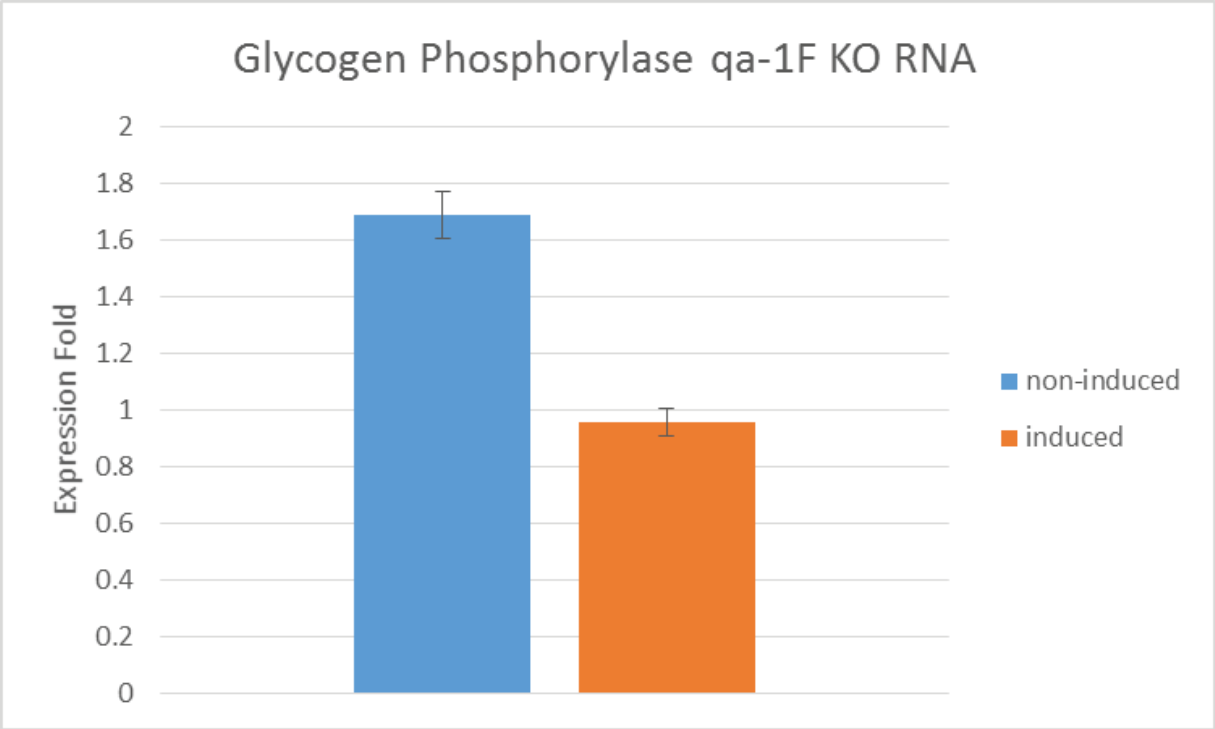

Figure 18: Glycogen Phosphorylase Expression Levels in FGSC 11034 qa-1F Knockout Non-induced and Induced RNA Samples. From the figure, it is clearly shown that in the non-induced sample (blue) the expression of the Glycogen Phosphorylase gene is greater than that of the Glycogen Phosphorylase gene in the induced sample (orange).

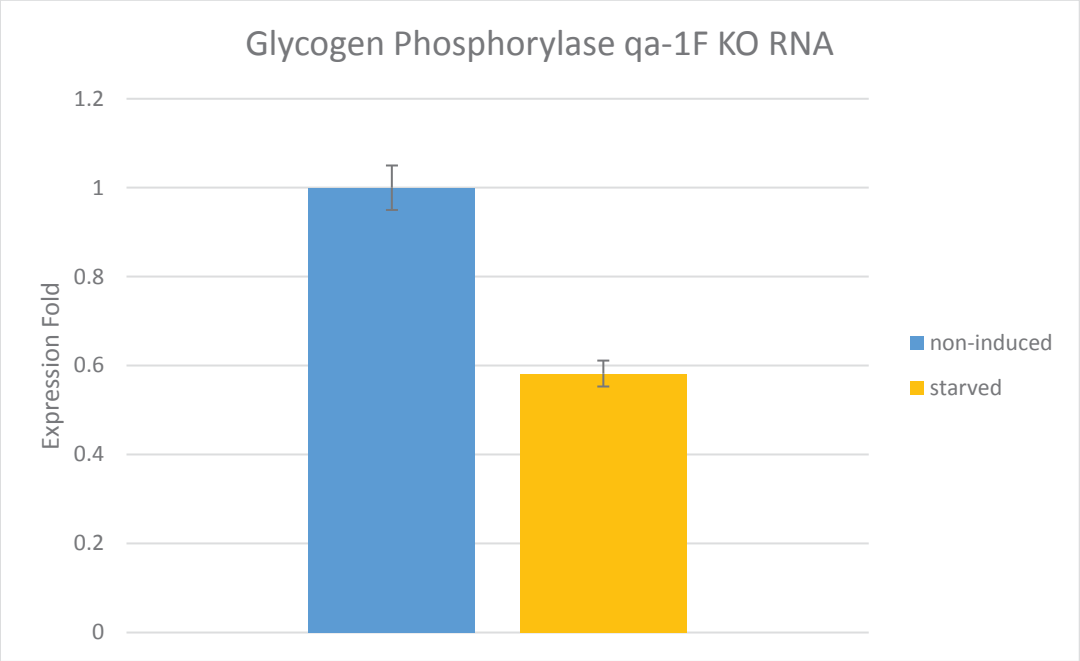

Figure 19: Glycogen Phosphorylase Expression Levels in FGSC 11034 qa-1F KO Non-induced and Starved Samples. From the figure, it is clearly shown that in the non-induced sample (blue) the expression of the Glycogen Phosphorylase gene is greater than that of the Glycogen Phosphorylase gene in the starved sample (yellow).

Peptidyl-prolyl Cis-trans Isomerase

Previous work showed that peptidyl-prolyl cis-trans isomerase was expressed at a heightened level in induced samples at the proteomic level (Allen K., 2010). Thus, the first analysis conducted with regard to NCU04140 Peptidyl-prolyl cis-trans Isomerase sought to analyze the expression of this gene in a non-induced induced wild-type (74A)

qRT-PCR trial (Figure 20) in order to determine the expression levels in the induced sample as compared to a non-induced variant.

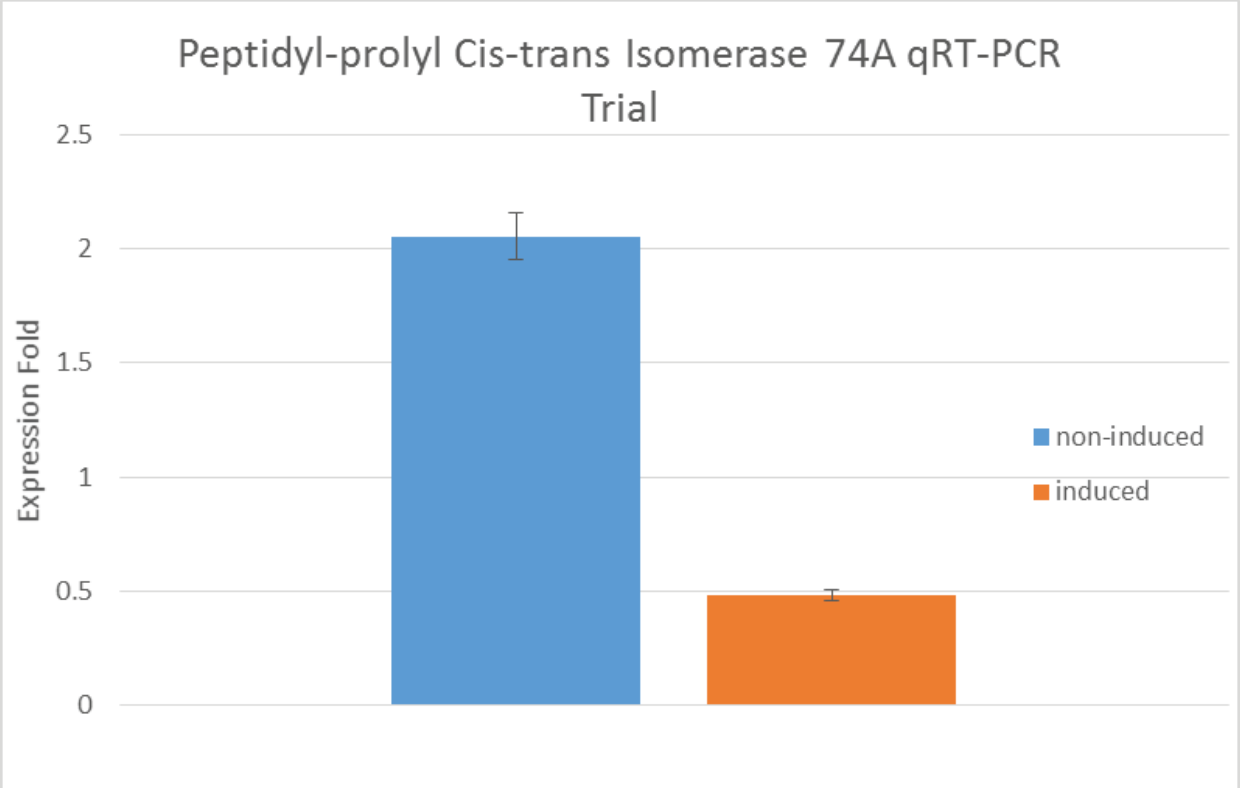

Figure 20: Peptidyl-prolyl Cis-trans Isomerase Expression Levels in 74A Non-induced and Induced RNA Samples. From the figure, it is clearly shown that in the non-induced sample (blue) the expression of the Peptidyl-prolyl cis-trans Isomerase gene is greater than that of the Peptidyl-prolyl cis-trans Isomerase gene in the induced sample (orange).

Since this result was did not correspond to the proteomic analysis that was conducted in Katie Allen's Master's thesis (Master's Thesis), a subsequent analysis analyzing Peptidyl-prolyl cis-trans Isomerase ensued analyzing the gene in a starved condition (less sucrose or quinic acid) with the goal of this analysis being to determine if the heightened translation of the gene that was seen during quinic acid metabolism at the proteomic level

in prior work was actually a result of starvation considering that quinic acid is not a preferred source of carbon (Figure 21).

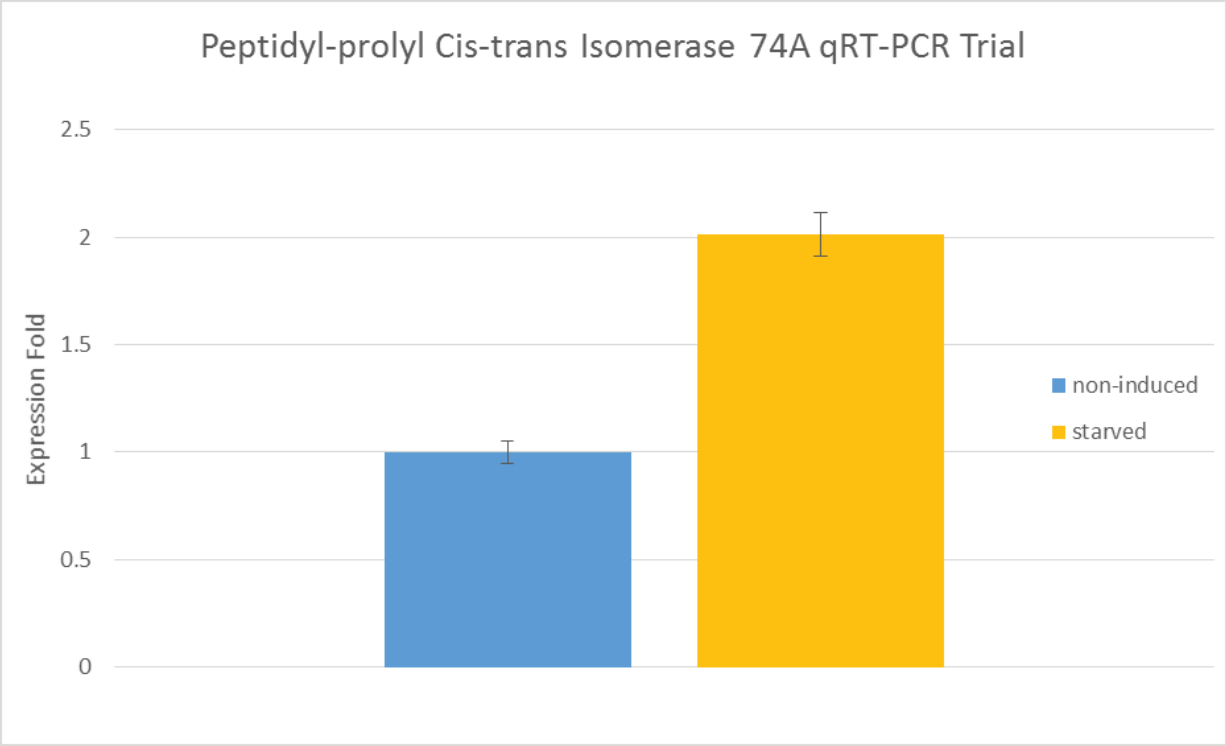

Figure 21: Peptidyl-prolyl Cis-trans Isomerase Expression Levels 74A Non-induced and Starved Samples. From the figure, it is clearly shown that in the starved sample (yellow) the expression of the Peptidyl-prolyl Cis-trans Isomerase gene is greater than that of the Peptidyl-prolyl Cis-trans Isomerase gene in the non-induced sample (blue).

From Figure 21, it can be hypothesized that since Peptidyl-prolyl cis-trans Isomerase showed a higher expression fold level in the starved sample that the transcription of this gene may indeed be reacting to starvation rather than quinic acid exposure or metabolism. Furthermore, it can be noted that a possibility for the protein product of this gene to be seen at the proteomic level may in fact be a stress response to starvation rather than a result of quinic acid metabolism. In order to determine if this was truly the case, further qRT-PCR gene expression analyses were conducted with FGSC 11034 qa-1F knock-out mutant samples under the same conditions set in the 74A trials (Figure 22 and 23).

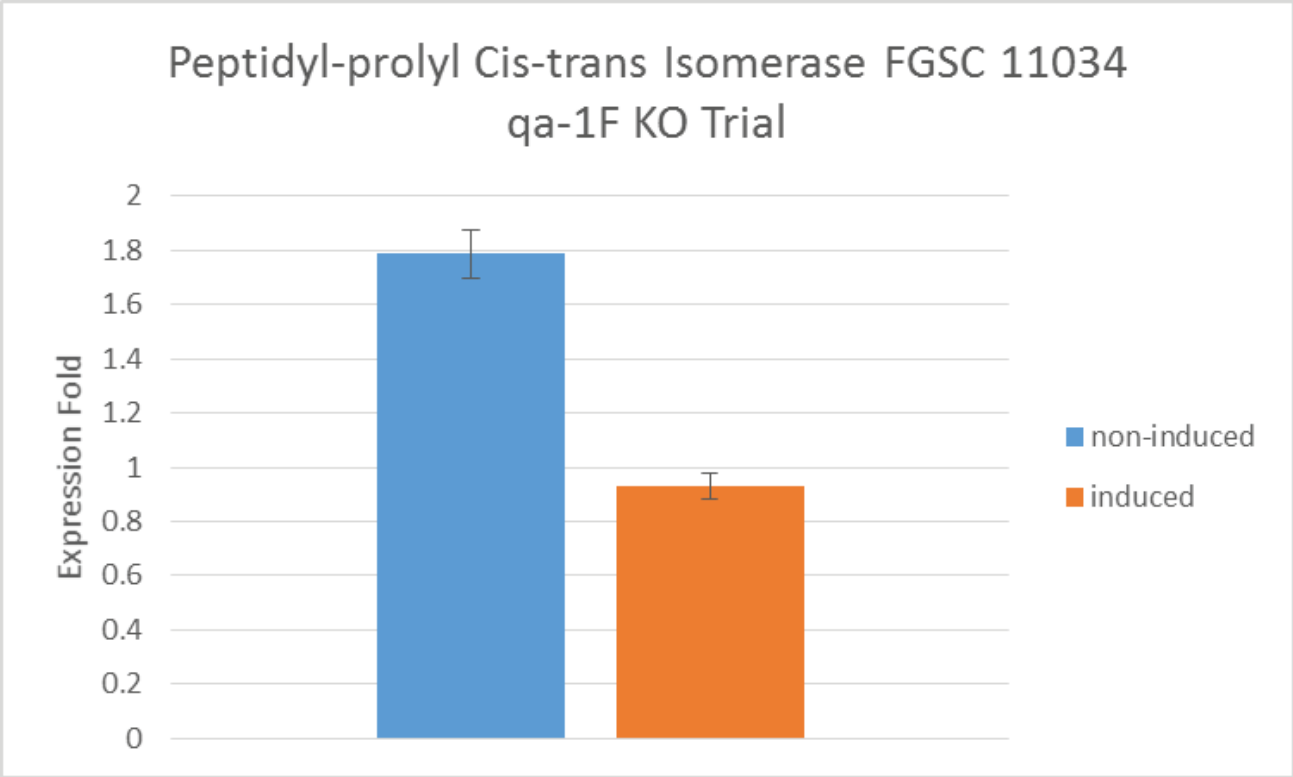

Figure 22: Peptidyl-prolyl Cis-trans Isomerase Expression Levels in FGSC 11034 qa-1F Knock-out Non-induced and Induced RNA Samples. From the figure, it is clearly shown that in the non-induced sample (blue) the expression of the Peptidyl-prolyl cis-trans Isomerase gene is greater than that of the Peptidyl-prolyl cis-trans Isomerase gene in the induced sample (orange).

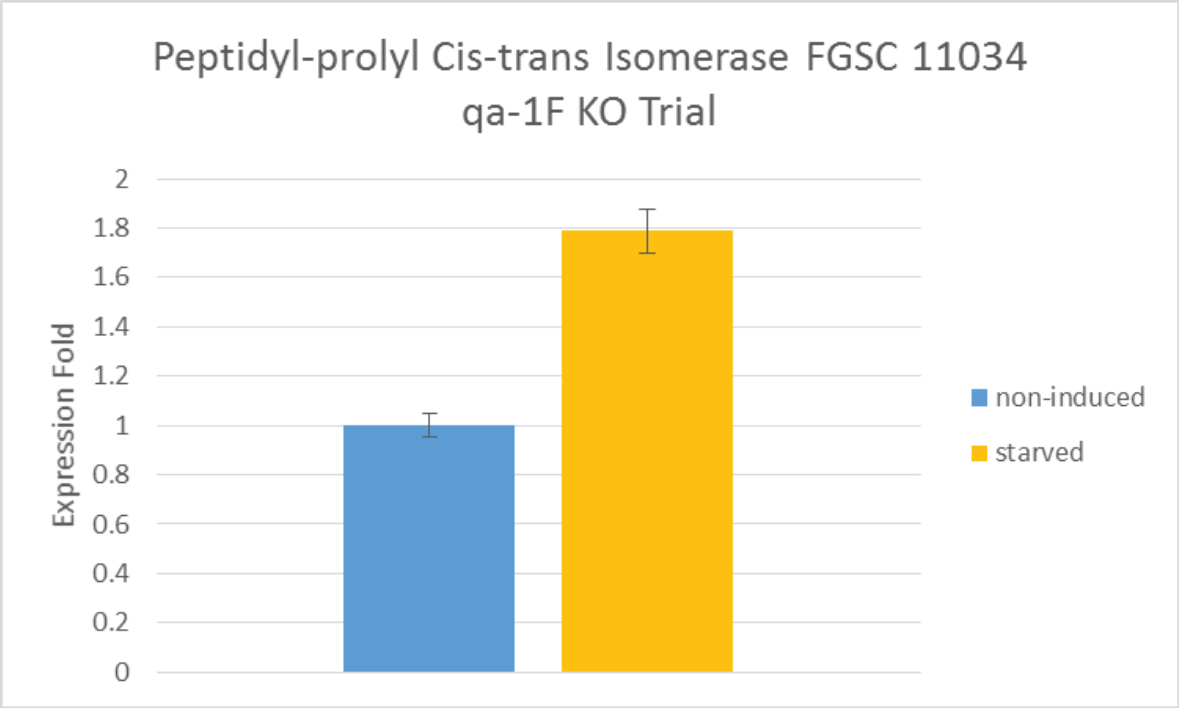

Figure 23: Peptidyl-prolyl Cis-trans Isomerase Expression Levels in FGSC 11034 qa-1F Knock-out Non-induced and Starved Samples. From the figure, it is clearly shown that in the starved sample (yellow) the expression of the Peptidyl-prolyl Cis-trans Isomerase gene is greater than that of the Peptidyl-prolyl Cis-trans Isomerase gene in the non-induced sample (blue).

Based upon the 74A trials (Figure 20 and 21) it is expected that similar results would be produced in the *qa-1F* KO samples (Figure 22 and 23). This was the case. Since it appeared that Peptidyl-prolyl Cis-trans Isomerase was a result of starvation and not a result of quinic acid metabolism, it can be hypothesized that in the mutant strain, knocking out the activator (qa-1F) of the quinic acid gene cluster would not have an effect on the expression of Peptidyl-prolyl Cis-trans Isomerase in induced or starved samples.

NCU08332 *Hex-1*: Woronin Body Major Protein

The *Hex-1*: Woronin Body Major Protein gene NCU08332 in *N. crassa* was shown in prior work to have heightened transcription at the proteomic level (Tirabassi D.M., 2013). Because of this, the initial qRT-PCR analysis aimed to determine the expression level of this gene in an induced sample of RNA as compared to a non-induced sample (Figure 24).

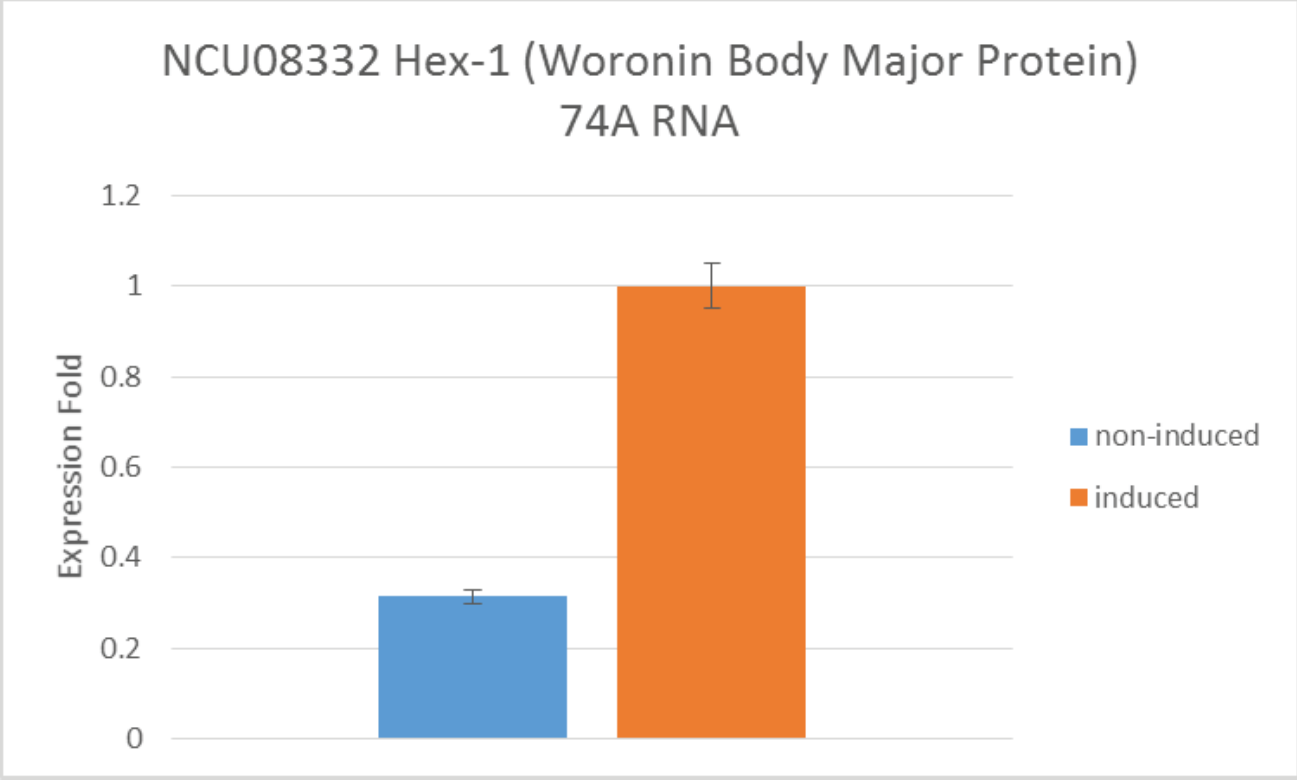

Figure 24: NCU08332 *Hex-1*: Woronin Body Major Protein Gene Expression Levels in 74A Non-induced and Induced Samples. From the figure, it is clearly shown that in the induced sample (orange) the expression of the NCU08332 *Hex-1*: Woronin Body Major Protein gene is greater than that of the NCU08332 *Hex-1*: Woronin Body Major Protein gene in the non-induced sample (blue).

This result corresponded to the prior proteomic analysis of this gene. From the data in Figure 24, it can be further hypothesized that NCU08332 *Hex-1*: Woronin Body Major Protein gene appears to have a correlation with quinic acid metabolism. In order to strengthen this hypothesis, a qRT-PCR analysis of NCU08332 *Hex-1*: Woronin Body Major Protein in a starved sample was conducted (Figure 25). This was done in order to rule out the possibility that the heightened expression seen at both the proteomic level (in prior studies) and at the genomic level (in this study) were indeed a result of quinic acid or quinic acid metabolism rather than a result of starvation considering that quinic acid is not the optimal carbon source for *N. crassa*.

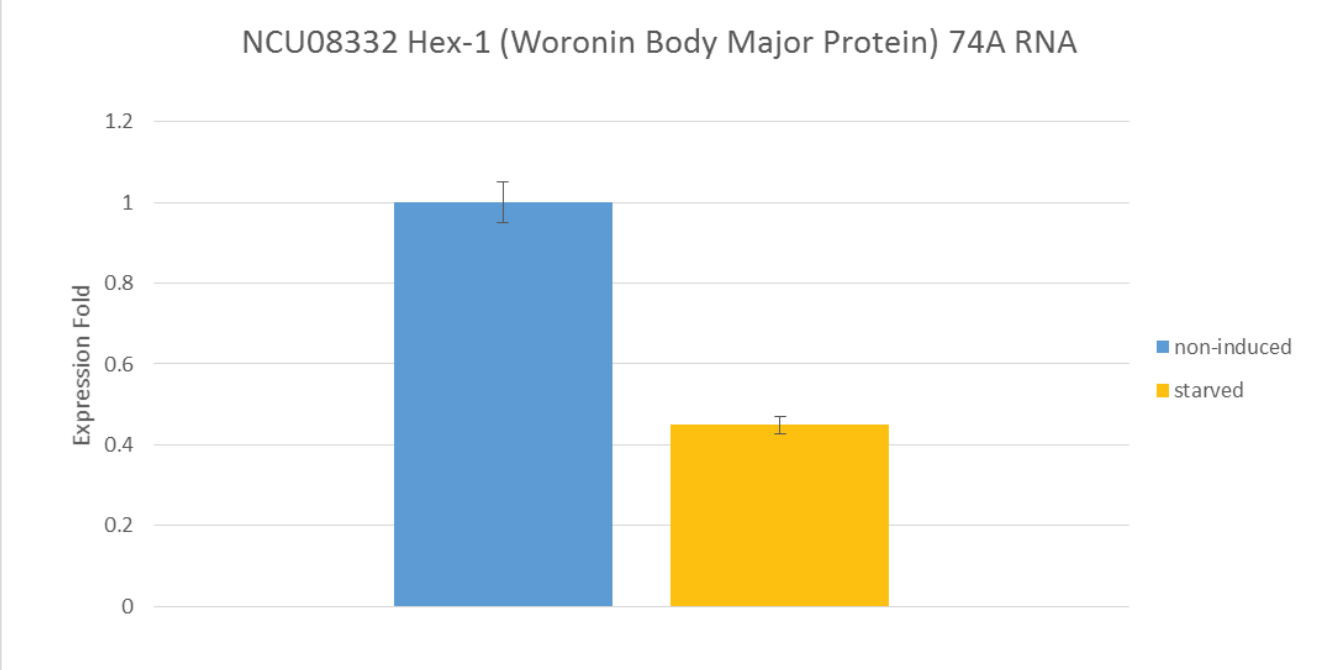

Figure 25: NCU08332 *Hex-1*: Woronin Body Major Protein Gene Expression Levels in 74A Non-induced and Induced Samples. From the figure, it is clearly shown that in the non-induced sample (blue) the expression of the NCU08332 *Hex-1*: Woronin Body Major Protein gene is greater than that of the NCU08332 *Hex-1*: Woronin Body Major Protein gene in the starved sample (yellow).

The results shown in Figure 25 show that in the starved RNA samples, the *Hex-1*: Woronin Body Major Protein gene (NCU08332) was not expressed at a higher level than that of what was seen in the non-induced sample. This further strengthened the hypothesis that the *Hex-1*: Woronin Body Major Protein gene (NCU08332) was indeed reacting to quinic acid metabolism rather than starvation. In order to further strengthen this hypothesis, a gene expression analysis of the *Hex-1*: Woronin Body Major Protein gene (NCU08332) was conducted utilizing FGSC 11034 qa-1F KO RNA under non-induced and induced conditions (Figure 26). Based on the results of the 74A qRT-PCR analyses, it was expected that expression in the induced FGSC 11034 qa-1F KO sample

would be less than that of the non-induced FGSC 11034 qa-1F KO sample since it was hypothesized that the *Hex-1*: Woronin Body Major Protein gene (NCU08332) was reacting to quinic acid metabolism. This was the case (Figure 26).

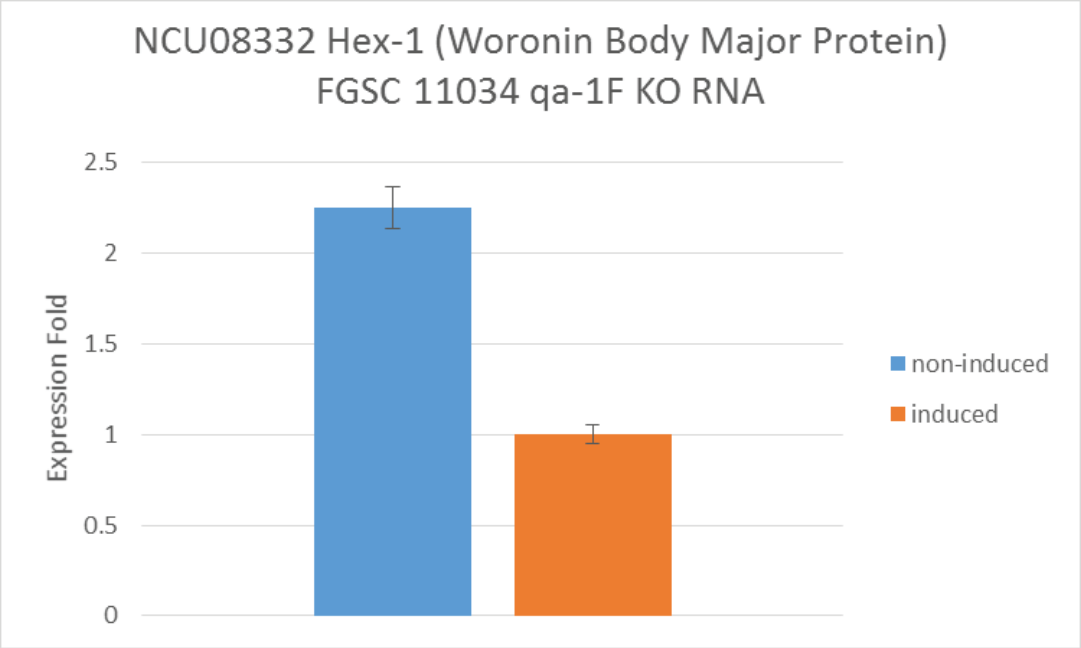

Figure 26: NCU08332 *Hex-1*: Woronin Body Major Protein Gene Expression Levels in FGSC 11034 qa-1F KO Non-induced and Induced Samples. From the figure, it is clearly shown that in the non-induced sample (blue) the expression of the NCU08332 *Hex-1*: Woronin Body Major Protein gene is greater than that of the NCU08332 *Hex-1*: Woronin Body Major Protein gene in the induced sample (orange).

The data from Figure 26 further supports the hypothesis by showing the opposite result that was seen in the 74A sample under the same conditions. Since *qa-1F* acts as an activator of the quinic acid gene cluster, in a induced *qa-1F* KO sample the expression of the *Hex-1*: Woronin Body Major Protein gene (NCU08332) should be suppressed assuming that the *Hex-1*: Woronin Body Major Protein gene (NCU08332) is responding to quinic acid. To further strengthen the hypothesis, a final qRT-PCR reaction testing the

expression of the *Hex-1*: Woronin Body Major Protein gene (NCU08332) in non-induced and starved FGSC 11034 *qa-1F* KO samples was conducted (Figure 27).

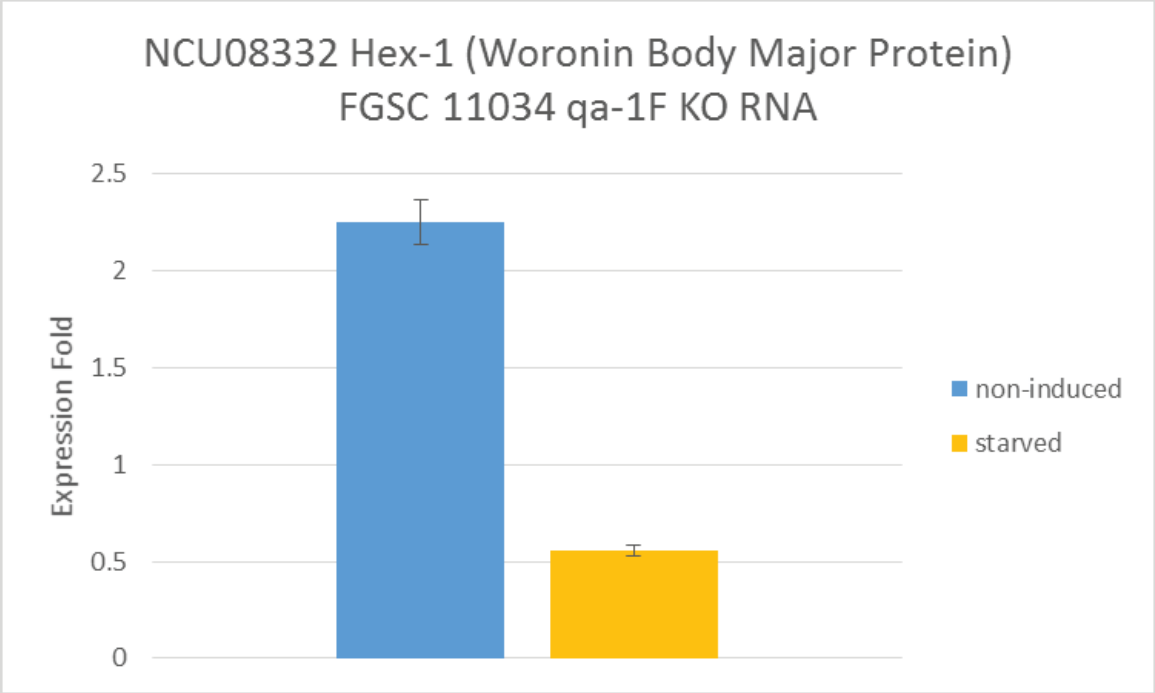

Figure 27: NCU08332 *Hex-1*: Woronin Body Major Protein Gene Expression Levels in 74A Non-induced and Induced Samples. From the figure, it is clearly shown that in the non-induced sample (blue) the expression of the NCU08332 *Hex-1*: Woronin Body Major Protein gene is greater than that of the NCU08332 *Hex-1*: Woronin Body Major Protein gene in the starved sample (yellow).

Bioinformatics Analysis of NCU08332:

Following qRT-PCR trials of the *Hex-1*: Woronin Body Major Protein gene (NCU08332), a bioinformatics analysis was conducted to further strengthen the hypothesis that the *Hex-1*: Woronin Body Major Protein gene (NCU08332) was actually responding to quinic acid and was activated by the *qa-1F* activator protein. In order to deduce why the *Hex-1*: Woronin Body Major Protein gene (NCU08332) was being

expressed at heightened levels during quinic acid metabolism, an alignment using ClustalX2 sequence alignment software was utilized to determine if the conserved 16 bp qa-1F binding site (5'-GGR-TAA-RYR-YTT-AYC-C-3') , as described by James A. Baum and his associates (Baum J.A., et al. 1986), was located within the 1 Kb sequence directly upstream of the start site of the *Hex-1*: Woronin Body Major Protein gene (NCU08332) (Figure 28). This may indicate a probable binding site near the *Hex-1* gene for the *qa-1F* activator protein.

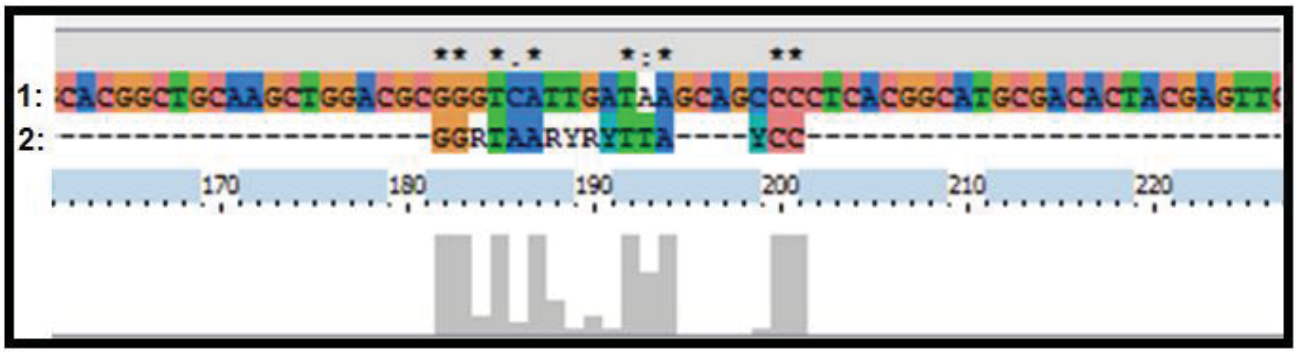

Figure 28: The Conserved qa-1F Binding Site Aligned With The 1 KB Sequence Upstream of The Start Site of The *Hex-1*: Woronin Body Major Protein Gene (NCU08332). The sequence labeled "1:" is the 1 Kb sequence upstream of the *Hex-1*: Woronin Body Major Protein gene (NCU08332) and the sequence labeled "2:" is the 16 bp conserved sequence as described by James Baum and his colleagues (Baum J.A., et al. 1986). The alignment by ClustalX2 showed that there was a similar binding site with 12 nucleotides being a probable match between -799 and -899 of the *Hex-1*: Woronin Body Major Protein gene (NCU08332). (i.e. 181-201 downstream from the start of the 1Kb sequence upstream of the *Hex-1*: Woronin Body Major Protein gene (NCU08332)) Asterisk (*) indicate identical nucleotide base matches between the sequences.

Chapter 4: Discussion

Although the genes that are presented in this study were not specifically noted as being among the genes that showed a relation to the quinic acid gene cluster via the microarray analyses that were analyzed in the study by Logan and his associates (Logan D.A., et al. 2007), prior thesis work in the Proteomic Genomic Research Laboratory at Youngstown State University showed that these three genes were generating protein products at a heightened rate during quinic acid metabolism rather than in the presence of glucose. This indicated that these may be three of the some 100 genes that were hypothesized to have some relation to the quinic acid gene cluster or to quinic acid metabolism itself. Along with a brief bioinformatics analysis, this study mainly utilized qRT-PCR to determine the expression of these genes under various conditions to determine if there is some correlation to quinic acid metabolism at the transcriptional level. Initial qRT-PCR analyses of primer efficiencies (Appendix) showed that the primer pairs that were utilized were relevant and useful for this study strengthening the results that were determined. Furthermore, analyses of *qa-y* and *qa-1F* in a non-induced induced trial of 74A wild-type RNA (Figures 14 and 15) indicated that the samples that were utilized were indeed induced (i.e. metabolizing quinic acid via expression of the *qa* gene cluster) further strengthening the results of this research.

Glycogen phosphorylase was predicted to have an elevated expression level in the presence of quinic acid as compared to that of glucose based on prior proteomic analyses. However, the results of this analysis showed that this was not the case. In the induced 74A qRT-PCR trial, the expression was much lower than of that in the non-

induced sample. Furthermore, the same result was produced in the qa-1f knockout samples (FGSC 11034) that were tested under the same conditions. From this, it can be inferred that the glycogen phosphorylase gene is not related to the quinic acid gene cluster. From the literature, this result makes sense considering that glycogen phosphorylase is an integral protein in glucose metabolism, but it does not provide a reason as to why this protein was seen at the proteomic level during quinic acid metabolism in the work of Kayla Brown (Master's thesis). The next step then sought to determine if the protein product that was originally seen may have been a response to metabolic stress rather than to quinic acid itself. In other words, the hypothesis was that maybe poorer carbon sources initiate a type of starvation response that may induce this gene at a heightened level. However, in both the 74A and *qa-1F* knockout non-induced starved trials this did not appear to be the case. Instead, in the starved samples the expression of this gene was suppressed as compared to its overall expression in the non-induced condition. So, it appears that this gene is not reacting to quinic acid or starvation, therefore it seems as though the cell is holding on to the glycogen phosphorylase protein that is produced during glucose metabolism as indicated by the work of Kayla Brown. Further proteomic analyses would need to be conducted to determine the absolute reason for this, especially considering that at the transcription level, this gene is not showing higher expression than what is seen in a non-induced sample. One guess may be that the cell is holding on to this protein in order to prepare itself in case there is a trace of glycogen that can be utilized, however there is not data to support this and further biochemical analyses would be needed to support this proposal.

qRT-PCR trials of peptidyl-prolyl cis-trans isomerase also did not correlate to the initial hypothesis of this work. From the data, it appeared that the peptidyl-prolyl cis-trans isomerase gene did not respond to induction in either the 74A or *qa-1F* knockout induced samples. This evidence is indicative that this gene is not related to the quinic acid gene cluster simply because it did not show heightened expression in a sample of *N. crassa* that was grown in a quinic acid rich media. Following this discovery, it was further hypothesized that the reason for the protein product being present in the proteomic analysis conducted by Katie Allen may be the product of a starvation response rather than that of a response to quinic acid. From the data collected from both the 74A and the *qa-1F* knockout starved trials this appears to be the case. Quinic acid is a poor carbon source, thus it induces metabolic stress to the organism. Although not to the same degree, starvation also causes metabolic stress, so it is thought that this gene is responding to this stress rather than to quinic acid specifically. This then would correlate to previous research showing that the protein product of this gene was present during quinic acid metabolism in *N. crassa.*

In this study, the only gene that showed results that are supported by the original hypothesis was the NCU08332 *Hex-1* Woronin body major protein gene. At the transcription level in the 74A non-induced induced trial, it was shown that the NCU08332 *Hex-1* Woronin body major protein gene had a heightened expression level in the induced sample. This suggested that the gene was related to the quinic acid gene cluster as suggested by prior work. Furthermore, in the *qa-1F* knockout sample that was utilized to analyze the NCU08332 *Hex-1* Woronin body major protein gene in a non-induced induced trial, the expression of the gene seemed to be suppressed in the induced

sample as compared to the non-induced. This further strengthened the hypothesis that this gene was reacting to quinic acid metabolism. To rule out then that this was a response to something else other than quinic acid metabolism several other analyses were conducted. First, the gene was analyzed again using qRT-PCR to determine if this response was a reaction to metabolic stress similar to that of starvation. In both the 74A and the *qa-1F* knockout trials looking at starvation, the expression of the NCU08332 *Hex-1* Woronin body major protein gene was lower than that in the non-induced sample indicating that this was not the case. Furthermore, it was shown that roughly -800 base pairs upstream of the start site of the NCU08332 *Hex-1* Woronin body major protein gene was a site that was similar to that of the 16 base pair conserved qa-1F binding site (Figure 28). Although, the function of this gene in quinic acid metabolism in *N. crassa* goes beyond the scope of this work, it can be inferred that this gene is related and induced by the quinic acid gene cluster and quinic acid metabolism.

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Appendix

RNA Nanodrop™ Spectrophotometer Results:

Sample	Concentration	260/280 Ratio
A	567.9 ng/µL	1.57
B	724.1 ng/µL	1.87
C	1429.3 ng/µL	1.87
D	2461.3 ng/µL	1.92

Table 21: Thermo Scientific NanoDrop© 2000c spectrophotometer Data Results For 74A Non-Induced RNA Samples Post-extraction. Sample B was utilized for further analysis.

Sample	Concentration	A260	A280	260/280	260/230
A	875.4 ng/µL	21.884	12.955	1.69	1.27
B	927.9 ng/µL	23.197	12.741	1.80	1.08
C	489.0 ng/µL	17.224	7.014	1.74	1.19
D	828.0 ng/µL	20.701	11.069	1.87	1.01

Table 22: Thermo Scientific NanoDrop© 2000c spectrophotometer Data Results For 74A Induced RNA Samples Post-extraction. Sample B was utilized for further analysis.

Sample	Concentration	260/280 Ratio
A	431.58 ng/µL	1.88
B	814.9 ng/µL	1.98
C	413.4 ng/µL	1.96
D	277.18 ng/µL	1.87

Table 23: Thermo Scientific NanoDrop© 2000c spectrophotometer Data Results For 74A Starved RNA Samples Post-extraction. Sample C was utilized for further analysis.

Sample	Concentration	260/280 Ratio
A	9,917.9 ng/µL	1.84
B	1033.7 ng/µL	1.91
C	797.6 ng/µL	1.96
D	5456.1 ng/µL	1.96

Table 24: Thermo Scientific NanoDrop© 2000c spectrophotometer Data Results for FGSC 11034 qa-1F KO Induced RNA Samples Post-extraction. Sample C was utilized for further analysis.

Sample	Concentration	260/280 Ratio
A	1,101.2 ng/µL	1.95
B	3,004.9 ng/µL	1.81
C	3,238.3 ng/µL	1.89
D	1779.5 ng/µL	1.83

Table 25: Thermo Scientific NanoDrop© 2000c spectrophotometer Data Results for FGSC 11034 qa-1F KO Non-induced RNA Samples Post-extraction. Sample A was utilized for further analysis.

Sample	Concentration	260/280 Ratio
A	149.5 ng/µL	1.80
B	4,616.4 ng/µL	1.02
C	1815.9 ng/µL	1.20
D	854.3 ng/µL	1.34

Table 26: Thermo Scientific NanoDrop© 2000c spectrophotometer Data Results for FGSC 11034 qa-1F KO Starved RNA Samples Post-extraction. Sample A was utilized for further analysis.

H3 Primer Efficiencies:

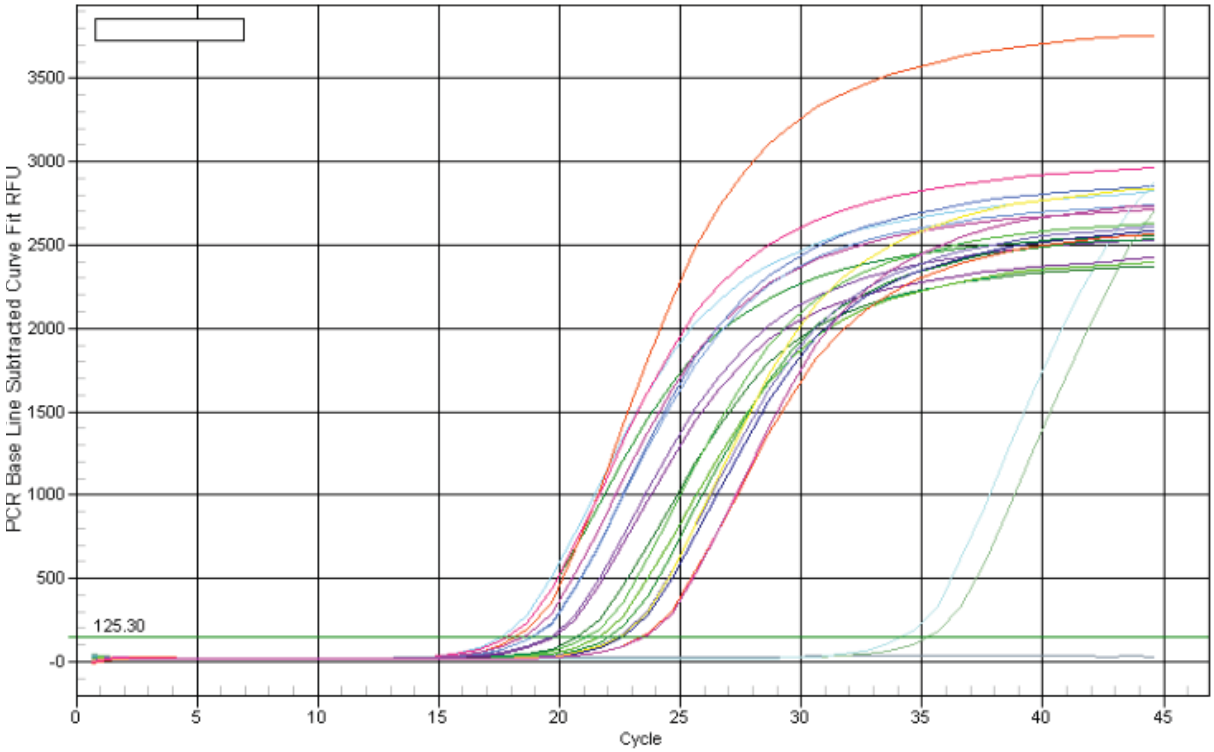

Figure 29: Standard Curves from a Primer Efficiency Test (Dilution Series) of The H3 Primer Pairs. Dilutions started with RNA concentrations of 1,000 ng/µL and were diluted down to 3.9 ng/µL. Each dilution was loaded in triplicate as seen in the figure. The green horizontal line parallel with respect to the X axis represents the threshold.

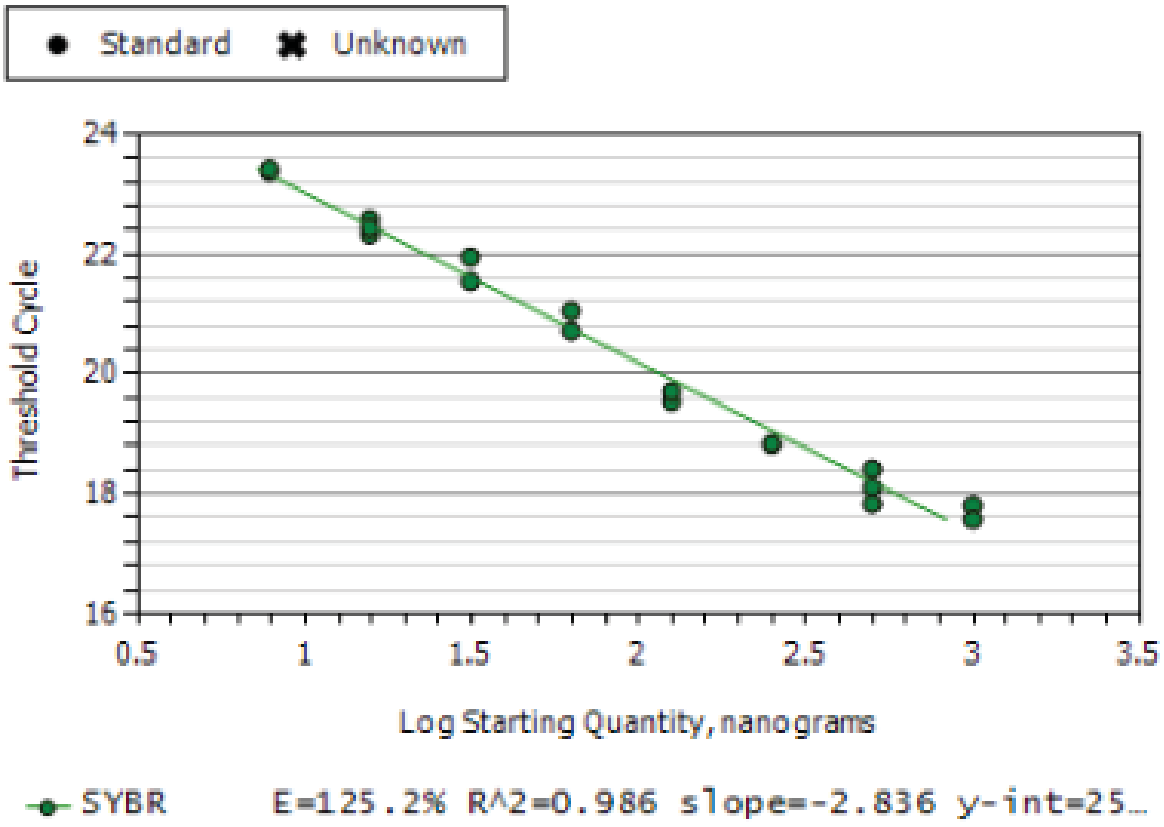

Figure 30: Variation of C_t With PCR Efficiency for H3 Primers. A slope of less than 0.1 indicates statistically comparable efficiencies. Furthermore, an R^2 value of greater than or equal to 0.99 provides good confidence in correlating two values.

Glycogen Phosphorylase Primer Efficiencies:

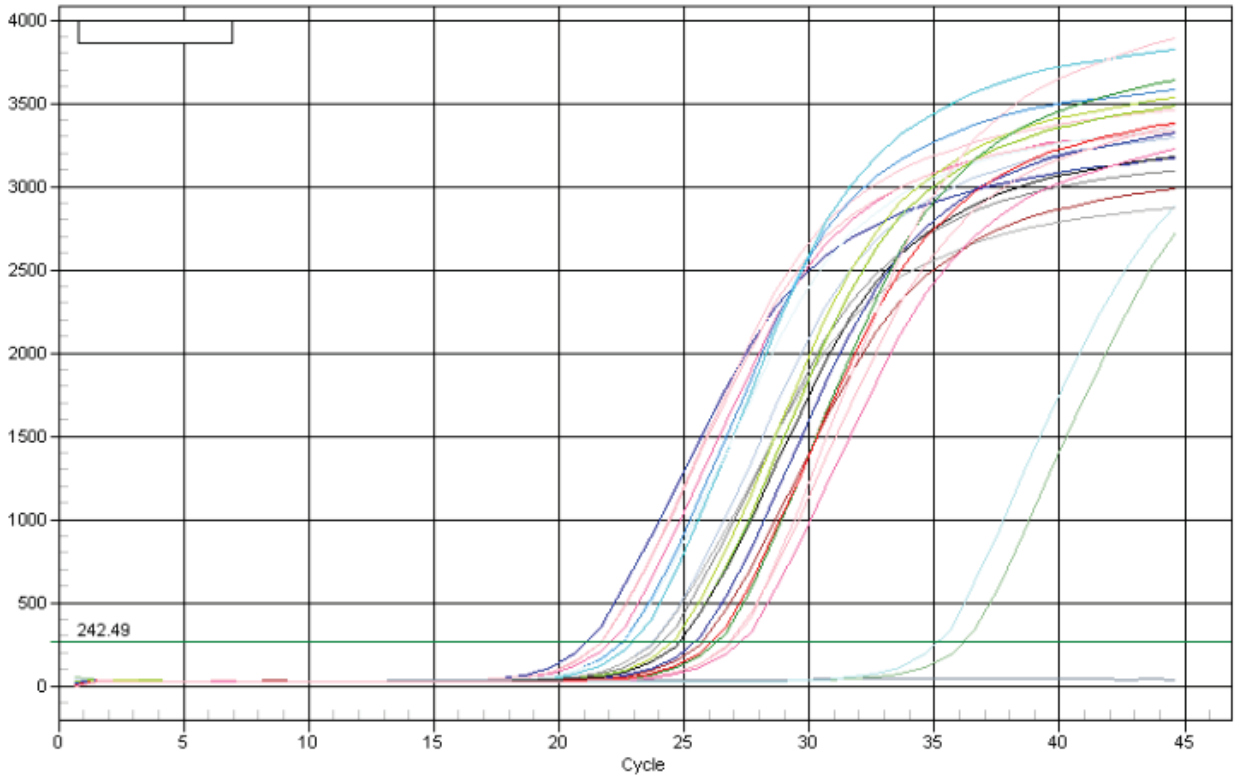

Figure 31: Standard Curves from a Primer Efficiency Test (Dilution Series) of The Glycogen Phosphorylase Primer Pairs. Dilutions started with RNA concentrations of 1,000 ng/µL and were diluted down to 3.9 ng/µL. Each dilution was loaded in triplicate as seen in the figure. The green horizontal line parallel with respect to the X axis represents the threshold.

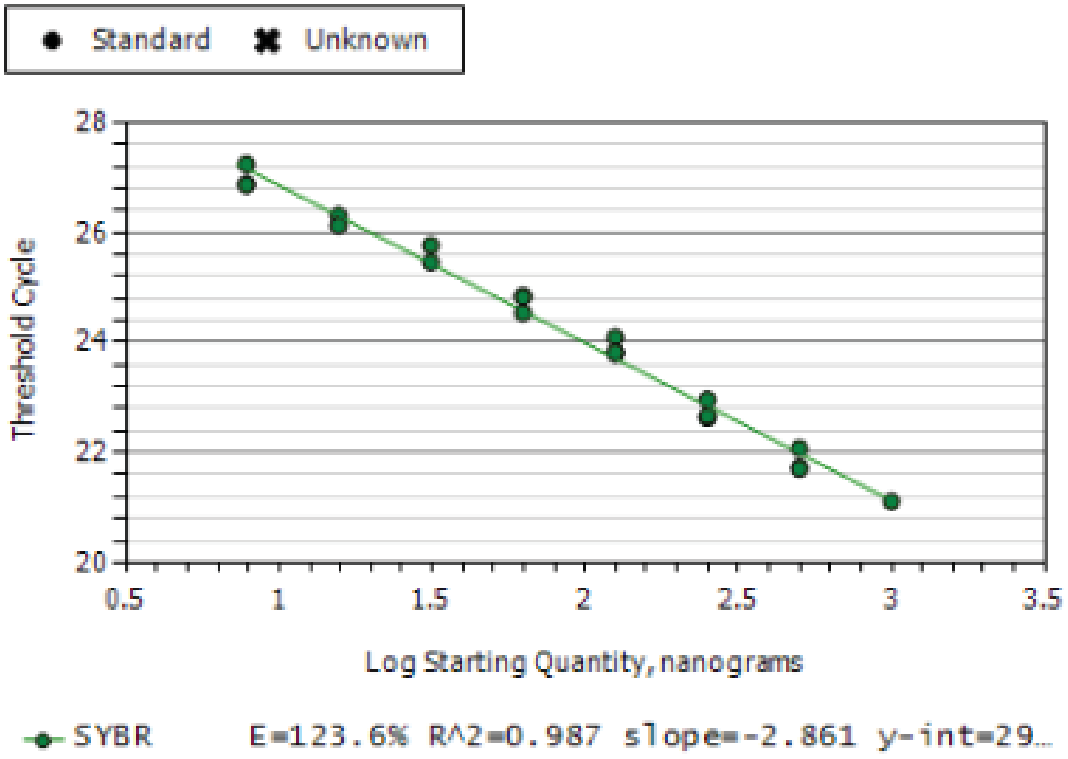

Figure 32: Variation of C_t With PCR Efficiency for Glycogen Phosphorylase Primers. A slope of less than 0.1 indicates statistically comparable efficiencies. Furthermore, an R^2 value of greater than or equal to 0.99 provides good confidence in correlating two values.

Peptidyl-prolyl Cis-Trans Isomerase Primer Efficiencies:

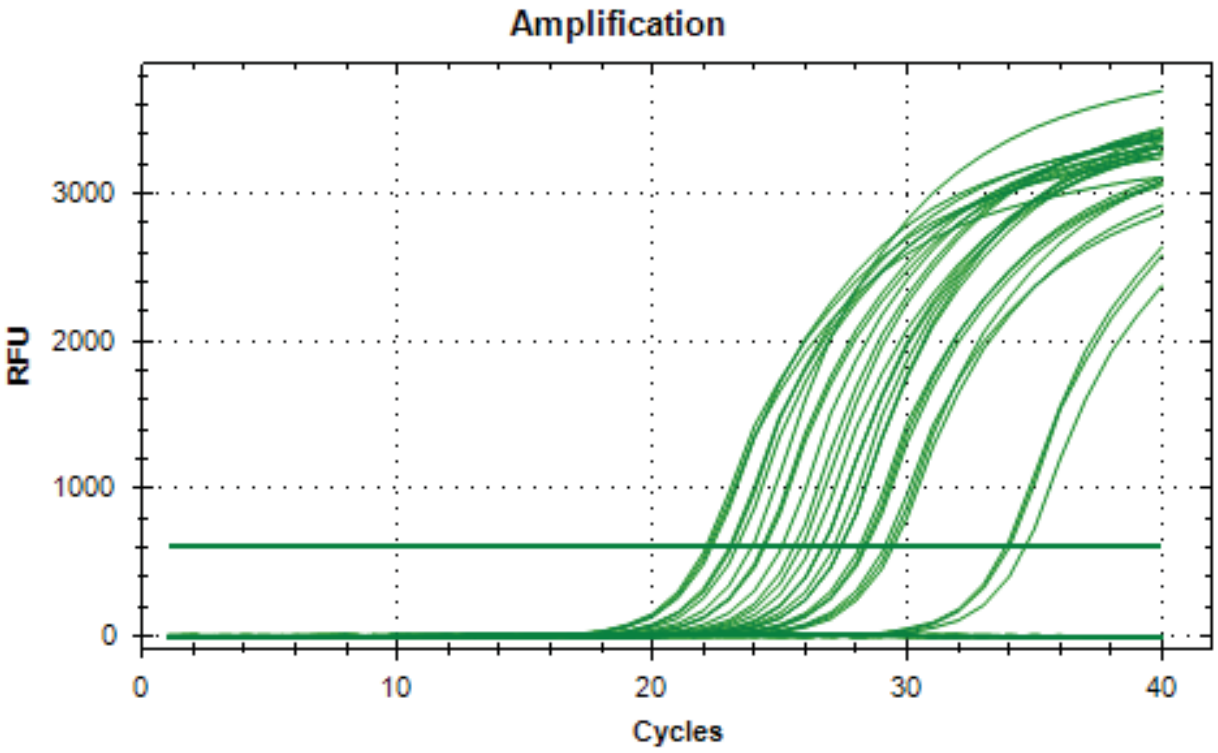

Figure 33: Standard Curves from a Primer Efficiency Test (Dilution Series) of The Peptidyl Prolyl cis-trans Isomerase Primer Pairs. Dilutions started with RNA concentrations of 1,000 ng/µL and were diluted down to 3.9 ng/µL. Each dilution was loaded in triplicate as seen in the figure. The green horizontal line parallel with respect to the X axis represents the threshold.

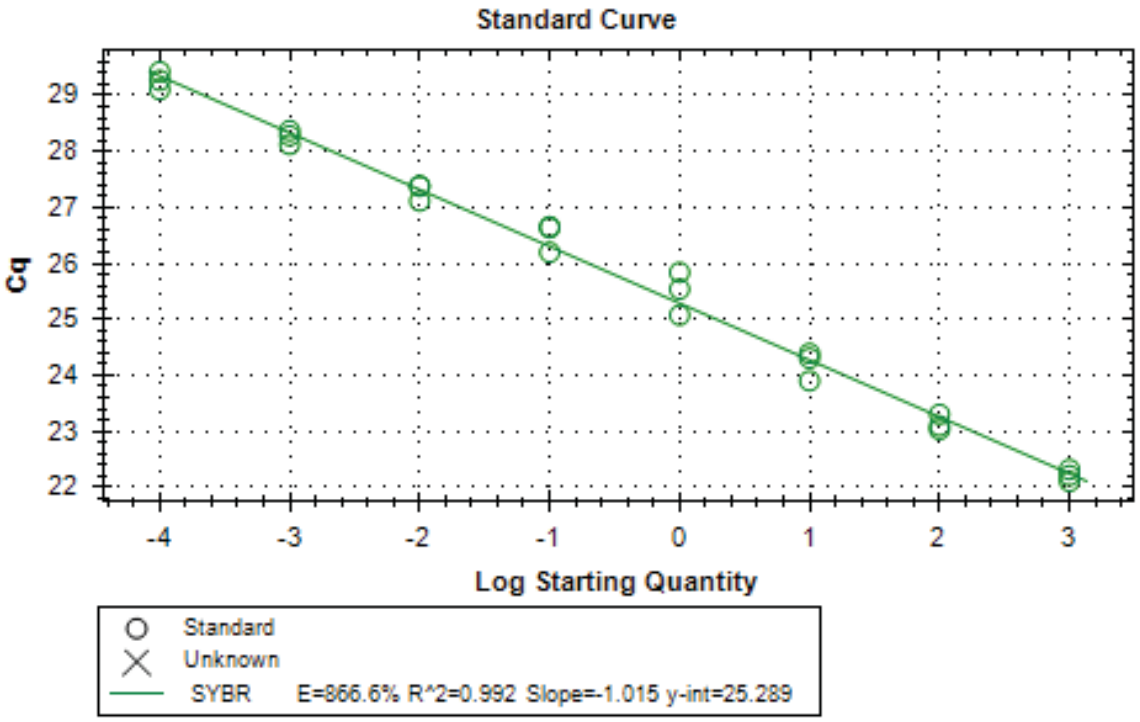

Figure 34: Variation of C_t With PCR Efficiency for Peptidyl Prolyl cis-trans Isomerase Primers. A slope of less than 0.1 indicates statistically comparable efficiencies. Furthermore, an R^2 value of greater than or equal to 0.99 provides good confidence in correlating two values.

***Hex-1*: Woronin Body Major Protein gene (NCU08332) Primer Efficiencies:**

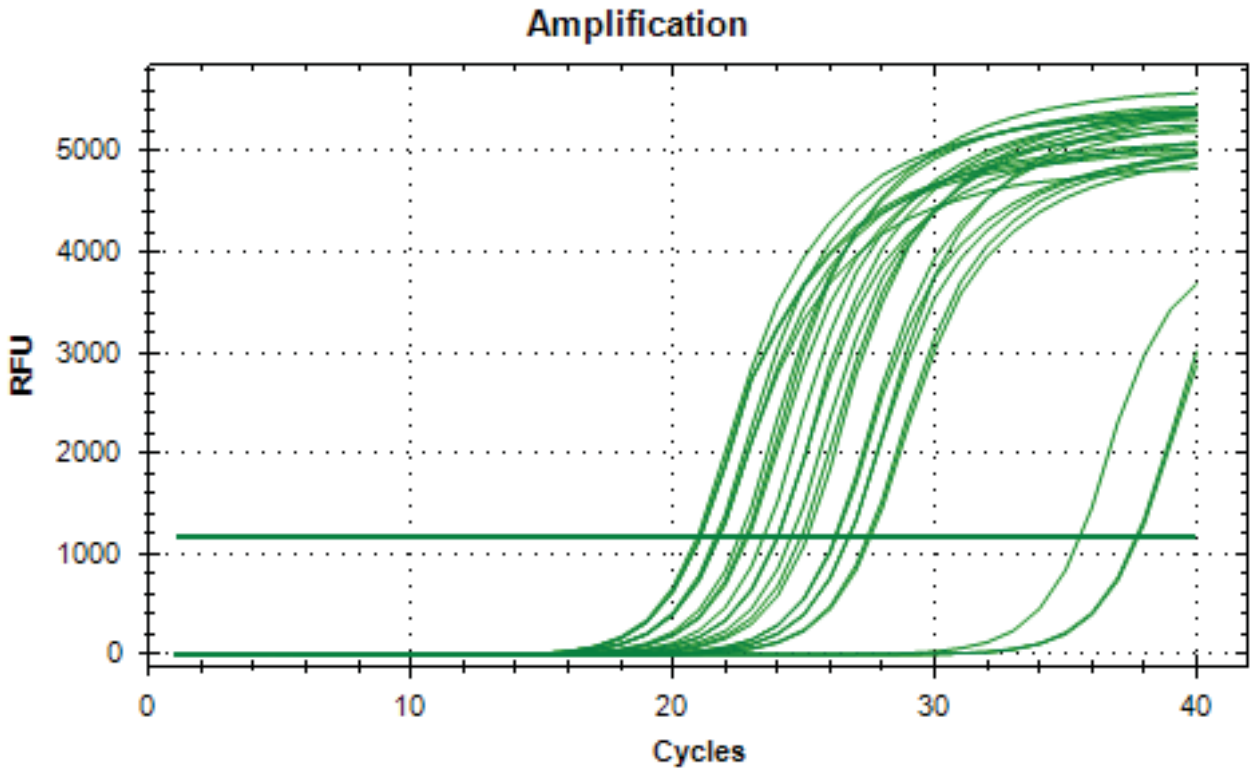

Figure 35: Standard Curves from a Primer Efficiency Test (Dilution Series) of The *Hex-1*: Woronin Body Major Protein gene (NCU08332) Primer Pairs. Dilutions started with RNA concentrations of 1,000 ng/µL and were diluted down to 3.9 ng/µL. Each dilution was loaded in triplicate as seen in the figure. The green horizontal line parallel with respect to the X axis represents the threshold.

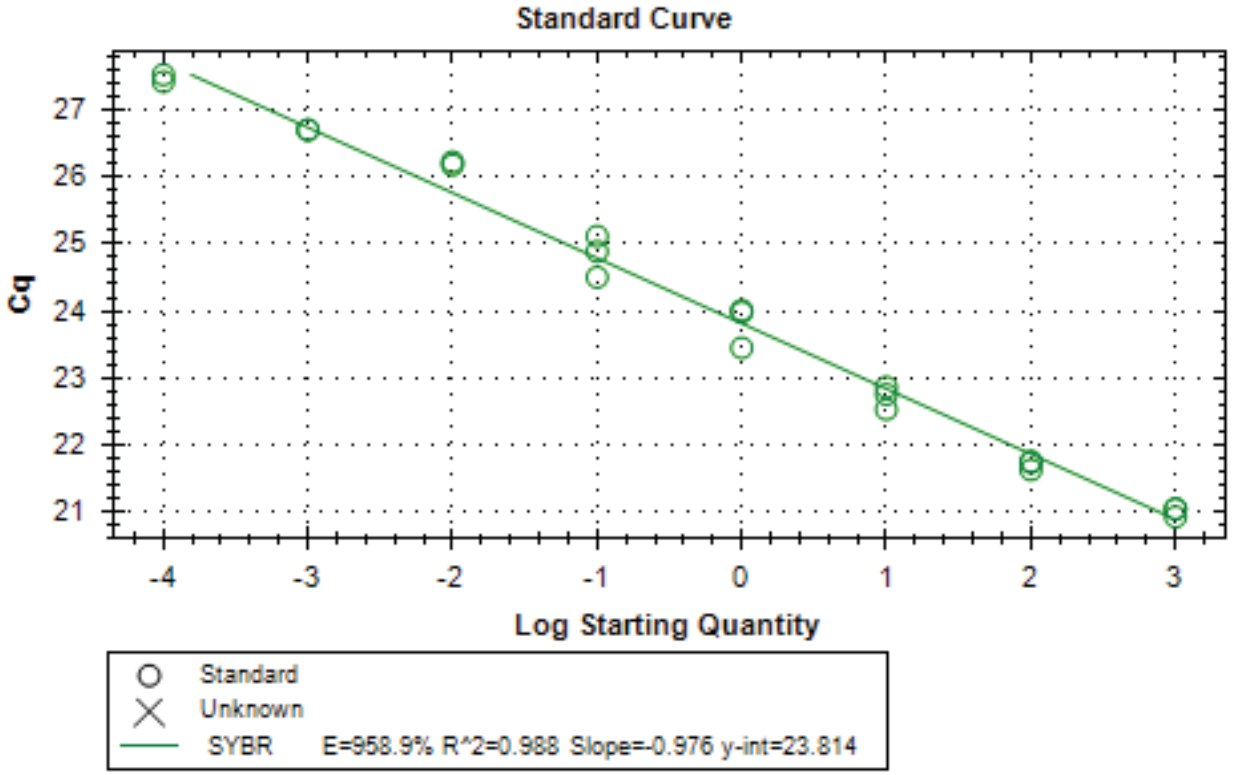

Figure 36: Variation of C_t With PCR Efficiency for *Hex-1*: Woronin Body Major Protein gene (NCU08332) Primers. A slope of less than 0.1 indicates statistically comparable efficiencies. Furthermore, an R^2 value of greater than or equal to 0.99 provides good confidence in correlating two values.