Effects of the *qa-1F* Activator Protein on the Expression of Quinic Acid Induced Genes in *Neurospora crassa*

by

Dana Marie Tirabassi

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Dana Tirabassi

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Dana Tirabassi, Student Date

Approvals:

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Dr. David Asch, Advisor Date

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Dr. Chet Cooper, Committee Member Date

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Dr. Gary Walker, Committee Member Date

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

Abstract

Neurospora crassa, like most fungi, is very flexible metabolically. When a preferred carbon source, such as dextrose, is unavailable, *N. crassa* has the ability to metabolize quinic acid. To do this, the quinic acid gene cluster is up-regulated by the *qa-1F* activator. This study uses a mutant form of *N. crassa* in which the *qa-1F* gene is knocked out. The protein profiles of *N. crassa* wild-type and *qa-1F* knockout when grown on both dextrose and quinic acid were analyzed and compared.

The wild-type and knockout strains were first grown on 2% dextrose and then shifted either to fresh dextrose or 0.3% quinic acid. The proteins from these tissues were then extracted, quantitated, and separated using two-dimensional gel electrophoresis (2DGE). The 2DGE gels were then analyzed using PDQUEST™. Cross-conditional comparisons were made and protein spots unique to each condition were identified. These gel comparisons show that, when grown on a preferred carbon source, nearly twice as many proteins are up-regulated than when grown on quinic acid. Also, the *qa-1F* knockout protein profiles had far fewer protein spots than their wild-type counterparts for both carbon sources.

Protein spots were then selected, excised, and sent to the Ohio State University for mass spectrometry and bioinformatic analysis. Two proteins affected by the presence of *qa-1F* when grown on quinic acid were identified as hypothetical proteins NCU 04072 and NCU 08332 likely be a catechol dioxygenase and a translational protein SH3-like protein, respectively.

Acknowledgement

First and foremost, I would like to thank my advising professor, Dr. David Asch. He has led me through this experience with patience and wisdom. In addition to all of his help in the laboratory, he helped me through writing my thesis with countless revisions and suggestions. Without his guidance, I would not have been able to successfully complete my work in a timely fashion. I also owe many thanks to my committee members: Dr. Gary Walker, who gave me indispensable help and advice in the laboratory and Dr. Chet Cooper who assisted me in receiving the financial aid that was necessary to allow me to focus on my studies.

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Introduction

I. Kingdom Fungi

As early as 1783, Fungi has been described as its own kingdom (Feofilova 2001). Mycology, the study of fungi, has traditionally been regarded as a subfield of botany, the study of plants. Though fungi and plants seem to be analogous, research has shown a greater similarity between fungi and animals. In 1993, a comparison of sequences of minor subunits of ribosomal RNA suggested that fungi and animals share a common ancestor: a flagellate protist (Feofilova 2001). About 80,000 species have been described of an estimated 1.5 million total species (Lutzoni, et al. 2004). With habitats ranging from the tropics to Antarctica, and from mountain rocks to sea water, they are found all over the world; these organisms make up 90% of the invertebrate biomass. Fungi's effect on the planet impacts virtually all life by decomposing organic materials and forming symbiotic relationships with plants, animals, and prokaryotes (Galagan, Henn, et al. 2005). Understanding filamentous fungi is extremely important as they play vital roles in the chemical and medical industries as well as being human and plant pathogens (Kim, Nandakumar and Marten 2007). They may be sexual or asexual, haploid or diploid, and unicellular or multicellular. True fungi have cell walls through all stages of life (Norvell 2002). There are four major phyla of fungi: Chytidiomycota, Zygomycota, Basidiomycota, and Ascomycota. As a simple eukaryote, fungi often serve as a model organism for more advanced eukaryotes. Fungal genomes contain few introns and are easily sequenced (Galagan, Henn, et al. 2005). The number of genomes sequenced have increased exponentially over the recent years. To date, approximately fifty fungal

genomes have been or are in the process of being sequenced (http://www.broad.mit.edu/annotation/fgi/).

II. Phylum Ascomycota

The name "Ascomycota" was first used in 1957 (Hibbett, et al. 2007). Ascomycota is presently the largest phylum of fungi with over 64,000 known species. Ascomycota are found in virtually all ecosystems, accounting for 8% of the Earth's land masses (Schoch, et al. 2009). These fungi lack flagella and are largely filamentous. In addition to chitin, the cell walls contain the polyaminosaccharide galactosamine (Feofilova 2001).

Basidiomycota, Ascomycota's sister taxa, is approximately 60 million years younger (Feofilova 2001). These two phyla both display a dikaryotic stage of life, with some species producing macroscopic fruiting bodies. Examples of fruiting bodies include cup fungi, gilled mushrooms, and coral fungi (Lutzoni, et al. 2004). These are produced during sexual reproduction and house a large number of asci. Some members of this phylum reproduce asexually, in which case they produce reproductive spores called conidia.

III. Neurospora crassa

Neurospora was first documented in 1843. During the previous year, a warm, humid summer caused the bread in the bakeries of Paris, France to become infected with a bright orange mold. Scientists of the time discovered that, when left in the dark, the mold is white. However, within as little as two hours of exposure to light, the fungus adopts a bright, orange color. It is for this reason that *N. crassa* has been given the nickname "orange bread mold." Later experiments revealed that this color change is due to increased carotenoid production when exposed to light. *Neurospora's* heat tolerance was

also studied by the French microbiologists. In fact, their results were cited by Louis Pasteur in his work on mold spores. *N. crassa's* association with heat has been observed for years. Burned fields and areas affected by volcanic eruptions have reported a bright orange growth covering the area. New Guinea tribesmen would see *Neurospora* growth after setting hillsides on fire to trap wild game. In Brazil, orange mold growing on burned vegetables was observed. We now know that sexually produced ascospores remain dormant until activated by heat (Perkins 1992).

It was not until the early twentieth century that *Neurospora* really found its place in the laboratory. A plant physiologist named F.A.F.C. Went observed bright orange cakes called oncham in Java, Indonesia. The oncham is *Neurospora crassa* grown on a peanut or soybean base. Went found this same fungus being used in Surinam to make an alcoholic beverage. Went returned to his homeland in the Netherlands and used *N. crassa* as a model system to study various enzymes.

In 1927, Bernard Dodge and C.L. Shear from the Department of Agriculture mycology and pathology laboratory in Virginia published a paper assigning this organism to a new genus. It previously held a few different labels, but upon discovery of their grooved ascospores, the organism found its proper home in *Neurospora.*

Several characteristics make *N. crassa* a great experimental organism. For example, it has a very high linear growth rate, as high as four millimeters an hour! Nutrition requirements are simple and were quickly identified (Perkins 1992). This multicellular, filamentous fungus paved the way for advancements in genetics and molecular biology. Work on this haploid organism led to the "one gene, one enzyme" hypothesis by Beadle and Tatum which states, as its name implies, that an enzyme is coded for by one

particular gene (Galagan, Calvo, et al. 2003). Around the same time this theory was being unraveled, Barbara McClintok revealed, using simple light microscopy, that the seven chromosomes of *Neurospora* were strikingly similar to those of plants and animals. This fungus was found to be eukaryote. This discovery quickly lead to studies and subsequent understanding of fungal chromosomes and meiosis (Perkins 1992).

As more was understood about mutants, fungi, and genetics, characteristics that once seemed unique to *Neurospora* were discovered in several other fungi. In spite of this, *N. crassa* remained a topic of interest. For example, *Neurospora* was used in gene recombination studies in the 1950s and gene conversion studies in the 1960s (Perkins 1992). In the 2000s, the *N. crassa* genome was completely sequenced. Many of the predicted proteins could not be matched when compared to genes known in yeast and other multicellular organisms. The genome is 43 megabases long with about 10,000 protein-coding genes. The average gene size is 1,673 basepairs. There is approximately 1 gene to every 3,700 basepairs. The genome has a 48% G-C content (Galagan, Calvo, et al. 2003, N. crassa Genome Statistics 2012).

IV. Carbon Source Metabolism

Metabolism describes all the chemical processes an organism uses to maintain life. Primary metabolism, prominent in the exponential growth phase of fungi, is predominantly involved in growth. This includes production of energy, nucleic acids, lipids, proteins, and carbohydrates. Secondary metabolism is not essential for growth. Its role, though less well defined, must play an important part in the organism's life in other aspects (Carlile, Watkinson and Gooday 2001).

Most fungi can metabolize sugars, such as dextrose, fairly easily. This is true for *Neurospora* via the usual metabolic pathways such as the glycolysis or the TCA cycle. An active transport system moves dextrose into the cell when it is in low concentration. At higher concentrations, the transport across the cell membrane is carried out by facilitated diffusion. When an ideal carbon source is unavailable, fungi will be forced to catabolize other sources of carbon, such as polysaccharides, hydrocarbons, lipids, or organic acids. Note, however, that not all fungi can metabolize all various forms of carbon sources.

V. Quinic Acid

In 1932, H.O.L. Fisher and G. Dangschat discovered the structure and stereochemistry of quinic acid, as depicted in figure 1 (Barco, et al. 1997). Quinic acid has the chemical formula $C_7H_{12}O_6$ and a molecular weight of 192.17 g/mol (CID: 6508 National Center for Biotechnology Information). It is quite common in the plant kingdom in dead vegetation. This aromatic molecule functions as a intermediate for secondary metabolites. It has been linked to regulation of the shikimate pathway. Quinic acid can also serve as a seconday, non-preferred carbon source for organisms capable of converting it to 3-dehydroquinate, such as *K. pneumonia* and *N. crassa* (Barco, et al. 1997). This is due to the quinate/shikimate pathway. Quinic acid or shikimate can be used as a sole carbon source by catabolising to protocatechuic acid which is then metabolised to succinate and acetate (Geever, et al. 1987).

VI. Quinic Acid Gene Cluster

The quinic acid genes are tightly clustered near one another on chromosome seven spanning over 17 kb in *Neurospora crassa*. Quinic acid or shikimic acid acts as an

inducer while glucose acts as a repressor. The gene cluster is made up of seven genes: two regulatory and five structural. Transcription takes place in varying directions as depicted in figure 2 (Giles, et al. 1991). Of the five structural genes, the three enzyme-coding genes are *qa-2*: catabolic dehydroquinase, *qa-3*: quiniate (or shikimate) dehydrogenase, and *qa-4*: dehydroshikimate dehydrase. The remaining two structural genes are *qa-x* of unknown function and *qa-y,* a quinate permease. The regulatory genes are *qa-1S* and *qa-1F* (Davis 2000).

VII. Quinic Acid Gene Cluster Regulation

When *Neurospora crassa* has access to glucose or another ideal sugar carbon source, transcription of the quinic acid genes is virtually nonexistent. The presence of quinic acid results in transcription of the genes. However, if quinic acid is present along with a preferred carbon source, the quinic acid metabolism will take a back seat to the favorable carbon source. This is accomplished by directly repressing the *qa-y* permease gene (Arnett, Lorimer and Asch 2009). Transcription of the quinic acid gene cluster remains at basal levels to allow the ideal carbon course to be utilized as long as possible. When quinic acid is the sole carbon source, the level of transcription is greatly increased and the enzymes are highly induced (Davis 2000). Only mutations in *qa-1F* result in very low levels of transcription (Geever, et al. 1987). The repressor protein, encoded by *qa-1S*, inhibits the activator by binding to the *qa-1F* activator protein. If the inducer (quinic acid) is present, it binds to the repressor gene and frees the activator. *Qa-1F* controls the expression of all genes in the cluster, including itself. The activator binds upstream to a symmetrical 16 base pair segment, activating the genes. There is a conserved DNA-

Figure 1: 2D Structure of Quinic Acid (National Center for Biotechnology Information, U.S. National Library of Medicine)

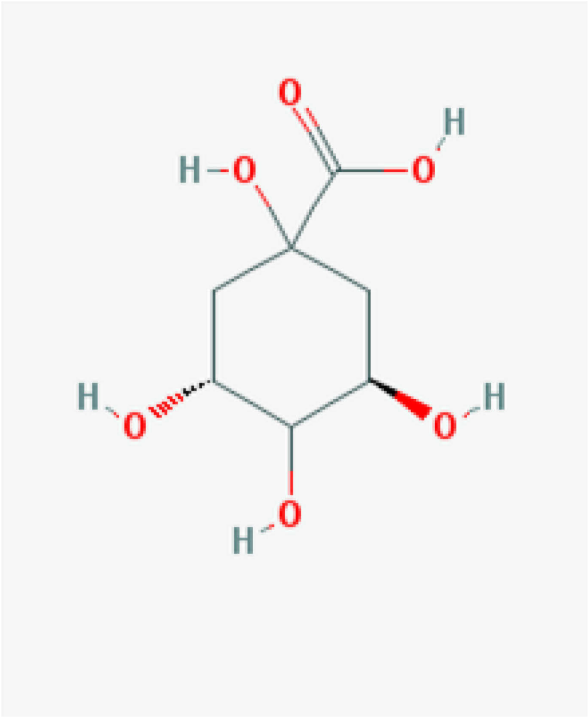
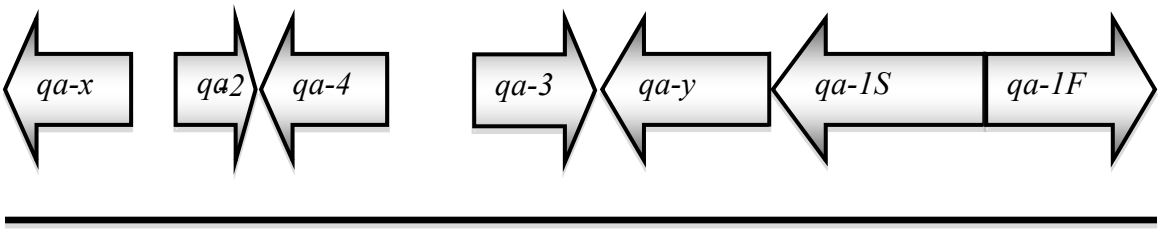

O
H
H
O
O
H
O
H
O
H
O
H

Figure 2: Quinic acid gene cluster (Giles, et al. 1991)


qa-x
qa2
qa-4
qa-3
qa-y
qa-1S
qa-1F

binding motif consisting of 28 amino acids that form a 6 cysteine zinc-binding motif located in the N-terminal region of the activator (Giles, et al. 1991). The whole *QA-1F* protein consists of 816 amino acids (Geever, et al. 1987).

VIII. Quinic Acid Catabolism

When quinic acid enters the cell, quinic acid dehydrogenase (*qa-3)* removes the hydrogen from the alcohol group attached to carbon 5 (see figure 3 for carbon numbers). This creates a double bond between the remaining oxygen and carbon 5. This new structure is known as dehydroquinic acid (DHQ). Catabolic dehydroquinase (*qa-2*) creates a reversible reaction transforming DHQ to dehydroshikimic acid (DHS). This is done by removing the alcohol group from carbon 1, thus creating a double bond between carbons 1 and 6. DHS dehydrase (*qa-4*) removes the hydrogen from carbon 2 and the alcohol group from carbon 3. By creating this water molecule, a double bond forms between carbons 2 and 3. DHS dehydrase also adds a hydrogen to the double bonded oxygen at carbon 5. This creates a single bond between the newly formed alcohol group and carbon 5 while simultaneously forming a double bond between carbons 4 and 5. This results in the structure Protocatechuic acid (PCA). After PCA is metabolized to succinate or acetate, the cell can finally utilize the molecule in general carbon metabolism, such as the TCA cycle (Geever, et al. 1987). This process is depicted in figure 4.

IX. *Qa-1F* Knockout Mutant

Neurospora returns a low frequency of homologous recombination after transformation. This has made gene knockouts historically rather difficult. *Qa-1F* is one of the few transcription factors studied in *Neurospora* (Colot, et al. 2006). Strain number

Figure 3: Quinic Acid with labeled carbons (ScienceDirect.com)

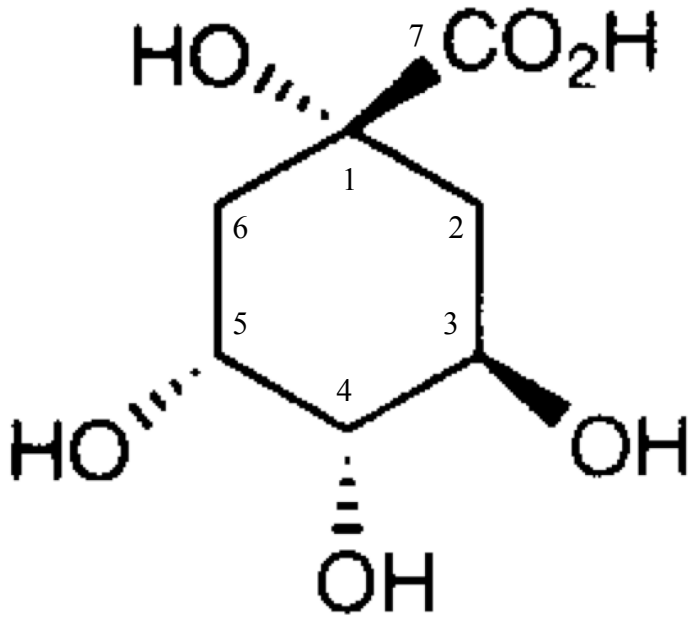
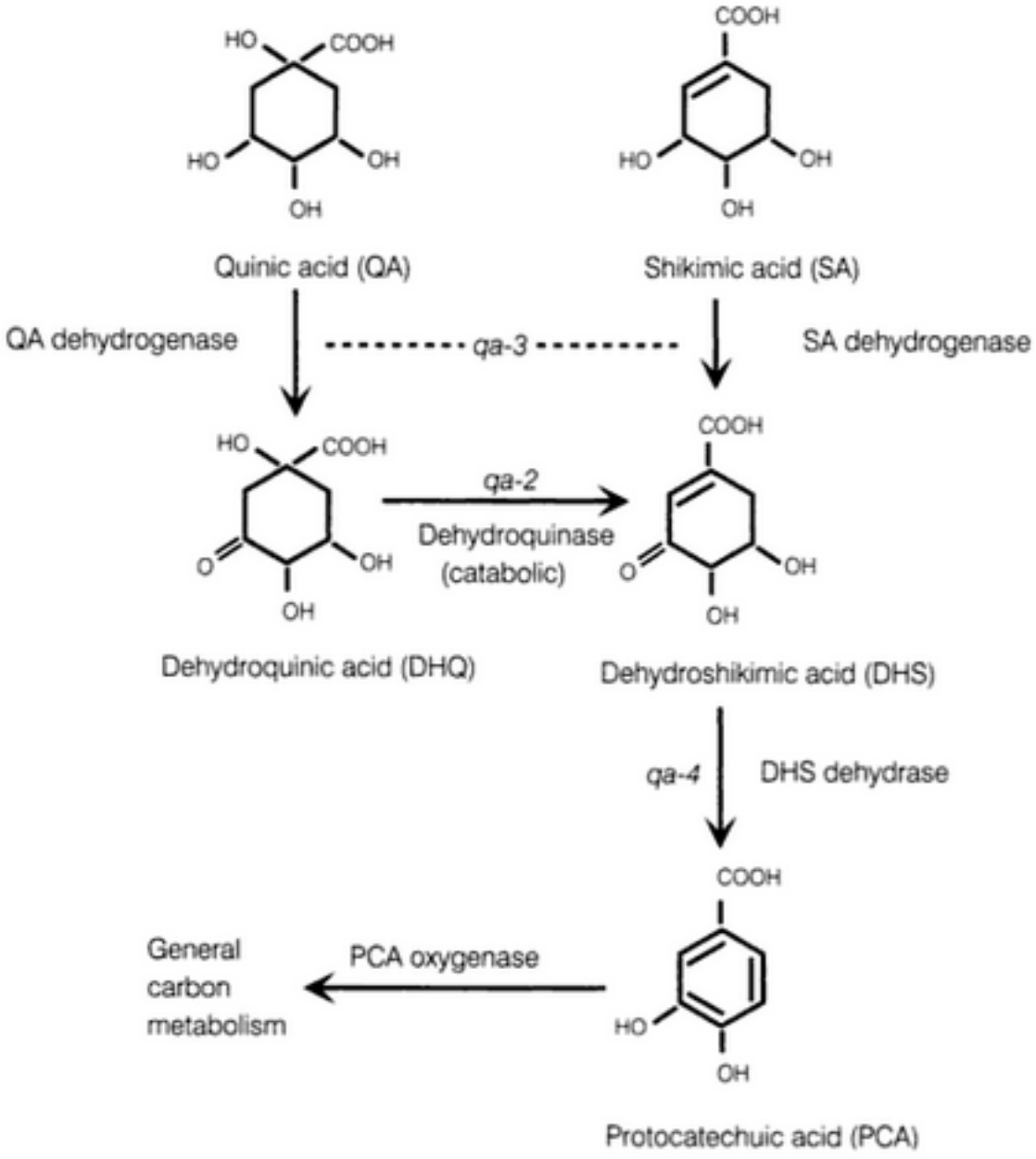

HO
7
CO2H
1
6
2
5
3
4
HO
OH
OH

Figure 4: Quinic acid catabolism pathway (David, 2000)

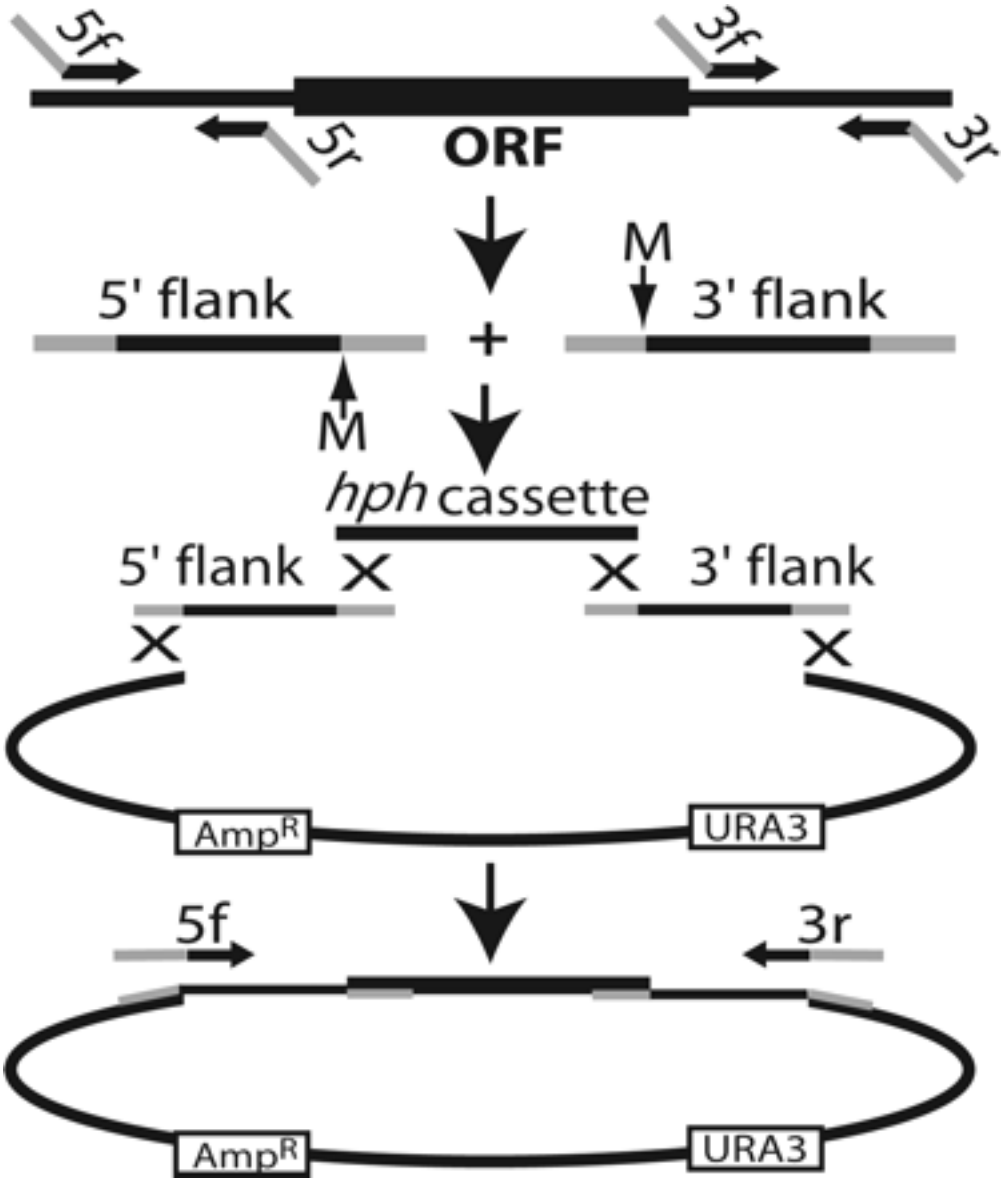

Quinic acid (QA)
Shikimic acid (SA)
QA dehydrogenase
qa-3
SA dehydrogenase
qa-2
Dehydroquinase
(catabolic)
Dehydroquinic acid (DHQ)
Dehydroshikimic acid (DHS)
qa-4
DHS dehydrase
General
carbon
metabolism
PCA oxygenase
Protocatechuic acid (PCA)

11034 (obtained from the Fungal Genetics Stock Center (FGSC)) was the *qa-1F* knockout mutant used in this study. The method for creating a deletion mutant is quite straightforward, as described in figure 5. First, the regions on either side of the target open reading frame (ORF) are amplified using polymerase chain reaction (PCR). The sides of the amplified fragments that originally faced the target ORF are made to incorporate *Mme*I sites that allow the fragments to bind to the *hph* cassette that is being inserted in place of the target gene being deleted. The *hph* is a widely used *trpC* promoter-*hph* cassette from *Aspergillus nidulans*. The opposite ends of the replicated fragments are made to be homologous to the yeast vector. The flanking fragments and *hph* cassette are then inserted into the yeast vector. Homologous recombination creates a circular construct. The linear deletion cassette is amplified using PCR and a split-marker technique results in the linear deletion cassette that is cotransformed into *Neurospora crassa* (Colot, et al. 2006).

X. Fungal Proteomics

Proteomics is the systematic analysis of the proteome. The proteome is the complete protein compliment to an organism's genome. Fungal proteomics aims to understand the expression, regulation, and function of fungal proteins (Bhadauria, et al. 2007). Due to the advances in proteomics in the recent years, there are a multitude of proteins that have been identified while their functions remain unknown. There are five main methods used to discover the proteins' functions: comparative proteomics, protein localization, coculture proteomics, *in silico* analysis, and immunoproteomics. Gene deletion studies, in which a mutant is designed to be missing a particular gene, is a common form of a

Figure 5: Method for creating deletion mutants (Colot, et al. 2006)


5f
3f
5r
ORF
3r
M
5' flank
3' flank
+
M
hph cassette
5' flank
3' flank
Amp^R
URA3
5f
3r
Amp^R
URA3

comparative study. The mutant is compared to the wild type to see what effect the mutant has on the proteome of the fungus. Comparative proteomics may also describe an analysis of the proteome after exposure to toxins (Doyle 2011). Protein localization is the study of the specific location of a protein within the cell. This is accomplished by tagging the protein's corresponding gene at either the N or C terminus. The terminus selected varies by the target gene as the tag must not interfere with the protein's function (Bhadauria, et al. 2007). Coculture proteomics refers to the analysis of protein expression when an organism shares a culture with a likely environmental partner. In silico refers to computer analysis and/or simulation. Popular in silico analyses include motif/domain structures and post-translational modification. Immunoproteomics researches the proteins involved in immune reactions. Mass spectrometry paired with immunoblotting is the most current method of studying immunoreactive fungal antigens (Doyle 2011).

XI. Qa-1F Responsive Genes

Previous studies have shown over 800 genes bear a *qa-1F* binding site. About half of these are of unknown function (Logan, et al. 2007). As shown by microarray analyses, 50 of these may be directly controlled by the *qa-1F* activator protein (Tang, et al. 2011). This is quite different from the original belief that the *QA-1F* protein only activated the 7 genes of the quinic acid gene cluster. We now know there are dozens, possibly hundreds, of genes impacted by the presence of quinic acid in the cell and, more specifically, *qa-1F*.

Materials and Methods

I. Strain

Wild-type *Neurospora crassa* 74A (Fungal Genetics Stock Center (FGSC) #2489) strain was obtained from laboratory stock collection. *Qa-1F* knockout *Neurospora crassa* mutant (FGSC# 11034) was obtained from the FGSC.

II. Tissue Growth

Neurospora crassa was inoculated on 50 ml Vogel's Minimal Media (1.5% Agar, 2% Sucrose, 1x Vogel's salts) in 250 ml Erlenmeyer flasks (Table 1). The flasks containing the *N. crassa* were grown at 30°C in an incubator two to three days. The flasks were then moved to fluorescent light at room temperature for two weeks. The conidia was harvested by adding 25 ml of 1 x Vogel's to each flask and swirling. The suspension was then poured through a sterile cheesecloth. The suspension that flowed through the cheesecloth was divided into two equal parts. Each half of the suspension was added to 50 ml 1 x Vogel's, 2% dextrose in 250 ml Erlenmeyer flasks. The cultures were grown for overnight at 30°C on an orbital shaker at 115 rpm. The mycelia from the overnight cultures were then collected by vacuum filtration through Whatman® filter paper. The mycelial pads were then transferred to a 250 ml Erlenmeyer flask containing 50 ml of sterile, distilled water and swirled. The washed mycelial pads were then refiltered, collected, and transferred to a medium of 50 ml 1 x Vogel's, 2% dextrose. The same procedure was carried out with the other culture from the overnight incubation; however, the mycelial pad were shifted to a medium of 50 ml 1 x Vogel's, 0.3% quinic acid. The cultures were then incubated for 3 hours on an orbital shaker at 30°C, 115 rpm. The mycelia were again collected by vacuum filtration and stored at -80°C.

Table 1: 50x Vogel's Minimal Media (1 Liter)

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

Table 2: Trace Element Solution

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

Table 3: Biotin Solution (50 ml)

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

III. Protein Isolation

The frozen mycelial pads were ground using liquid nitrogen, baked sand, and a mortar and pestle. The ground tissue was transferred to 1.5 ml Eppendorf tubes until the tube was approximately half full. Lysis buffer (760 µl lysis buffer solution (200 mM Tris-HCl, 10 mM NaCl, 0.5 mM deoxycholate) plus 40 µl liquid Promega Protease Inhibitor) was added to each tube. The tubes were vortexed for 60 seconds and iced for 2 minutes. The vortex/ice process was repeated 3 additional times. The tubes were centrifuged for 10 minutes at 12,000 rpm at 4°C. The supernatant was collected and transferred to a new, sterile 1.5 ml Eppendorf tube. To remove any nucleotides from the proteins, the samples were sonicated at power 5 for 10 seconds followed by a 5 second break three times, consecutively. The samples were stored at -20°C.

IV. Protein Check

Test gels (12%) were prepared to check the presence of protein in the isolation samples (Table 4). A small amount of butanol was added to the mini gel mold tank, filling from the bottom, to help the acrylamide solution stay level. The acrylamide solution was added to the tank, still filling from the bottom, to the appropriate height. The gels solidified overnight. A stacking gel was added to the top of the gel using a Pasteur pipette and a comb was inserted to form the wells when the stacking gel solidifies (Table 5). After the gel solidifiied for 10-20 minutes, the comb was removed.

Ten µl of each protein sample was combined with 10 µl 2x SDS-PAGE buffer. The new solutions were then loaded into the wells of the gel. The gel was loaded into an electrophoresis tank that was filled with TGS buffer to the fill-line. The electrophoresis ran at 100 volts for about 1 hour. The gel was removed from the tank and the mold was

Table 4: 12% SDS-PAGE Test Gels

Distilled H_2O	44 ml
40% Acrylamide	30 ml
Gel Buffer Solution (1.5 M Tris + 10% SDS)	25 ml
10% Ammonium Persulfate	1 ml
TEMED	0.04 ml

Distilled H_2O	5.9 ml
40% Acrylamide	1.5 ml
Stacking Gel Buffer Solution (1.5 M Tris + 10 % SDS)	2.5 ml
10% Ammonium Persulfate	0.1 ml
TEMED	0.008 ml

opened to extract the gel. The gel sat in Bio-Rad® 0.4% coomassie stain on an orbital shaker for 2 hours. The gel was then transferred to a high-destain solution for 1 hour. The gel was transferred to low destain (10% methanol, 6% acetic acid) for additional time if necessary to clearly see protein bands.

V. Protein Quantitation

Ten µl of each protein sample was combined with 10 µl 2x SDS-PAGE buffer. These samples were then loaded into a 12% Criterion™TGX™ Precast Gel from Bio-Rad® with 18 wells. Ten µl of Amresco® Blue-Step Protein Marker (Broad Range) was loaded onto the first well of the gel, followed by BSA standards (200 ng, 400 ng, 600 ng, 800 ng, and 1000 ng), followed again by another well of the ladder, followed by the protein samples. The protein samples were loaded with 2x SDS-PAGE buffer in a 1:1 ratio; 10 µl of protein sample plus 10 µl of 2x SDS-PAGE. Any remaining wells were left empty. The gel was loaded into the Criterion™ electrophoresis tank. The tank was filled with TGS buffer to the fill line. The inner buffer well of the gel mold was also filled with TGS buffer. The electrophoresis ran at 100 volts for approximately one hour (until the dye front reaches a centimeter above the bottom of the gel). The gel was then broken out of the case and stained in Bio-Rad® 0.4% coomassie stain for 2 hours on an orbital shaker. The gel sat in a high destain (40% methanol, 10% acetic acid) for one hour. The gel was photographed using Bio-Rad® Pharos FX™ Plus Molecular Imager and the Bio-Rad® Quantity One® software program. Using ImageJ software, the image was analyzed and the protein was quantitated.

VI. Two-Dimensional Gel Electrophoresis

Neurospora crassa protein samples (100 ug) were transferred to a sterile microfuge tube. Enough modified sample buffer (MSB) rehydration buffer (7M urea, 2M thiourea, 4% CHAPS, 1% DTT) was added to bring the sample to 200 µl. Bio-Rad® Electrode Wicks were placed onto of the electrodes at either end of the wells in a Protean® IEF tray. A drop of water was added to the wicks to moisten them. The samples were loaded into the wells of the tray and 11 cm pH 5-8 Bio-Rad® ReadyStrip™ IPG strips were placed gel-side down on top of the samples, making sure to align the positive and negative ends of the strip with the positive and negative electrodes of the IEF tray. The gel strips sat like this for 45 minutes to absorb the protein. Three ml of mineral oil was added to the wells, on top of the strips. The IEF tray were then covered with its plastic lid and the covered tray was then placed into the Bio-Rad® Protean® IEF Cell. The IEF cell was switched on and programmed to the linear, preset method for passive rehydration of an 11 cm strip with a focus temperature of 20°C. The focus hours was 20-35,000 volt-hours with a 500 volt hold. The number of strips in the IEF tray was entered and the program was set to run.

The following day, once the program was complete, the IEF tray was removed from the IEF cell. The gel strips were moved from the tray and held vertically over a paper towel to allow the mineral oil to drain. The down-ward facing end of the strip was touched to the paper towel to attempt to gently remove as much mineral oil as possible. The strips were placed in an equilibration tray gel-side up. The wells with gel strips were filled with equilibration buffer I (6M urea, 2% SDS, 0.375M Tris-HCL (pH 8.8), 20% glycerol, 2% DTT) and the tray was placed on an orbital shaker for 10 minutes. The

equilibration buffer I was drained from the tray, tapping the tray to remove as much as possible. Equilibration buffer II (6M urea, 2% SDS, 0.375M Tris-HCL (pH 8.8), 20% glycerol, 2.5% iodoacetamide) was then added to the wells. The tray was returned to the orbital shaker for an additional 10 minutes. The equilibration buffer II was drained from the tray, tapping the tray to remove as much as possible. The strips were dipped into a 25 ml graduated cylinder filled with enough 1x TGS buffer. The excess buffer was allowed to drip off of the gel strip onto a paper towel before loading the strip into the 8-16% Criterion™TGX™ precast gel – 11 cm IPG + 1 well comb. The positive end of the gel strip was loaded towards the ladder well in the gel to maintain symmetry across the gels. A layer of liquid overlay agarose (0.5 g agarose, 100 ml 1x TGS buffer, 1 grain bromophenol blue) was layered on top to the gel strip in the well, making sure to leave the small well for the ladder empty. Once this solidified (after 10-20 minutes), the gel was loaded into the Criterion™ Electrophoresis tank. The tank was filled with 1x TGS buffer to the fill line. The inner buffer well of the gel mold was also filled with 1x TGS buffer. Five ul Amresco® Protein Molecular Weight Marker, Wide Range was loaded into the small well of the gel. The electrophoresis ran at a 40 amps per gel constant for an average of 1 hour and 25 minutes.

After the electrophoresis is complete, the Criterion™ gels were cracked out of their plastic molds and placed in fixing solution (40% methanol, 10% acetic acid) on an orbital shaker at room temperature for 1 hour. The fixing solution was then removed from the container and enough SYPRO® stain was added to the container to cover the gels. The gels sat in the SYPRO® stain on an orbital shaker at room temperature overnight before

being transferred to deionized H_2O for 20 minutes before imaging. The gels were stored in 5% acetic acid.

VII. 2D Gel Imaging and Analysis

Using Bio-Rad® PharosFX™ Plus Molecular Imager, gels were scanned one at a time into the Bio-Rad® Quantity One (version 4.6.9) software program. Using PD-QUEST (version 7.3) software, the images were compared to one another to detect variations in protein spots.

VIII. Protein Spot Excision

In order to see the spots of interest, the gels were placed in Coomassie stain for 1 hour 30 minutes on an orbital shaker in plastic containers. The stain was then removed from the containers and replaced with high destain. The gels sat in the high destain on an orbital shaker for another 1 hour 30 minutes. The high destain was drained and replaced with low destain. The gels sat in the low destain on an orbital shaker over night. The next day, the gels were allowed to rehydrate in distilled water for 10 minutes. The gels were placed one at a time on a clean, white surface and the protein spots of interest were excised using sterile disposable glass Pasteur pipettes. The gel spots were placed into a sterile eppendorf tube containing 5% acetic acid. The tubes were sent to Ohio State University for mass spectrometry analysis.

IX. In Gel Digestion

The Ohio State University Mass Spectrometry and Proteomics Facility carried out the following procedure. Gels were digested with sequencing grade trypsin from Promega (Madison WI) or sequencing grade chymotrypsin from Roche T (Indianapolis, IN) using

the Multiscreen Solvinert Filter Plates from Millipore (Bedford, MA). Briefly, bands were trimmed as close as possible to minimize background polyacrylamide material. Gel pieces are then washed in nanopure water for 5 minutes. The wash step is repeated twice before gel pieces are washed and or destained with 1:1 v/v methanol:50 mM ammonium bicarbonate for ten miuntes twice. The gel pieces were dehydrated with 1:1 v/v acetonitrile: 50 mM ammonium bicarobonate. The gel bands were rehydrated and incubated with dithiothreitol (DTT) solution (25 mM in 100 mM ammonium bicarbonate) for 30 minute prior to the addition of 55 mM Iodoacetamide in 100 mM ammonium bicarbonate solution. Iodoacetamide was incubated with the gel bands in dark for 30 min before removed. The gel bands were washed again with two cycles of water and dehydrated with 1:1 v/v acetonitrile: 50 mM ammonium bicarobonate. The protease is driven into the gel pieces by rehydrating them in 12 ng/ml trypsin in 0.01% ProteaseMAX Surfactant for 5 minutes. The gel piece is then overlaid with 40 ml of 0.01% ProteaseMAX surfactant:50 mM ammonium bicarbonate and gently mixed on a shaker for 1 hour. The digestion is stopped with addition of 0.5% TFA. The MS analysis is immediately performed to ensure high quality tryptic peptides with minimal non-specific cleavage or frozen at -80oC until samples can be analyzed.

X. Mass Spectrometry

The Ohio State University Mass Spectrometry and Proteomics Facility then performed mass spectrometry analysis via the following procedure. Capillary-liquid chromatography tandem mass spectrometry (Cap-LC/MS/MS) was performed on a Thermo Finnigan LTQ mass spectrometer equipped with a CaptiveSpray source (Bruker Michrom Billerica, MA) operated in positive ion mode. The LC system was an

UltiMate™ 3000 system from Dionex (Sunnyvale, CA). The solvent A was water containing 50mM acetic acid and the solvent B was acetonitrile. 5 mL of each sample was first injected on to the m-Precolumn Cartridge (Dionex, Sunnyvale, CA), and washed with 50 mM acetic acid. The injector port was switched to inject and the peptides were eluted off of the trap onto the column. A 0.2x150mm, 3u, 200A, Magic C18 (Bruker Michrom Billerica, MA) was used for chromatographic separations. Peptides were eluted directly off the column into the LTQ system using a gradient of 2-80%B over 45 minutes, with a flow rate of 2ul/min. The total run time was 65 minutes. The MS/MS was acquired according to standard conditions established in the lab. Briefly, a CaptiveSpray source operated with a spray voltage of 3 KV and a capillary temperature of 200PoPC is used. The scan sequence of the mass spectrometer was based on the TopTen™ method; the analysis was programmed for a full scan recorded between 350 – 2000 Da, and a MS/MS scan to generate product ion spectra to determine amino acid sequence in consecutive instrument scans of the ten most abundant peak in the spectrum. The AGC Target ion number was set at 30000 ions for full scan and 10000 ions for MSn mode. Maximum ion injection time was set at 20 ms for full scan and 300 ms for MSn mode. Micro scan number was set at 1 for both full scan and MSn scan. The CID fragmentation energy was set to 35%. Dynamic exclusion was enabled with a repeat count of 2 within 10 seconds, a mass list size of 200, an exclusion duration 350 seconds, the low mass width was 0.5 and the high mass width was 1.5.

Sequence information from the MS/MS data was processed by converting the .raw files into a merged file (.mgf) using an in-house program, RAW2MZXML_n_MGF_batch (merge.pl, a Perl script). The resulting mgf files were

searched using Mascot Daemon by Matrix Science version 2.3.2 (Boston, MA) and the database searched against the full SwissProt database version 2012_06 (536,489 sequences; 190,389,898 residues) or NCBI database version 20120515 (18,099,548 sequences; 6,208,559,787 residues). The mass accuracy of the precursor ions were set to 1.8 Da and the fragment mass accuracy was set to 0.8 Da. Considered variable modifications were methionine oxidation and deamidation NQ. Fixed modification for carbamidomethyl cysteine was considered. Two missed cleavages for the enzyme were permitted. A decoy database was searched to determine the false discovery rate (FDR) and peptides were filtered according to the to the FDR and proteins identified required bold red peptides. Protein identifications were checked manually and proteins with a Mascot score of 50 or higher with a minimum of two unique peptides from one protein having a -*b* or -*y* ion sequence tag of five residues or better were accepted.

Results

The purpose of this study was to identify the difference in the proteome of *Neurospora crassa* when grown on the non-preferred carbon source quinic acid and, more specifically, which proteins are dependent on activation by the *qa-1F* gene. This was accomplished by first acquiring a knockout mutant of *N. crassa* in which the *qa-1F* gene was removed (Colot, et al. 2006). Both the wild-type (74A) and the *qa-1F* knockout mutant were grown under standard conditions (see materials and methods). The mycelia of both strains were harvested and grown on a 2% dextrose media overnight. The conidia was then transferred to either new 2% dextrose media or to 0.3% quinic acid media to grow to three hours. As described in the Materials and Methods section, the protein was then extracted. In order to be sure the protein was extracted properly and present in samples, 12% acrylamide test gels were used in one-dimensional gel electrophoresis (1DGE) and stained with coomassie stain (figure 6). If present, the protein was then quantitated via 1DGE on a Criterion™TGX™ Precast 8-16% gel. The gel was stained with SYPRO®, scanned with the Bio-Rad® PharosFX™ Plus Molecular Imager, and analyzed with ImageJ software (figure 7). Once the amount of protein in each sample was known, protein profiles were created via two-dimensional gel electrophoresis (2DGE). The protein samples used were as follows: dextrose wild-type sample #2, dextrose *qa-1F* knockout sample #1, quinic acid wild-type sample #1, and quinic acid *qa-1F* knockout sample #1. These were done using 11 cm Bio-Rad ReadyStrips™ IPG strips pH 5-8 and Bio-Rad Criterion™TGX™ Precast Gels 8-16% acrylamide in triplicate. These gels were all stained in SYPRO® stain and scanned with the Bio-Rad® PharosFX™ Plus Molecular Imager (figures 8-11).

Figure 6: Test gel (12% acrylamide) of *Neurospora crassa* protein grown on either 2% dextrose or 0.3% quinic acid. Sample load indicated on image.

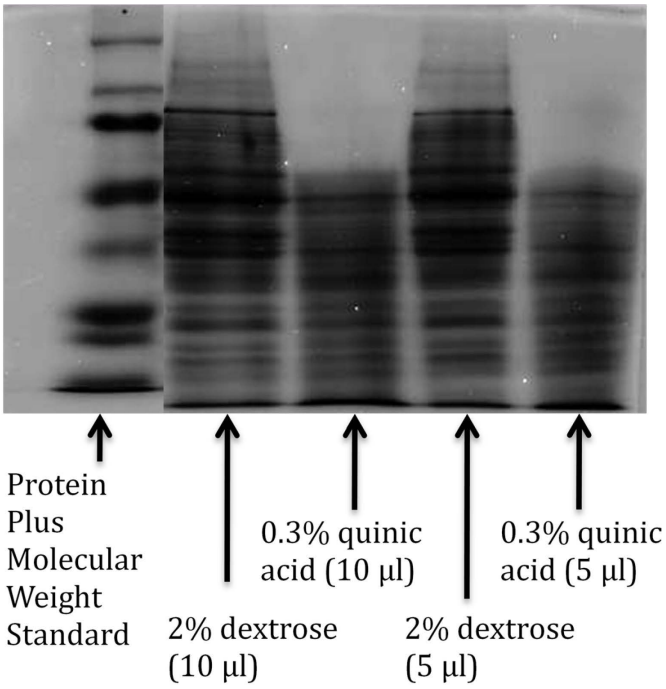

Protein
Plus
Molecular
Weight
Standard
2% dextrose
(10 µl)
0.3% quinic
acid (10 µl)
2% dextrose
(5 µl)
0.3% quinic
acid (5 µl)

Figure 7: Coomassie stained Criterion™TGX™ Precast 8-16% gel quatitation 1DGE gel of *N. crassa* grown on 2% dextrose or 0.3% quinic acid.

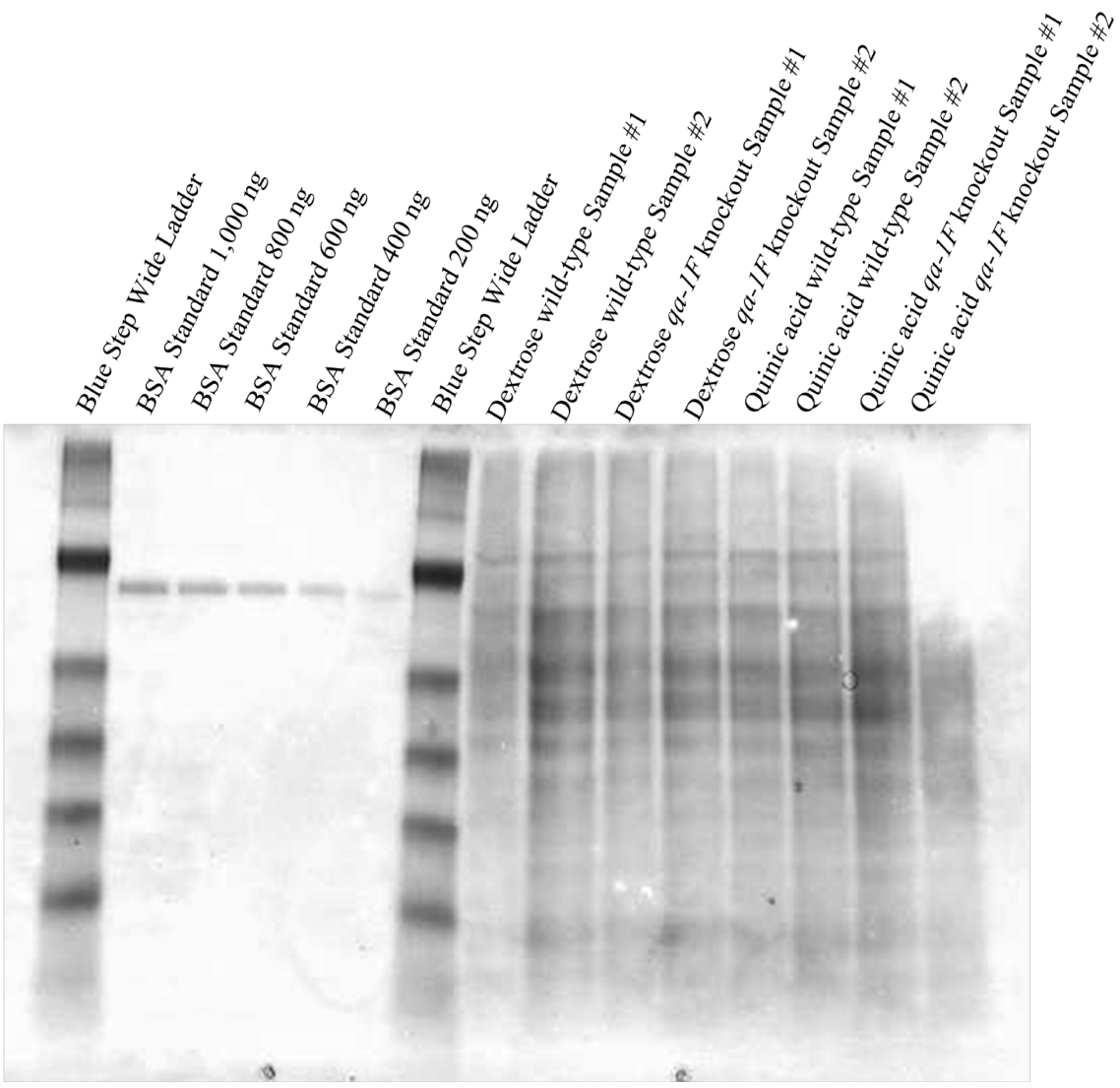
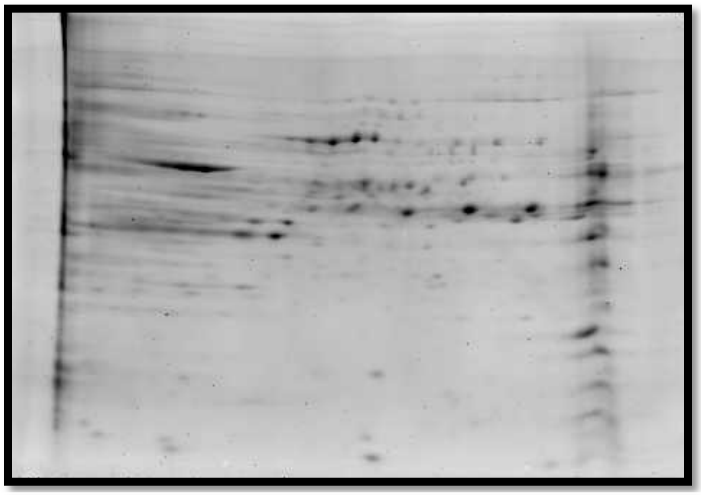
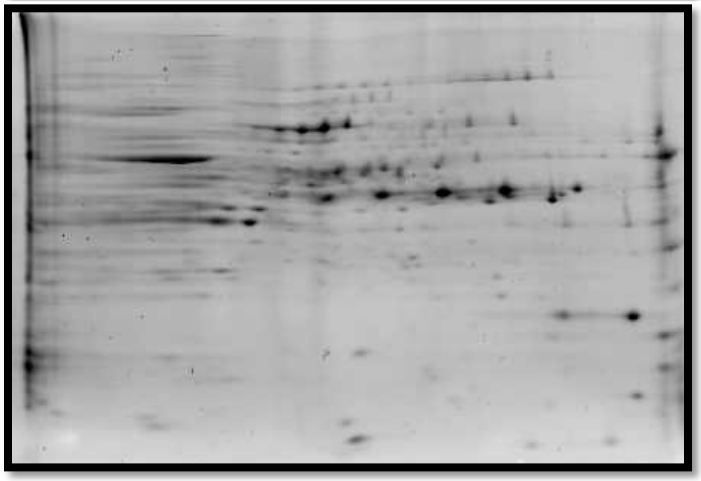

Blue Step Wide Ladder
BSA Standard 1,000 ng
BSA Standard 800 ng
BSA Standard 600 ng
BSA Standard 400 ng
BSA Standard 200 ng
Blue Step Wide Ladder
Dextrose wild-type Sample #1
Dextrose wild-type Sample #2
Dextrose qa-1F knockout Sample #1
Dextrose qa-1F knockout Sample #2
Quinic acid wild-type Sample #1
Quinic acid wild-type Sample #2
Quinic acid qa-1F knockout Sample #1
Quinic acid qa-1F knockout Sample #2

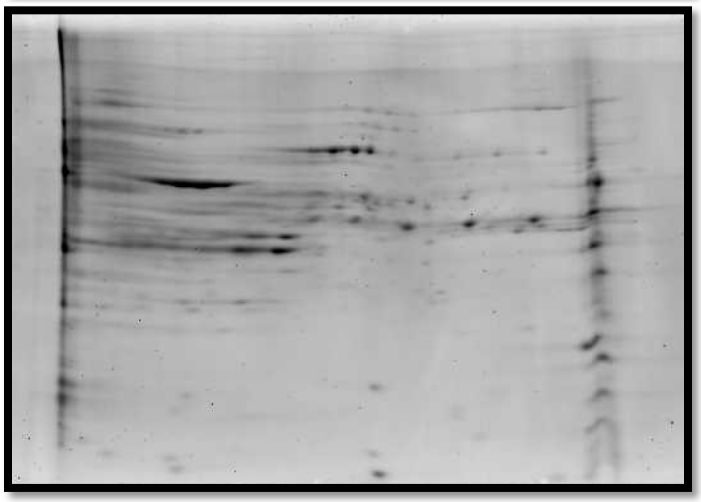
Figure 8: Criterion™TGX™ Precast 8-16% gels (length = 11 cm; pI 5-8) stained in SYPRO of wild-type *N. crassa* proteins grown on 2% dextrose resolved in two-dimensional gel electrophoresis. Gels were done in triplicate.



Dextrose wild-type gel A

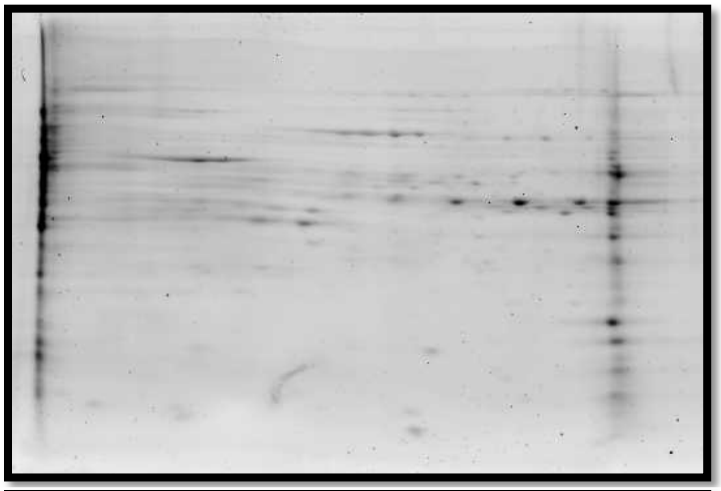


Dextrose wild-type gel B

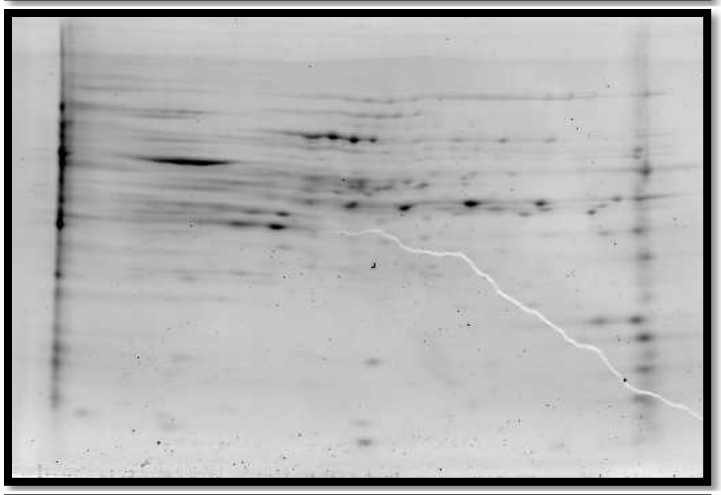


Dextrose wild-type gel C

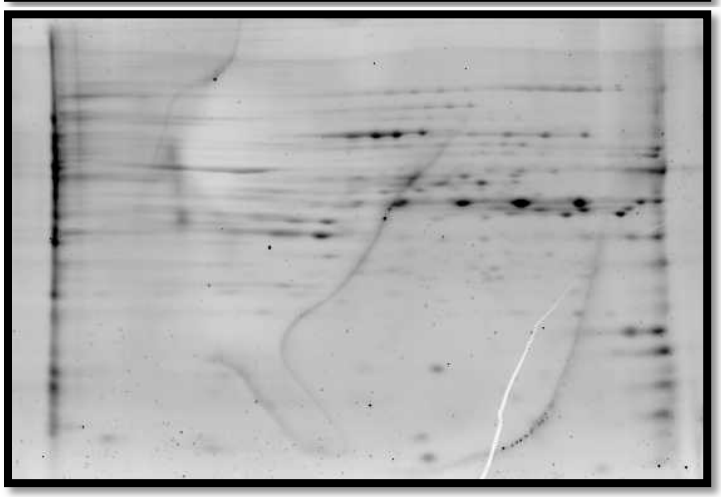
Figure 9: Criterion™TGX™ Precast 8-16% gels (length = 11 cm; pI 5-8) stained in SYPRO of *qa-1F* knockout *N. crassa* proteins grown on 2% dextrose resolved in two-dimensional gel electrophoresis. Gels were done in triplicate.



Dextrose *qa-1F* knockout gel A

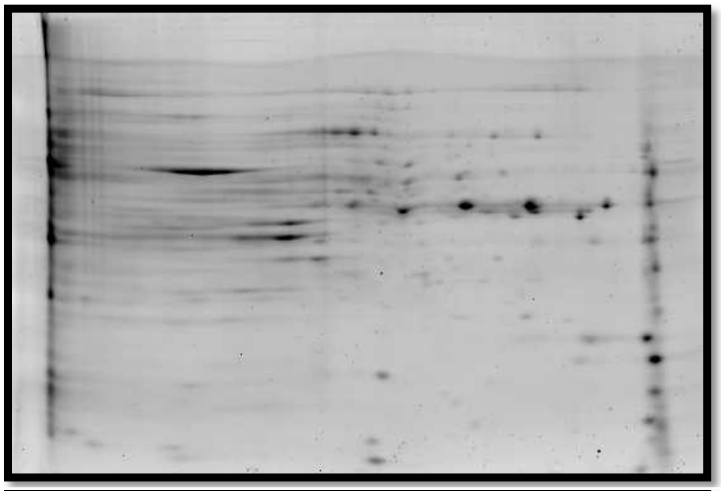


Dextrose *qa-1F* knockout gel B

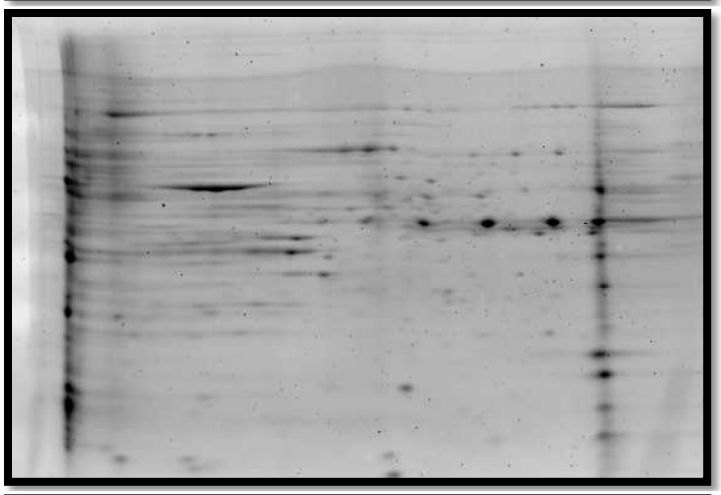


Dextrose *qa-1F* knockout gel C

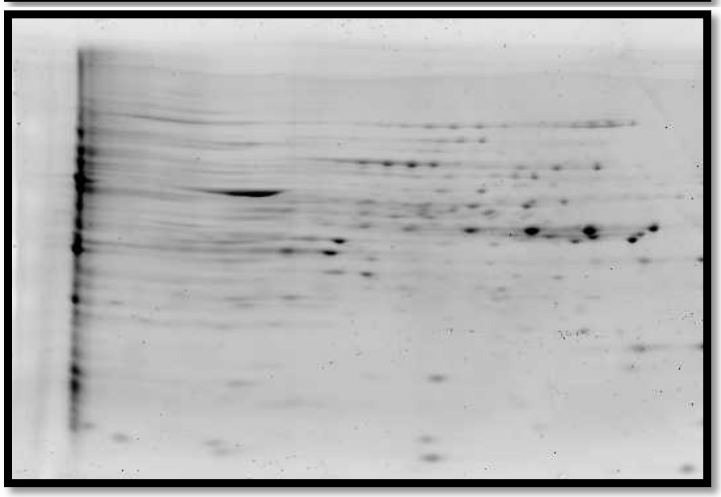
Figure 10: Criterion™TGX™ Precast 8-16% gels (length = 11 cm; pI 5-8) stained in SYPRO of wild-type *N. crassa* proteins grown on 0.3% quinic acid resolved in two-dimensional gel electrophoresis. Gels were done in triplicate.



Quinic Acid wild-type gel A

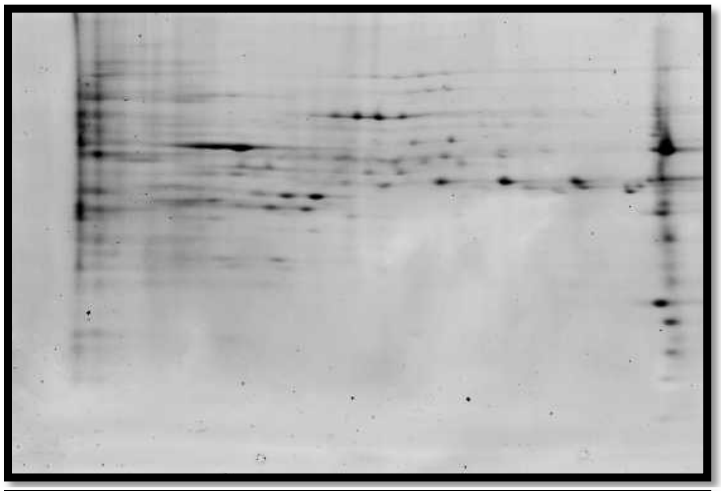


Quinic Acid wild-type gel B

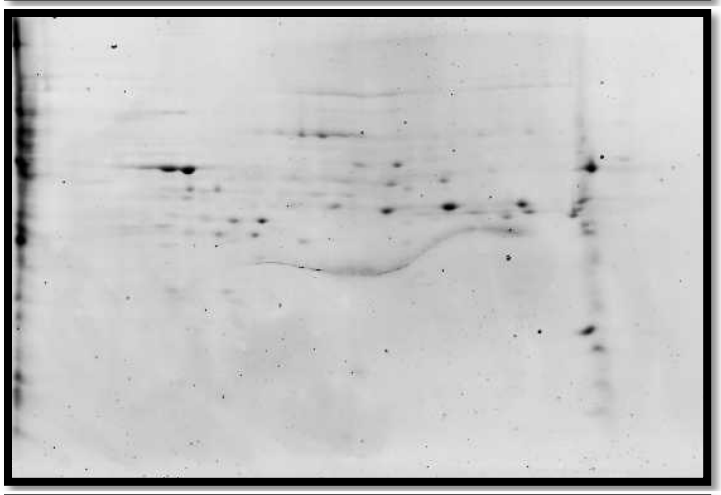


Quinic Acid wild-type gel C

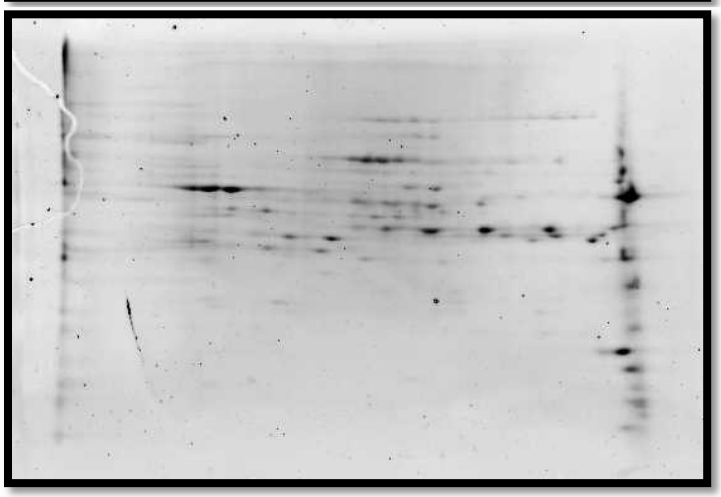
Figure 11: Criterion™TGX™ Precast 8-16% gels (length = 11 cm; pI 5-8) stained in SYPRO of *qa-1F* knockout *N. crassa* proteins grown on 0.3% quinic acid resolved in two-dimensional gel electrophoresis. Gels were done in triplicate.



Quinic acid *qa-1F* knockout gel A



Quinic acid *qa-1F* knockout gel B



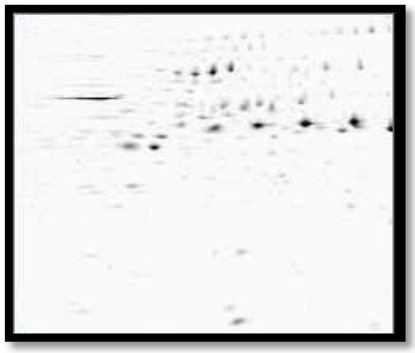
Quinic acid *qa-1F* knockout gel C

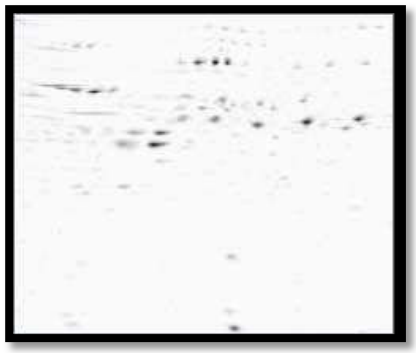
Figure 8 is the 2DGE gel image of *N. crassa* wild-type grown on 2% dextrose. Figure 9 is the 2DGE gel image of *N. crassa qa-1F* knockout grown on 2% dextrose. Figure 10 is the 2DGE gel image of *N. crassa* wild-type grown on 0.3% quinic acid. Figure 11 is the 2DGE gel image of *N. crassa qa-1F* knockout grown on 0.3% quinic acid.

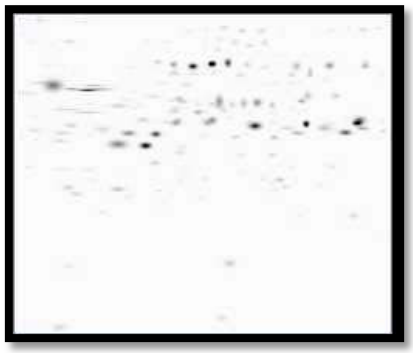
Once these protein profiles were created, Bio-Rad® PDQuest™ 2-D analysis software was used to analyze the data. To begin, PDQuest™ analyzes the 2DGE images and creates a filtered image in which any specks, streaks, and shadows that do not belong are removed. The filtered images are then further processed to create Guassian images. These Guassian images are an ideal representation of actual scanned spots. Then, matchsets were created of each of the four conditions to create a composite image, called the master. The filtered and guassian images of the matchsets and masters of each condition are shown in figures 12-19. Figure 12 is the protein profile of *N. crassa* wild-type grown on 2% dextrose matchset filtered and Gaussian images. Figure 13 is the master image of the wild-type *N. crassa* gown on 2% dextrose matchset. Figure 14 is the *N. crassa qa-1F* knockout grown on 2% dextrose matchset. Figure 15 is the master image of the *N. crassa qa-1F* knockout grown on 2% dextrose matchset. Figure 16 is the *N. crassa* wild-type grown on 0.3% quinic acid matchset. Figure 17 is the master image of the *N. crassa* wild-type grown on 0.3% quinic acid matchset. Figure 18 is the *N. crassa qa-1F* knockout grown on 0.3% quinic acid matchset. Figure 19 is the master image of the *N. crassa qa-1F* knockout grown on 0.3% quinic acid matchset. Using the masters of these matchsets, higher level matchsets were created to compare conditions. These new matchsets have their own masters. The higher level matchsets are shown in images figures 20-31. Figure 20 shows the higher level dextrose matchset in which the

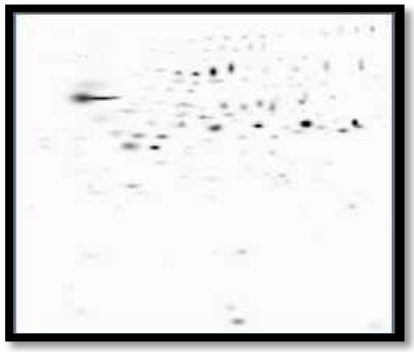
Figure 12: Matchset of wild-type *N. crassa* grown on 2% dextrose gels created using PDQuest™. a) Filtered image of dextrose, wild-type set gel A. b) Guassian image of dextrose, wild-type set gel A. c) Filtered image of dextrose, wild-type set gel B. d) Guassian image of dextrose wild-type set gel B. e) Filtered image of dextrose, wild-type set gel C. f) Guassian image of dextrose, wild-type set gel C.

a. 

b. 

c. 

d. 

e. 

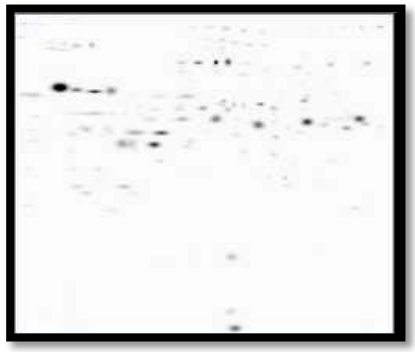
f. 

Figure 13: Master image of wild-type *N. crassa* grown on 2% dextrose matchset created using PDQuest™.

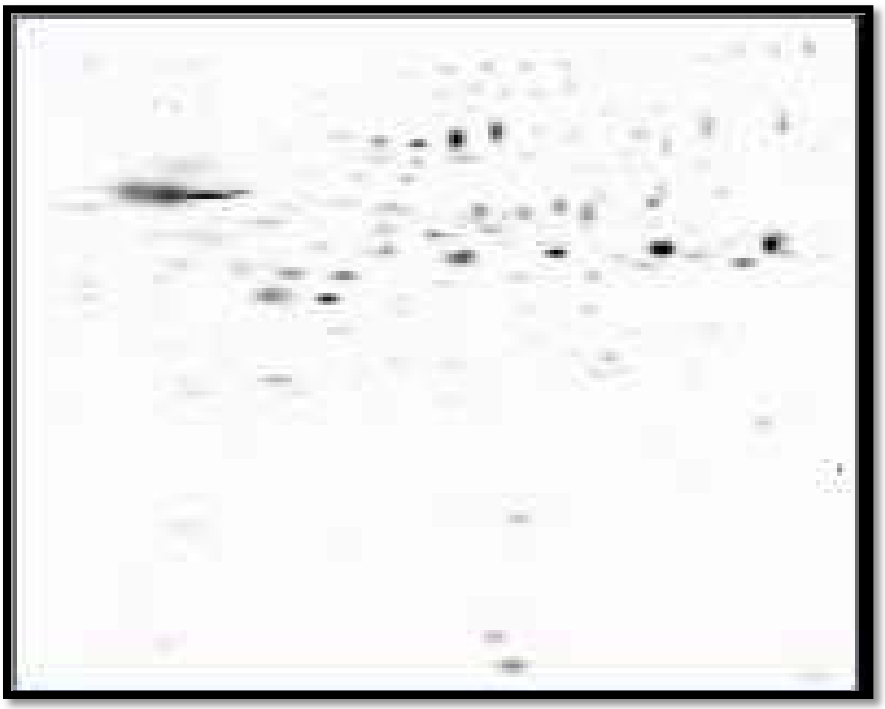
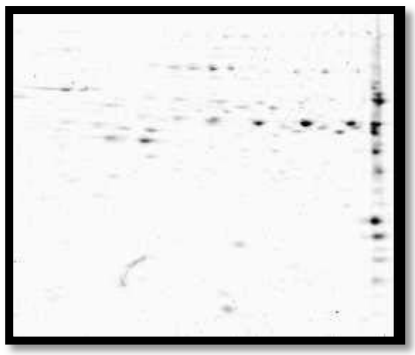
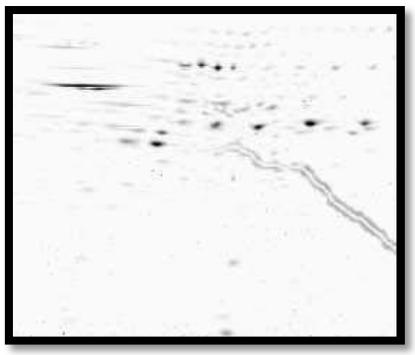
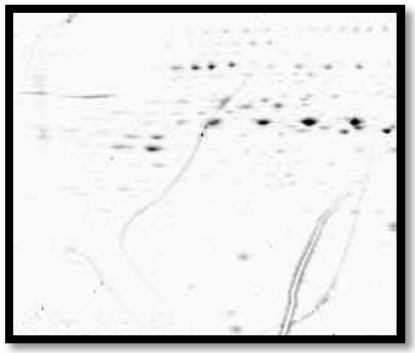
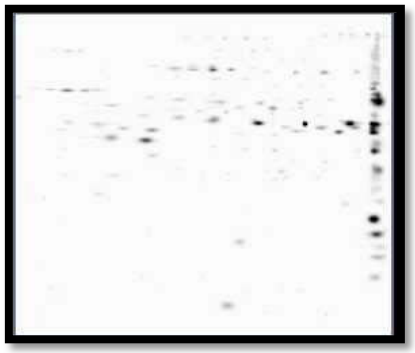


Figure 14: Matchset of *qa-1F* knockout *N. crassa* grown on 2% dextrose gels created using PDQuest™. a) Filtered image of dextrose, *qa-1F* knockout set gel A. b) Guassian image of dextrose, *qa-1F* knockout set gel A. c) Filtered image of dextrose, *qa-1F* knockout set gel B. d) Guassian image of dextrose *qa-1F* knockout set gel B. e) Filtered image of dextrose, *qa-1F* knockout set gel C. f) Guassian image of dextrose, *qa-1F* knockout set gel C.

a. 

b. 

c. 

d. 

e. 

f. 

Figure 15: Master image of *qa-1F* knockout *N. crassa* grown on 2% dextrose matchset created using PDQuest™.

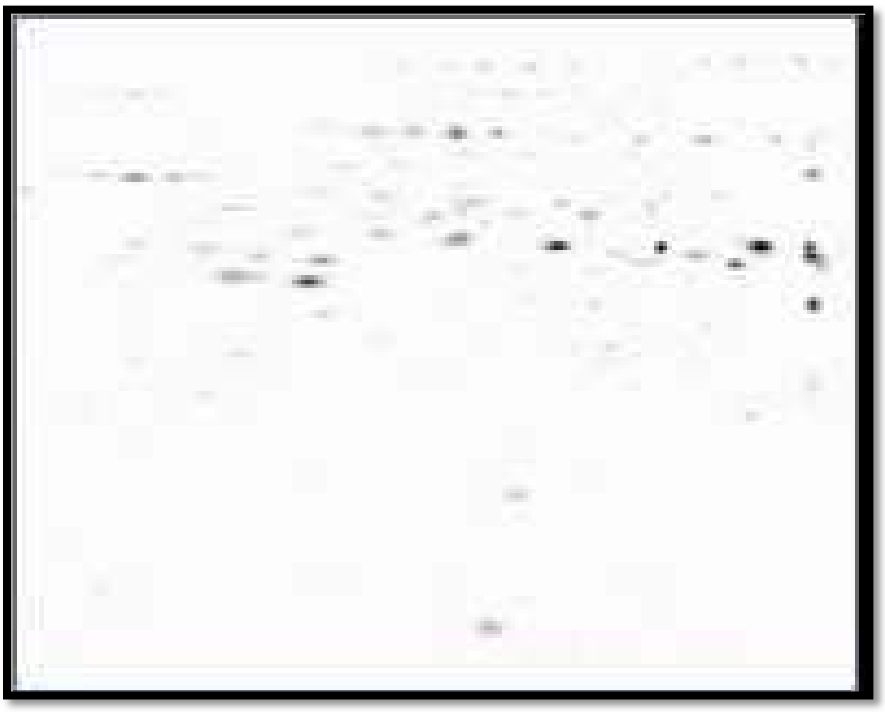
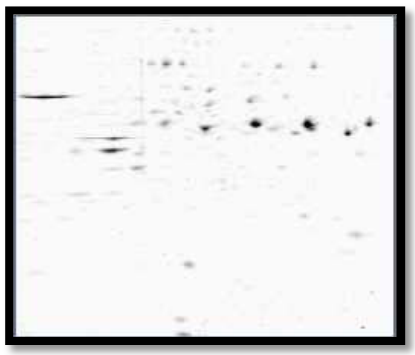
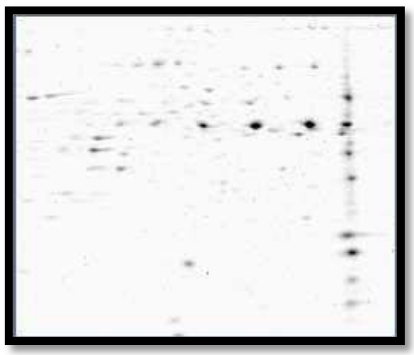
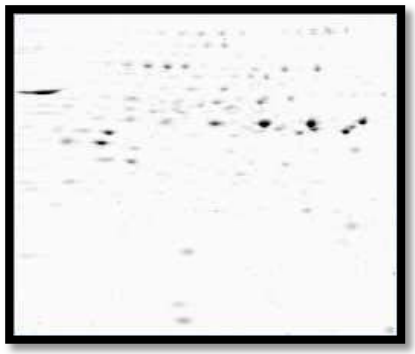
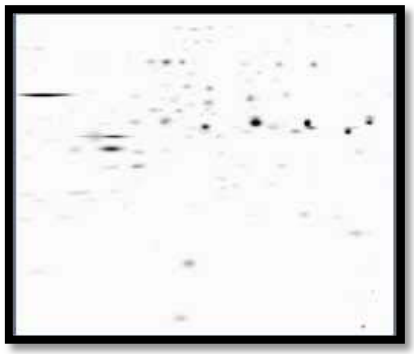


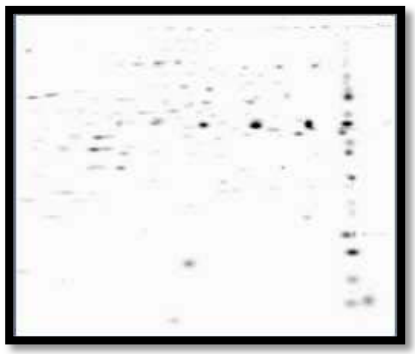
Figure 16: Matchset of wild-type *N. crassa* grown on 0.3% quinic acid gels created using PDQuest™. a) Filtered image of quinic acid, wild-type set gel A. b) Guassian image of quinic acid, wild-type set gel A. c) Filtered image of quinic acid, wild-type set gel B. d) Guassian image of quinic acid wild-type set gel B. e) Filtered image of quinic acid, wild-type set gel C. f) Guassian image of quinic acid, wild-type set gel C.

a. 

b. 

c. 

d. 

e. 

f. 

Figure 17: Master image of wild-type *N. crassa* grown on 0.3% quinic acid matchset created using PDQuest™.

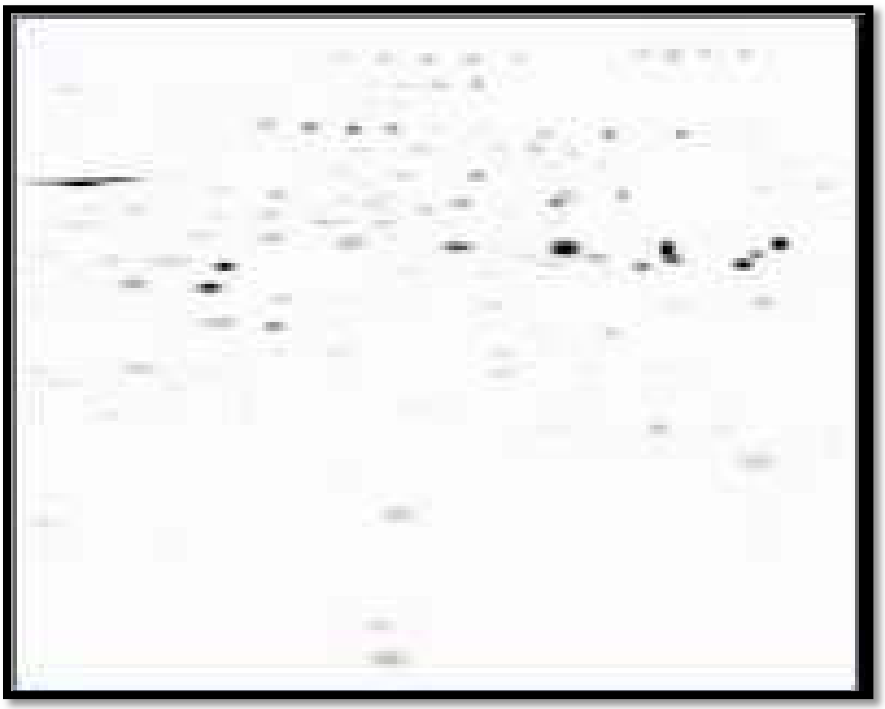
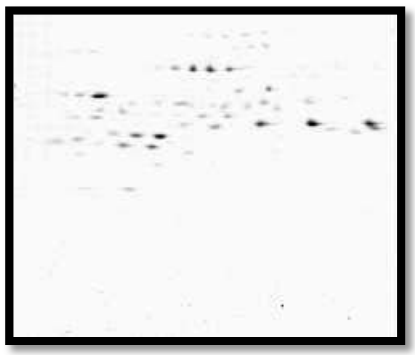
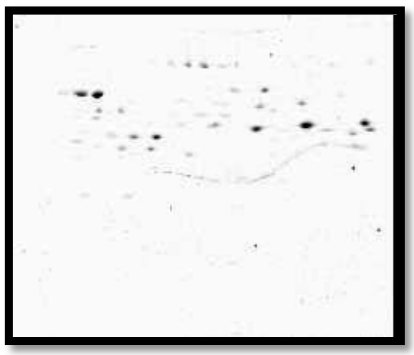
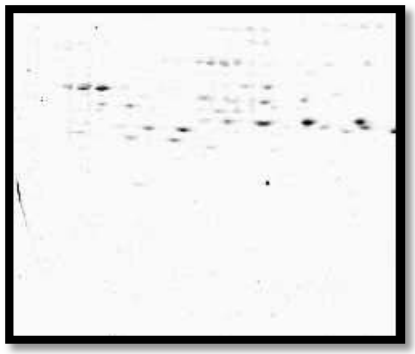
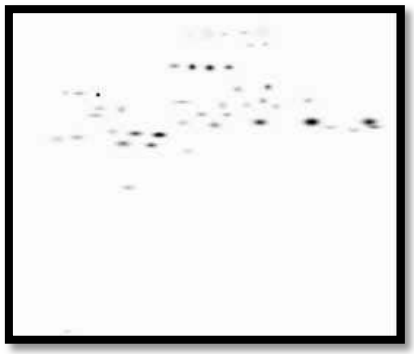


Figure 18: **Matchset of *qa-1F* knockout *N. crassa* grown on 0.3% quinic acid gels created using PDQuest™. a) Filtered image of quinic acid, *qa-1F* knockout set gel A. b) Guassian image of quinic acid, *qa-1F* knockout set gel A. c) Filtered image of quinic acid, *qa-1F* knockout set gel B. d) Guassian image of quinic acid *qa-1F* knockout set gel B. e) Filtered image of quinic acid, *qa-1F* knockout set gel C. f) Guassian image of quinic acid, *qa-1F* knockout set gel C.**

a. 

b. 

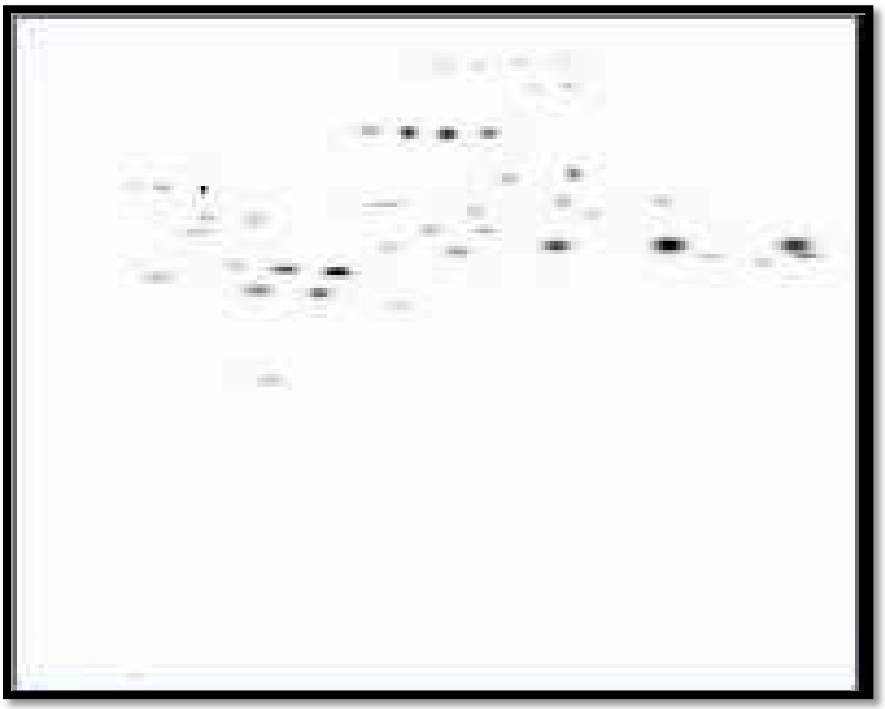
c. 

d. 

e. 

f. 

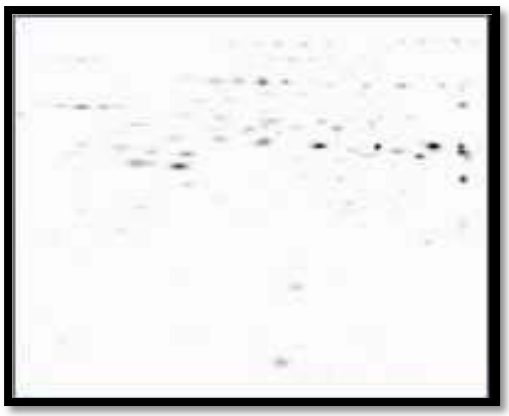
Figure 19: Master image of *qa-1F* knockout *N. crassa* grown on 0.3% quinic acid matchset created using PDQuest™.



dextrose wild-type master and the dextrose *qa-1F* knockout master are compared. Figure 21 shows the same images as figure 20 with the addition of squares indicating the protein spots that match on both masters being compared. Figure 22 is a Venn diagram depicting the number of protein spots on each master and the number of spots in common. Figure 23 shows the higher level quinic acid matchset in which the quinic acid wild-type master and the quinic acid *qa-1F* knockout master are compared. Figure 24 shows the same images as figure 23 with the addition of squares indicating the protein spots that match on both masters being compared. Figure 25 is a Venn diagram describing the number of protein spots on each master and the number of spots in common. Figure 26 shows the higher level wild-type matchset in which the dextrose wild-type and quinic acid wild-type masters are compared. Figure 27 shows the same images as figure 26 with the addition of squares indicating the protein spots that match on both masters. Figure 28 is a Venn diagram illustrating the number of protein spots on each master and the number of spots in common. Figure 29 shows the higher level *qa-1F* knockout matchset in which the dextrose *qa-1F* knockout and quinic acid *qa-1F* knockout masters are compared. Figure 30 shows the same images as figure 29 with the addition of squares indicating the spots shared between the two masters. Figure 31 is a Venn diagram depicting the number of protein spots on each master and the number of spots in common. From there, an even higher level matchset of the higher level dextrose master and the higher level quinic acid master was created (figure 32). This figure includes the squares denoting the shared protein spots. Figure 33 is a Venn diagram illustrating the number of protein spots on each master and the number of spots in common. Figure 34 is another higher level matchset of the higher level wild-type master and the higher level *qa-1F* knockout master

Figure 20: Higher level matchset of *N. crassa* grown on 2% dextrose created using PDQuest™. a) Master of dextrose wild-type matchset (figure 13). b) Master of dextrose *qa-1F* knockout matchset (figure 15). c) Master of higher level dextrose matchset.

a. 

b. 

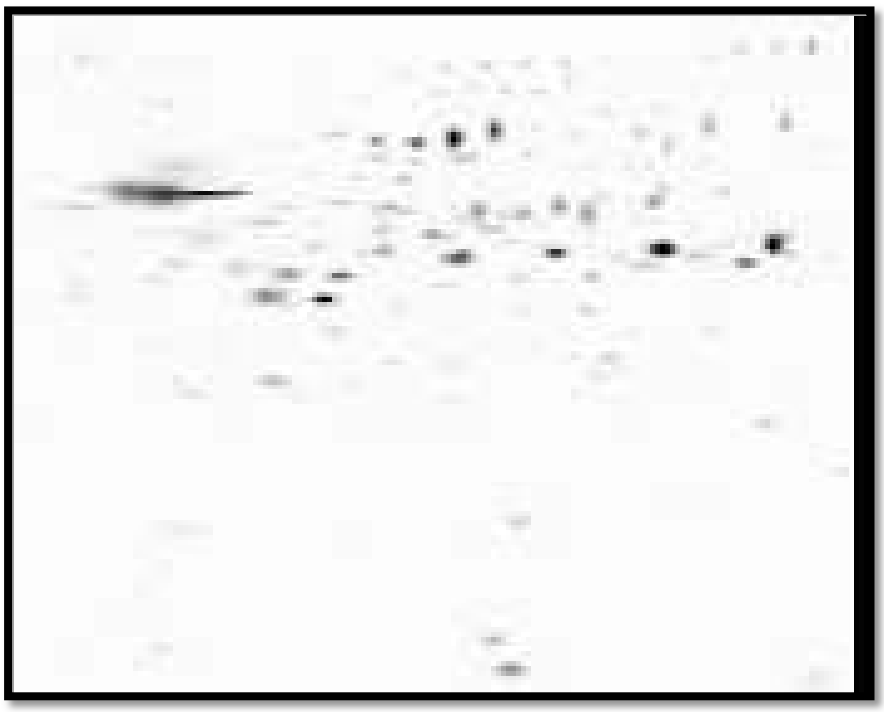
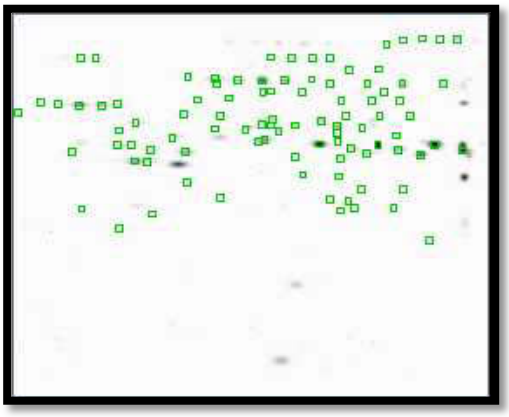
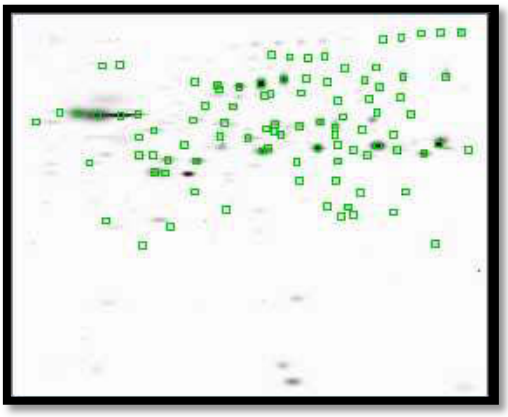
c. 

Figure 21: Higher level matchset of *N. crassa* grown on 2% dextrose created using PDQuest™. Squares indicate matching spots on each gel. a) Master of dextrose wild-type matchset (figure 13). b) Master of dextrose *qa-1F* knockout matchset (figure 15). c) Master of higher level dextrose matchset.

a. 

b. 

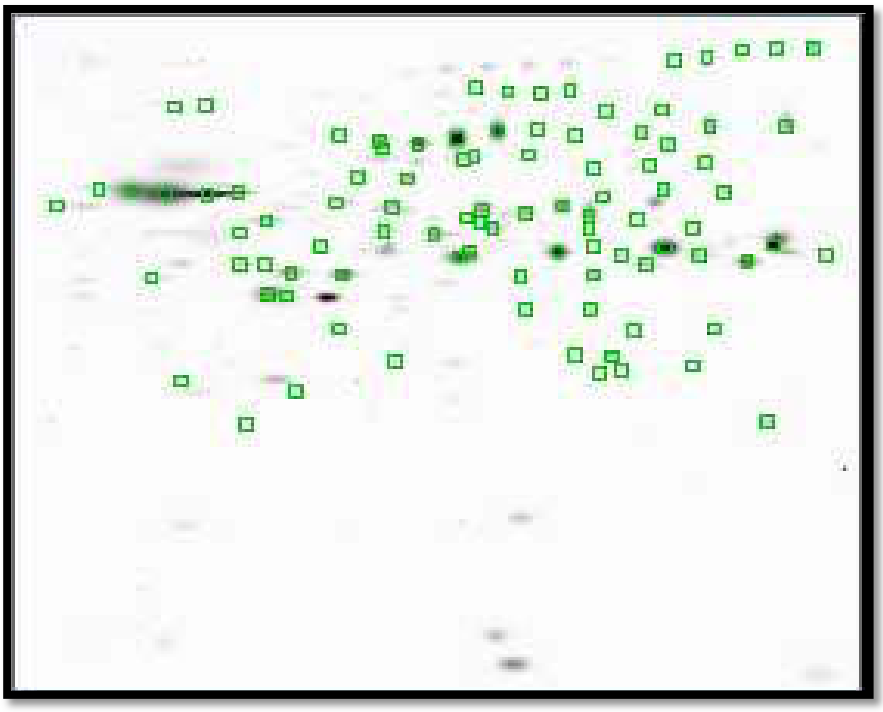
c. 

Figure 22: Diagram of number of spots in Dextrose wild type matchset master (figure 13) and Dextrose *qa-1F* knockout matchset master (figure 15) and how many of those spots are shared between the two.

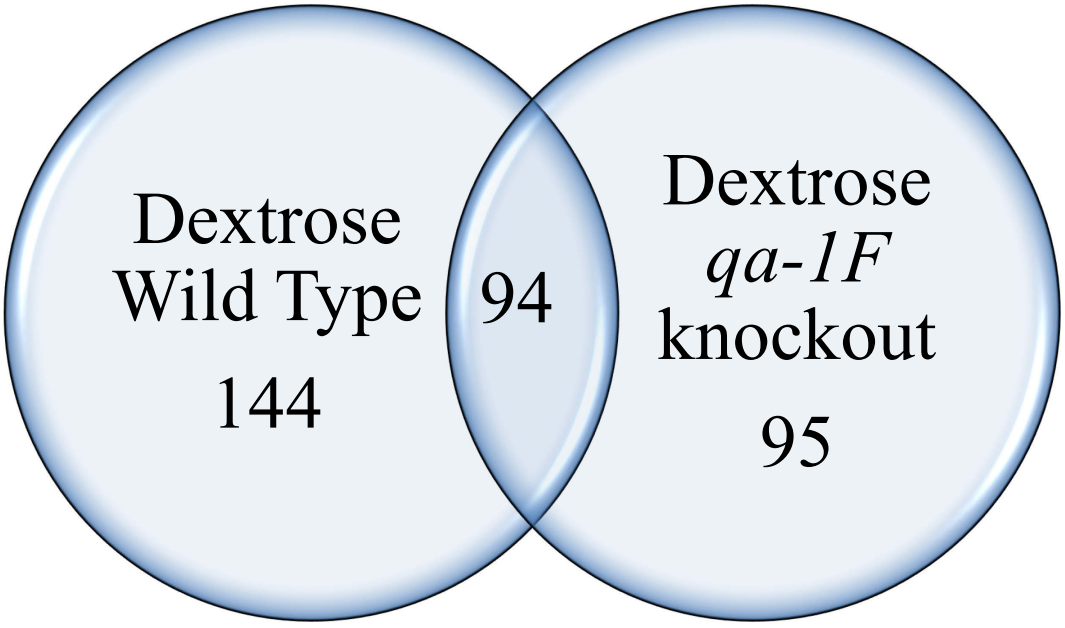

Dextrose
Wild Type
144
94
Dextrose
qa-1F
knockout
95

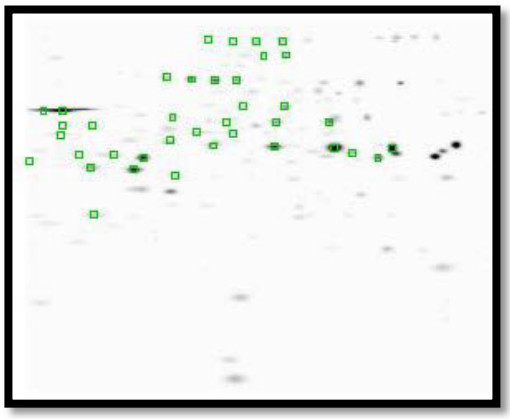
Figure 23: Higher level matchset of *N. crassa* grown on 0.3% quinic acid created using PDQuest™. a) Master of quinic acid wild-type matchset (figure 17). b) Master of quinic acid *qa-1F* knockout matchset (figure 19). c) Master of higher level quinic acid matchset.

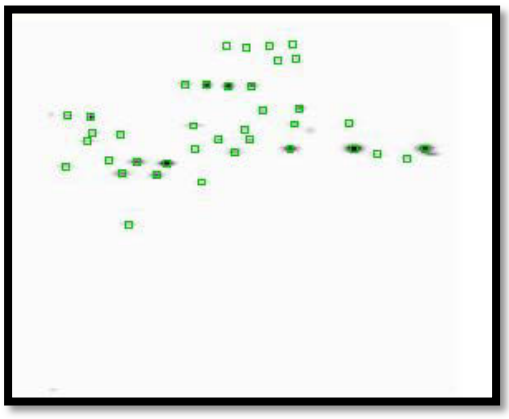
a. 

b. 

c. 

Figure 24: Higher level matchset of *N. crassa* grown on 0.3% quinic acid created using PDQuest™. Squares indicate matching spots on each gel. a) Master of quinic acid wild-type matchset (figure 17). b) Master of quinic acid *qa-1F* knockout matchset (figure 19). c) Master of higher level quinic acid matchset.

a. 

b. 

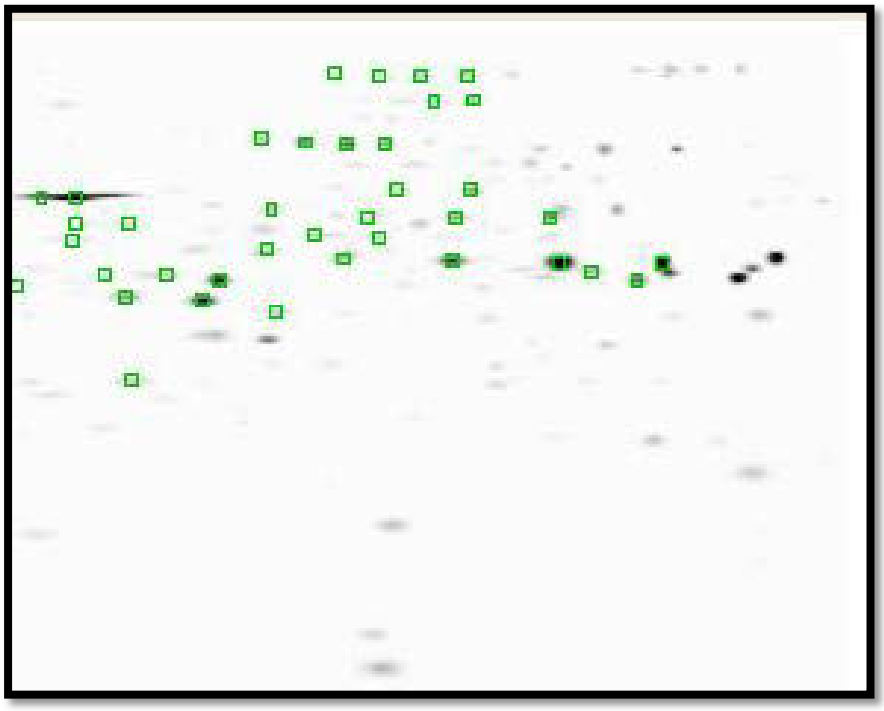
c. 

Figure 25: Diagram of number of spots in Quinic acid wild type matchset master (figure 17) and Quinic acid *qa-1F* knockout matchset master (figure 19) and how many of those spots are shared between the two.

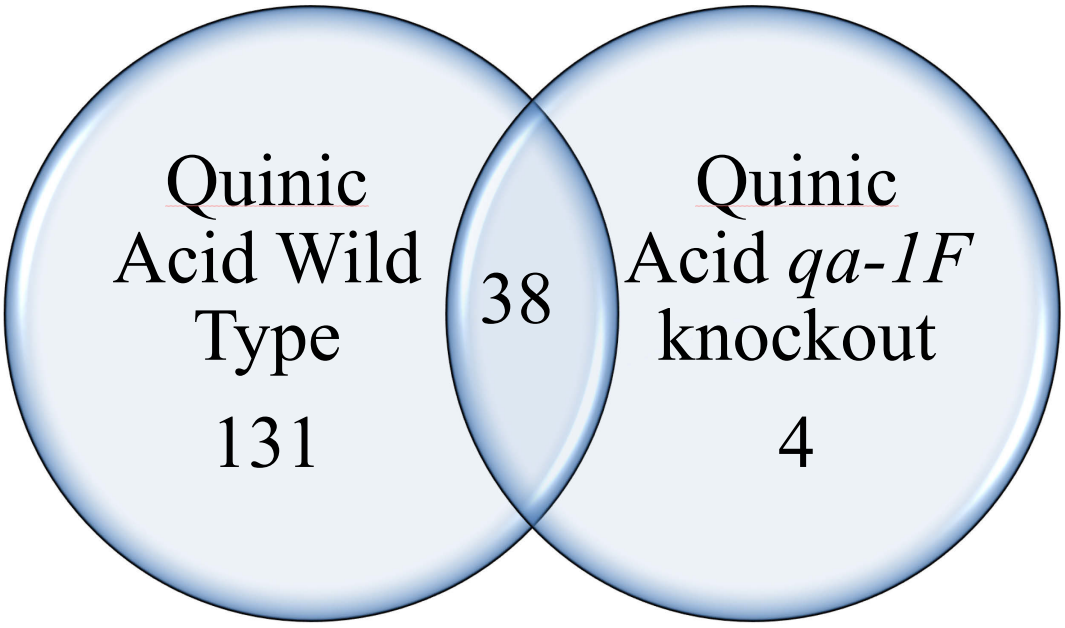

Quinic Acid Wild Type
131
38
Quinic Acid *qa-1F* knockout
4

Figure 26: Higher level matchset of wild-type *N. crassa* grown on 2% dextrose or 0.3% quinic acid created using PDQuest™. a) Master of dextrose wild-type matchset (figure 13). b) Master of quinic acid wild-type matchset (figure 17). c) Master of higher level wild-type matchset.

a. 

b. 

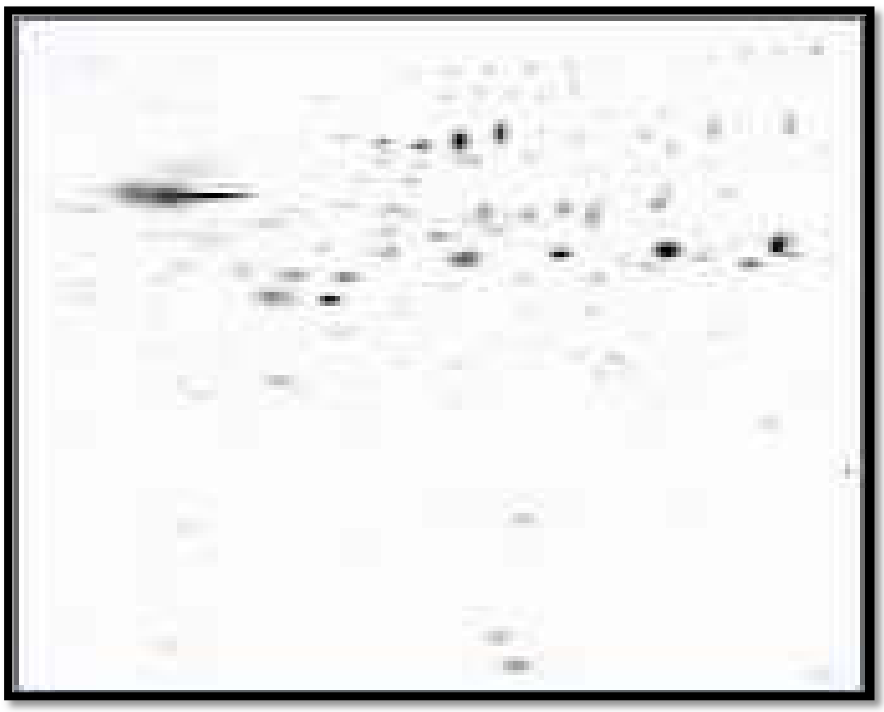
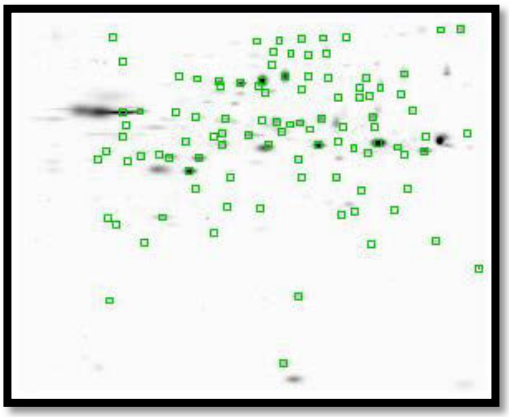
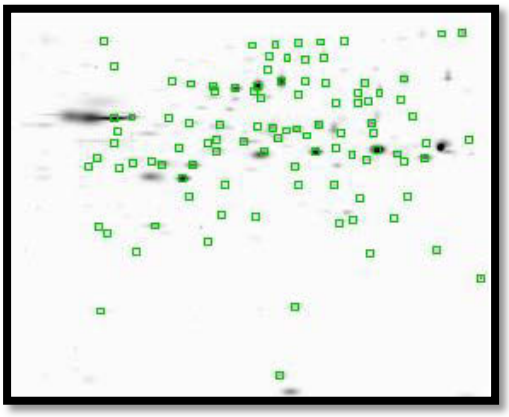
c. 

Figure 27: Higher level matchset of wild-type *N. crassa* grown on 2% dextrose or 0.3% quinic acid created using PDQuest™. Squares indicate matching spots on all gels. a) Master of dextrose wild-type matchset (figure 13). b) Master of quinic acid wild-type matchset (figure 17). c) Master of higher level wild-type matchset.

a. 

b. 

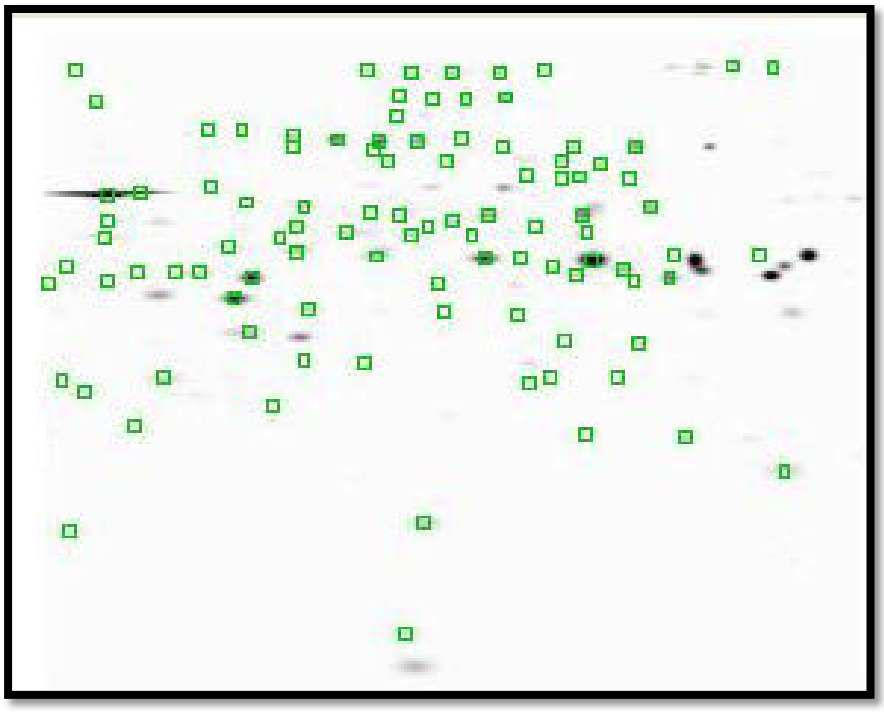
c. 

Figure 28: Diagram of number of spots in Dextrose wild type matchset master (figure 13) and Quinic acid wild type matchset master (figure 17) and how many of those spots are shared between the two.

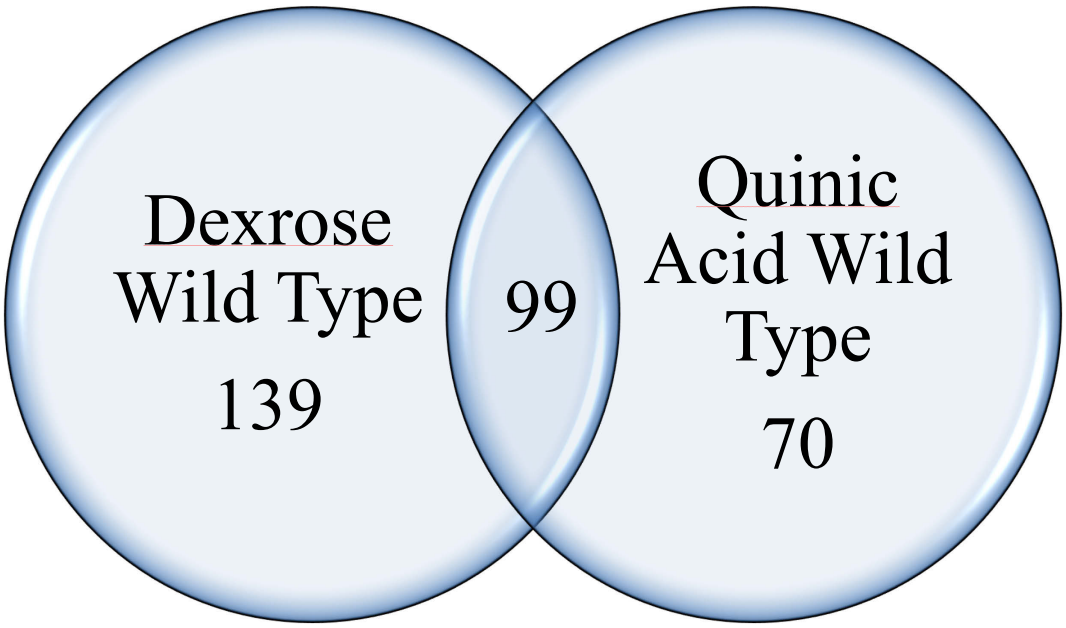
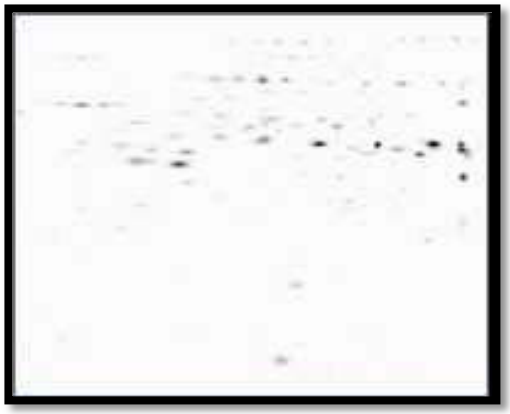

Dexrose
Wild Type
139
99
Quinic
Acid Wild
Type
70

Figure 29: Higher level matchset of *N. crassa qa-1F* knockout grown on 2% dextrose or 0.3% quinic acid created using PDQuest™. a) Master of dextrose *qa-1F* knockout matchset (figure 15). b) Master of quinic acid *qa-1F* knockout matchset (figure 19). c) Master of higher level *qa-1F* knockout matchset.

a. 

b. 

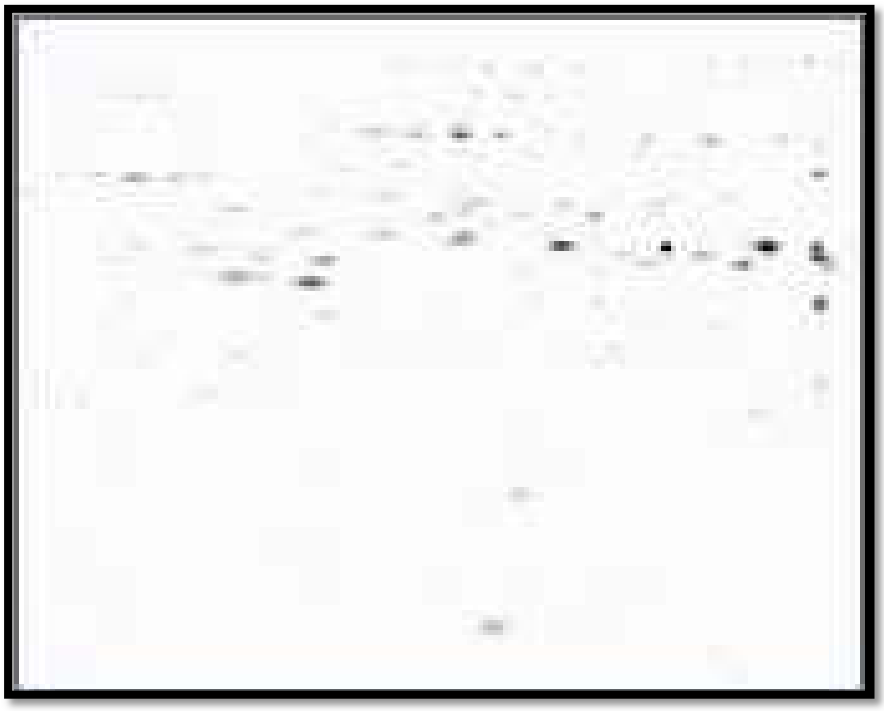
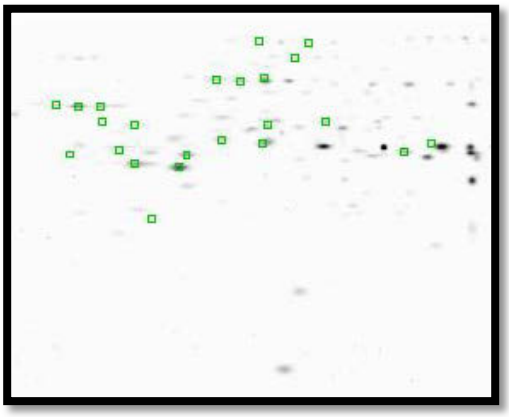
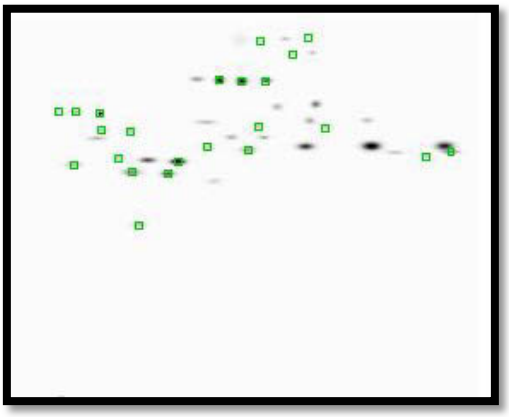
c. 

Figure 30: Higher level matchset of *N. crassa qa-1F* knockout grown on 2% dextrose or 0.3% quinic acid created using PDQuest™. Squares indicate matching spots on all gels. a) Master of dextrose *qa-1F* knockout matchset (figure 15). b) Master of quinic acid *qa-1F* knockout matchset (figure 19). c) Master of higher level *qa-1F* knockout matchset.

a. 

b. 

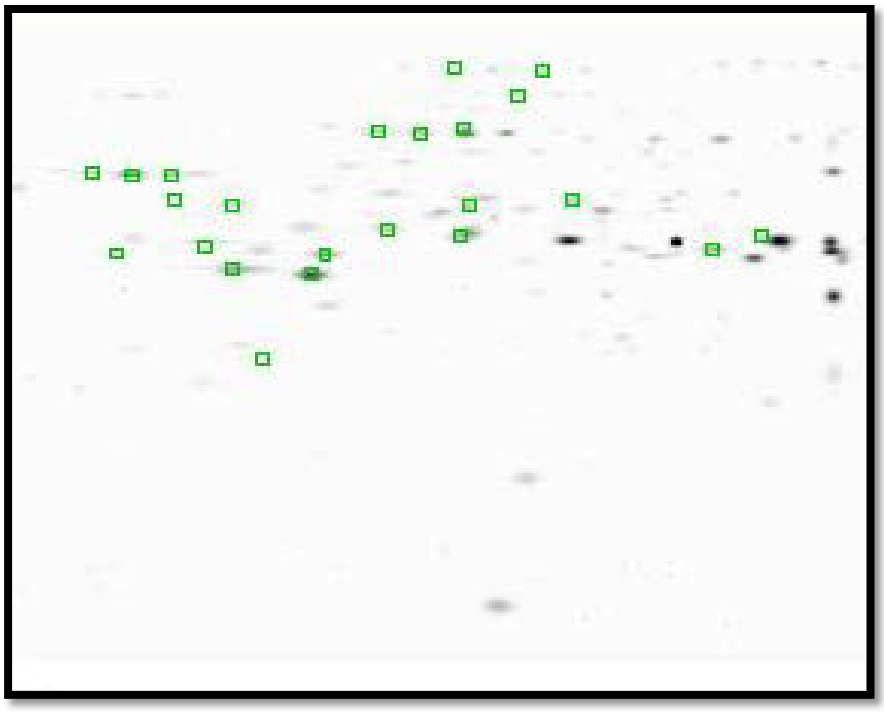
c. 

Figure 31: Diagram of number of spots in Dextrose *qa-1F* knockout matchset master (figure 15) and Quinic acid *qa-1F* knockout matchset master (figure 19) and how many of those spots are shared between the two.

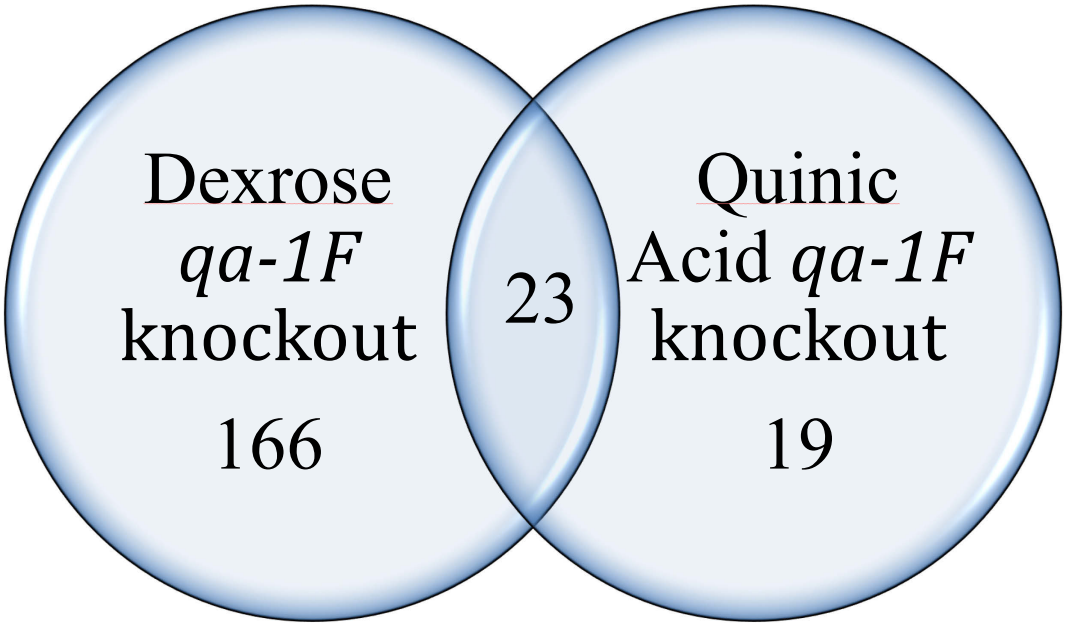
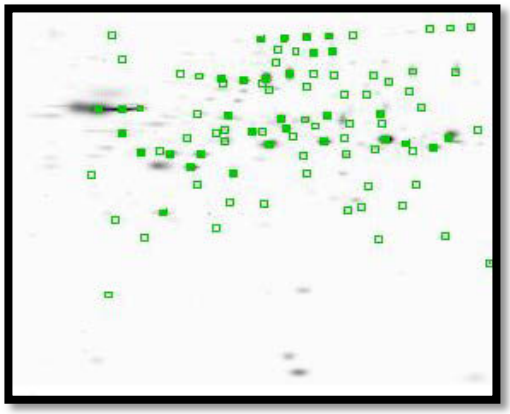
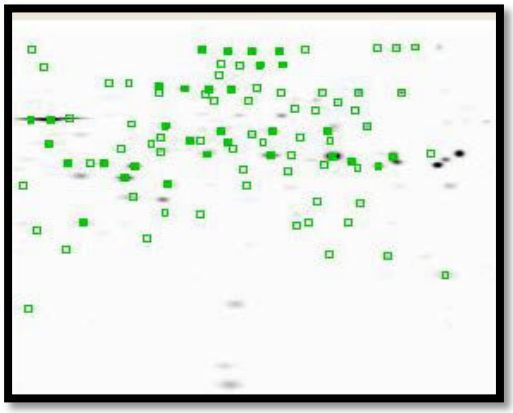

Dexrose
qa-1F
knockout
166
23
Quinic
Acid *qa-1F*
knockout
19

Figure 32: Higher level matchset of dextrose higher level matchset master and quinic acid higher level matchset master created using PDQuest™. Squares indicate matching spots on all gels. a) Master of dextrose higher level matchset (figure 20). b) Master of quinic acid higher level matchset (figure 23). c) Master of higher level matchset.

a. 

b. 

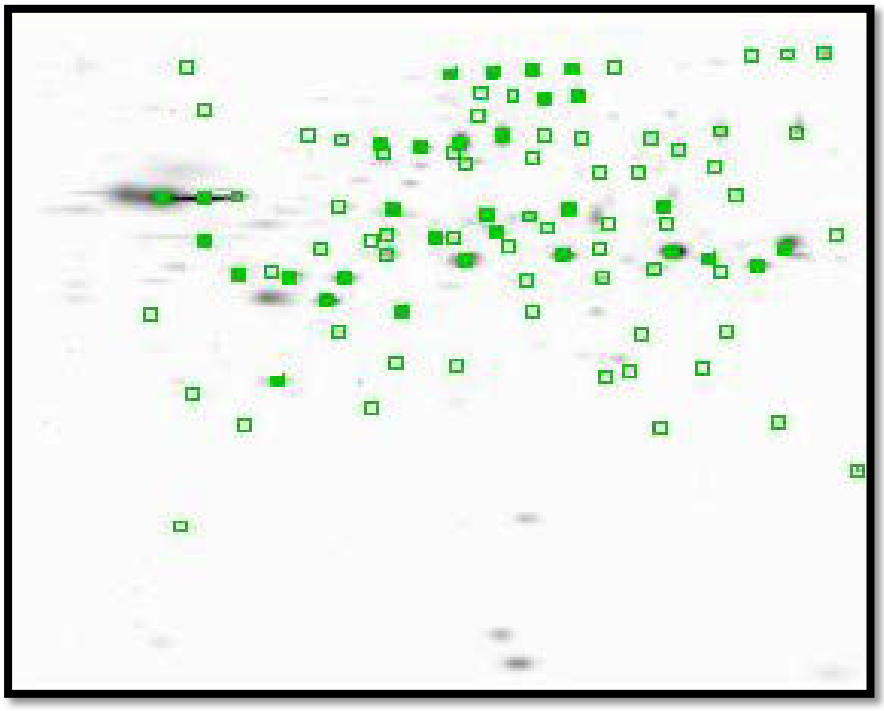
c. 

Figure 33: Diagram of number of spots in Dextrose matchset master (figure 20) and Quinic acid matchset master (figure 23) and how many of those spots are shared between the two.

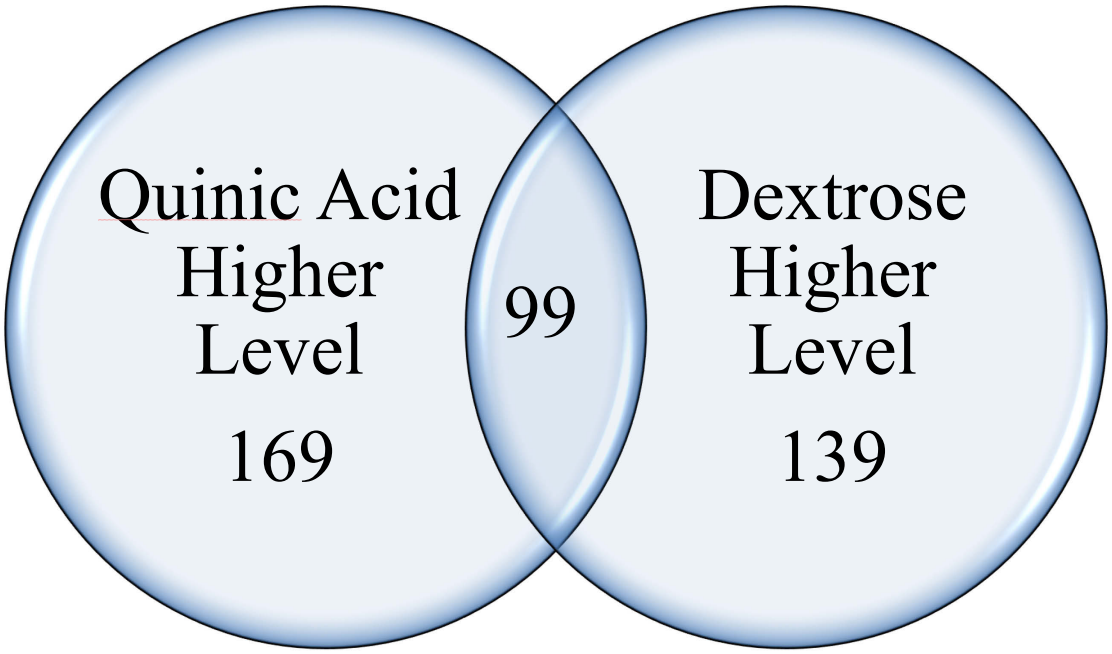
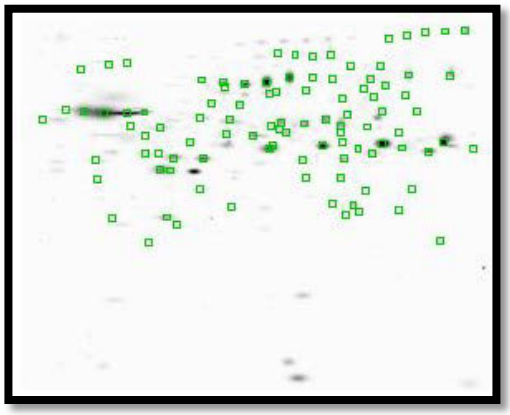
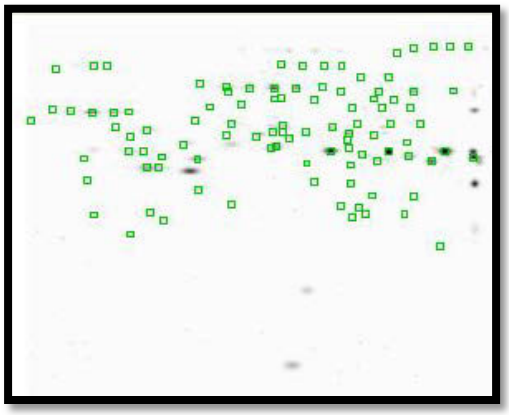

Quinic Acid
Higher
Level
169
99
Dextrose
Higher
Level
139

Figure 34: Higher level matchset of wild-type higher level matchset master and *qa-1F* knockout higher level matchset master created using PDQuest™. Squares indicate matching spots on all gels. a) Master of wild-type higher level matchset (figure 26). b) Master of *qa-1F* knockout higher level matchset (figure 29). c) Master of higher level matchset.

a. 

b. 

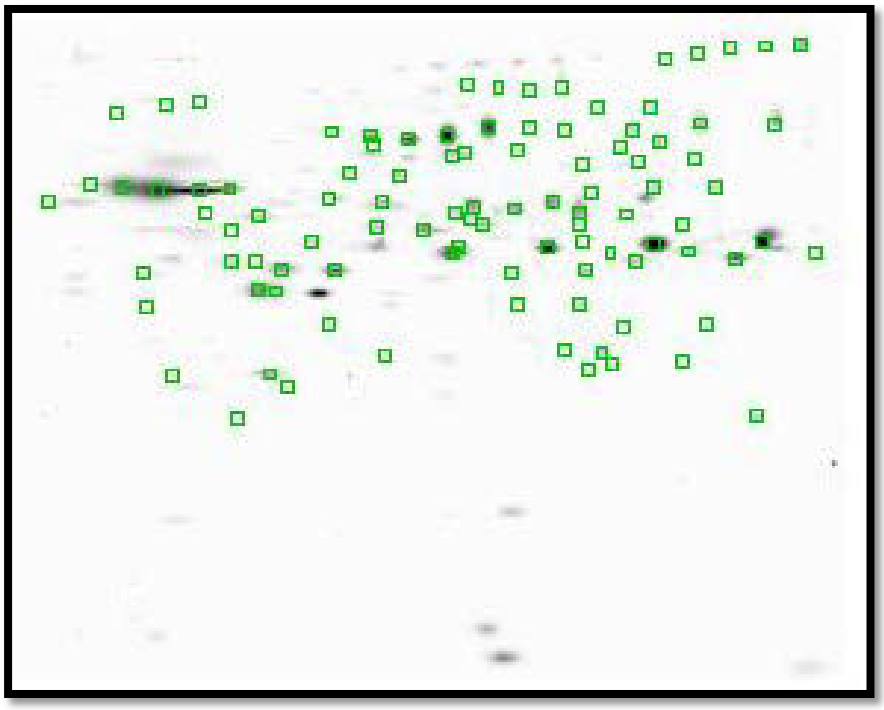
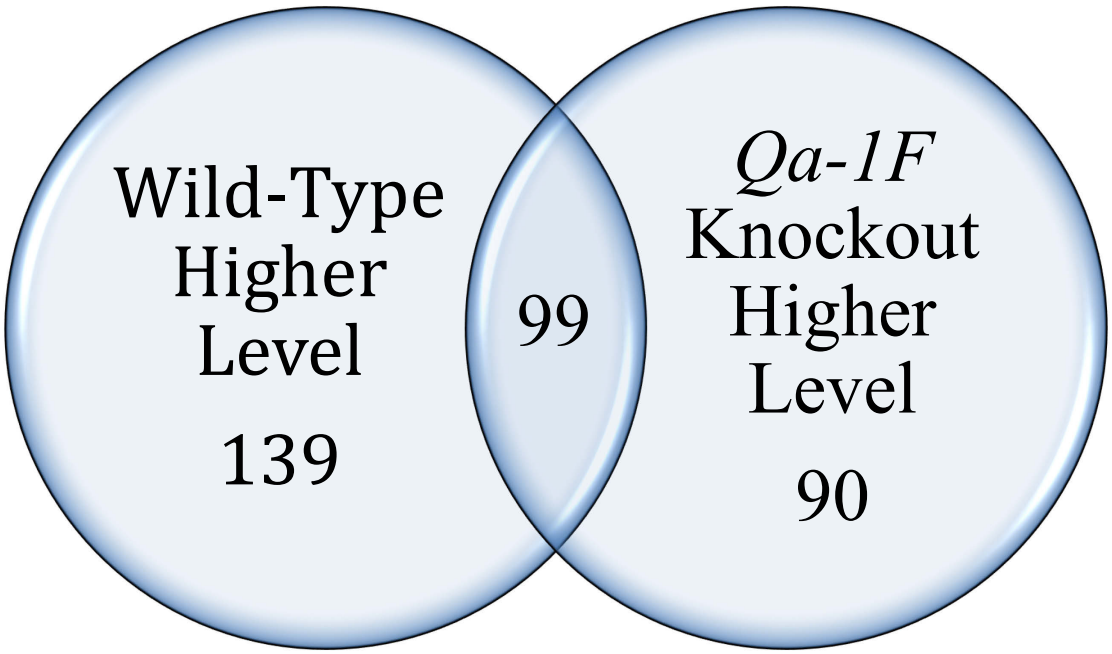
c. 

Figure 35: Diagram of number of spots in wild-type matchset master (figure 26) and *qa-1F* knockout matchset master (figure 29) and how many of those spots are shared between the two.

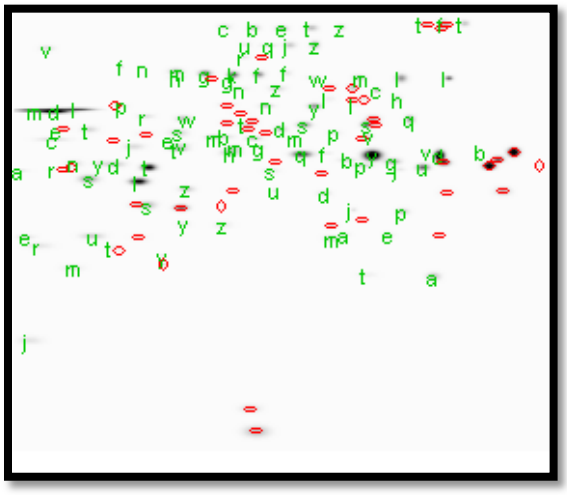

Wild-Type
Higher
Level
139
99
Qa-1F
Knockout
Higher
Level
90

with squares indicating the shared spots. Figure 35 is a Venn diagram describing the number of protein spots on each master and the number of spots in common.

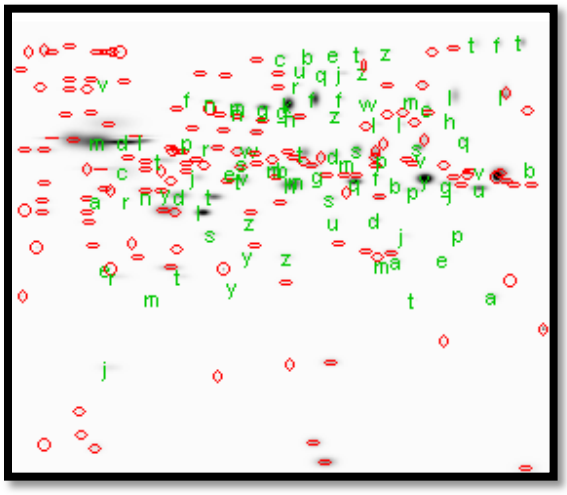
In order to pinpoint which proteins are affected by the presence of quinic acid and/or controlled by the *QA-1F* protein, a three-way matchset of dextrose wild-type, quinic acid wild-type, and quinic acid *qa-1F* knockout was created and analyzed using PDQuest™. Theoretically, there should be no change between the protein profiles of *N. crassa* wild-type grown on dextrose and *qa-1F* knockout grown on dextrose, so those images were not used here. Figure 36 shows the master images from these three conditions and the master image generated for this matchset by PDQuest™. The green letters and purple square indicate shared protein spots. Any red circles represent a spot not matched to all three images. The red circles may also be an area that PDQuest™ detected as a spot, but was later determined to be too faint for consideration. Figure 37 is a Venn diagram illustrating the number of protein spots on each master and the number of spots shared. With this analysis, spots of particular interest could be identified for further analysis by sending them to the Ohio State University Mass Spectrometry and Proteomics Facility for partial sequencing and potential identification. Since no protein spots from *N. crassa qa-1F* knockout grown on quinic acid appeared to be really unique, spots were selected from the wild-type strains grown on both dextrose and quinic acid (figure 38). First, spots truly unique to growth on quinic acid with a functioning *qa-1F* gene were indentified and labeled with blue circles. Spots absent in the *qa-1F* knockout growth on quinic acid that PDQuest™ ruled as unique to quinic acid wild-type growth were marked with pink circles. A group of spots that were present in all conditions except the quinic acid *qa-1F* knockout was circled in green. Spots present in all four

Figure 36: Higher level matchset of wild-type dextrose matchset master, wild-type quinic acid matchset master, and *qa-1F* knockout quinic acid matchset master created using PDQuest™. Green letters and purple squares indicate matching spots on all gels. Any red circles indicate spots unmatched by PDQuest™ a) Master of this higher level matchset. b) Master of dextrose wild-type matchset (figure 13). c) Master of quinic acid *qa-1F* knockout matchset (figure 19). d) Master of quinic acid wild-type matchset (figure 17).

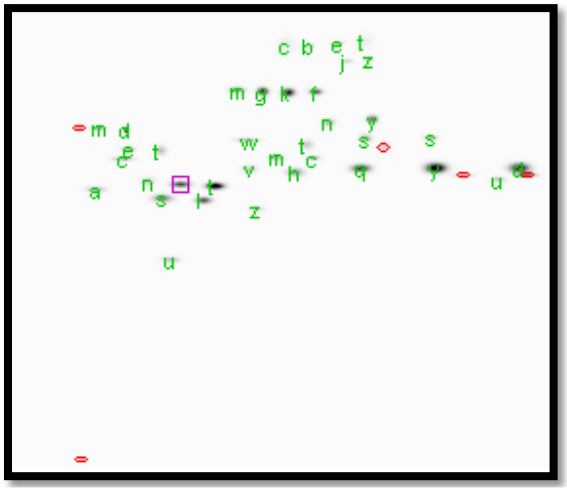
a.



b.



c.



d.

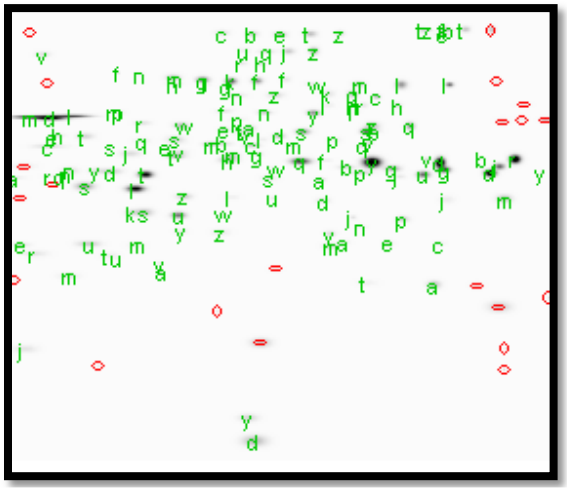
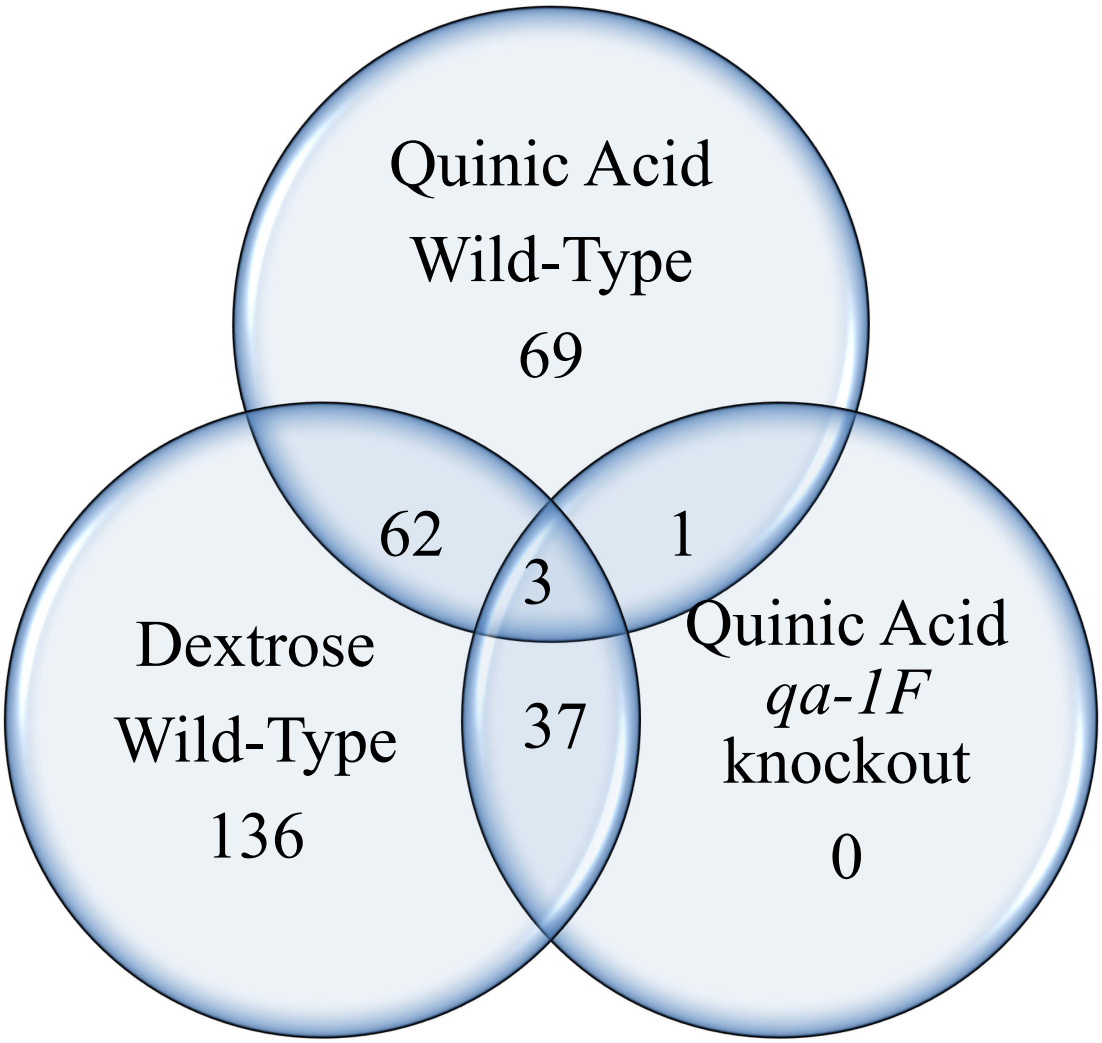


Figure 37: Diagram of number of spots in dextrose wild-type master (figure 13), quinic acid wild-type matchset master (figure 17), and quinic acid *qa-1F* knockout matchset master (19) and how many of those spots are shared between the three.


Quinic Acid
Wild-Type
69
62
1
3
Dextrose
Wild-Type
136
37
Quinic Acid
qa-1F
knockout
0

conditions were labeled with orange boxes. On the dextrose wild-type master image, yellow boxes were placed around unique to wild-type *N. crassa* grown on dextrose. The selected spots were then assigned names (visible in figure 38) and excised from all three of the original triplicate of two-dimensional Criterion™TGX™ Precast 8-16% gels. Spot names were then followed by an A, B, or C to track from which of the three gels it originated. The spots determined to be the most pertinent to this study were SB1, SB2, SG2, SO1, and SY2. The best extractions of these spots were SB1A, SB1B, SG2C, SO1A, SO1B, and SY2B.

These spots were sent to the Ohio State University Mass Spectrometry and Proteomics Facility and analyzed as described in materials and methods. Some of the information returned is represented in table 5. The analysis of SY2B did not return any results with high confidence, and therefore is not discussed further. Table 5 includes the spots name, the identified protein name and NCBI accession number, the percent sequence coverage, and the Mascot score. Mascot uses a probability based scoring system; the score describes the probability that the match is a random event. The higher the score, the less likely the match is random. SB1A and SB1B are the same protein since they are the same spots extracted from duplicate gels. This is also true of SO1A and SO1B. This proves that extraction and analysis methods were carried out properly.

The SB1 and SB2 spots are unique to protein profiles of wild-type *Neurospora crassa* grown on quinic acid. These proteins are therefore up-regulated due to the presence of quinic acid in the cell and are likely activated by *qa-1F*. The peptide sequences are in figure 39. The hypothetical protein sequence is shown with the peptides matching the query in bold. The SB1 proteins match a hypothetical *N. crassa* protein called NCU

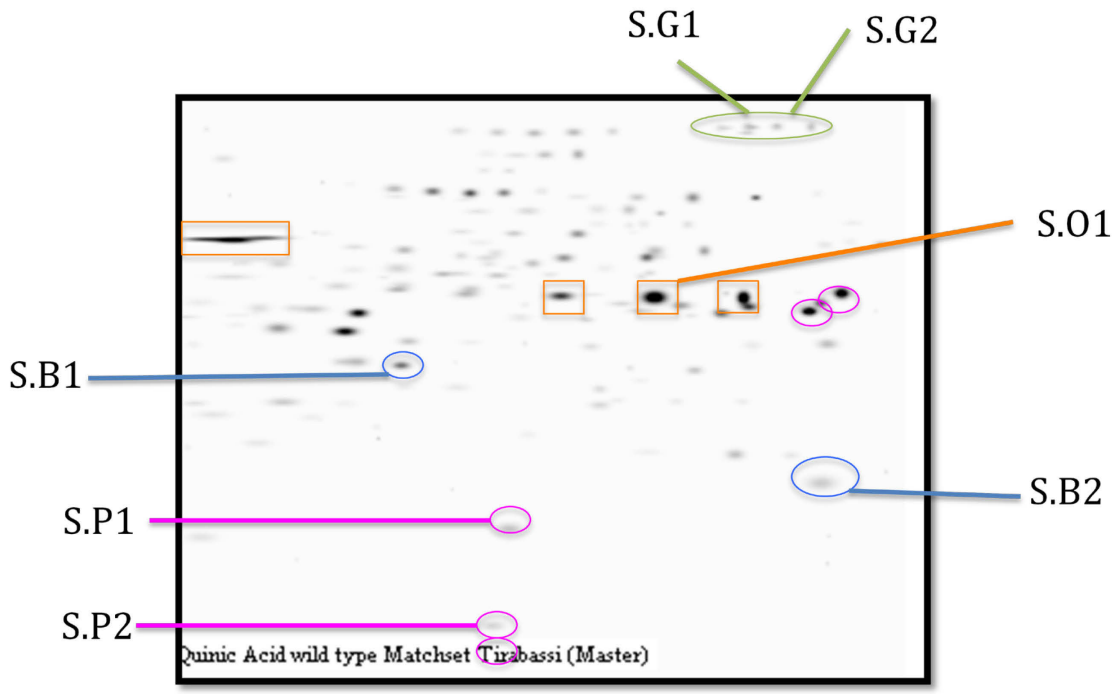
04072. A Basic Local Alignment Search Tool (BLAST) search returned several catechol dioxygenase protein matches of other fungal species. This enzyme is used to cleave the aromatic ring of the catechol molecule(Dodge 2008). The SB2 protein match a hypothetical *N. crassa* protein called NCU 08332. A BLAST search revealed that this protein sequence is very similar to another known protein from *Neurospota tetrasperma*: the translational protein SH3-like protein.

Spot SG2 was a protein spot present in all conditions except *N. crassa qa-1F* knockout grown on quinic acid. This suggests this protein is only regulated by *qa-1F* when quinic acid is present. When an ideal carbon source is present, another protein must regulate its transcription as it was present in both dextrose wild-type and *qa-1F* knockout conditions. The sequence of this protein revealed it to be 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase. This protein catalyzes the reaction 5-methyltetrahydropteroyltri-L-glutamate + L-homocysteine → L-methionine + tetrahydropteroyltri-L-glutamate(Whitfield, Steers and Weissbach 1970). The peptide sequence is shown in figure 40.

Lastly, the SO1 spots are proteins present in all conditions and, therefore, a protein important to sustaining the life of the cell. As expected, the analysis revealed this to be a housekeeping protein: glyceraldehyde-3-phosphate dehydrogenase. This protein plays a key role in glycolysis . The peptide sequences are shown in figure 41.

Figure 38: a) Master image of *N. crassa* wild-type grown on 0.3% quinic acid. b) Master image of *N. crassa* wild-type grown on 2% dextrose. Spots circled or squared were chosen for excision. Name given to spot indicated at end of line connecting to spot. If no line connects a name to the spot, the spot was considered but not chosen.

a)


S.G1
S.G2
S.O1
S.B1
S.B2
S.P1
S.P2
Quinic Acid wild type Matchset Tirabassi (Master)

b)

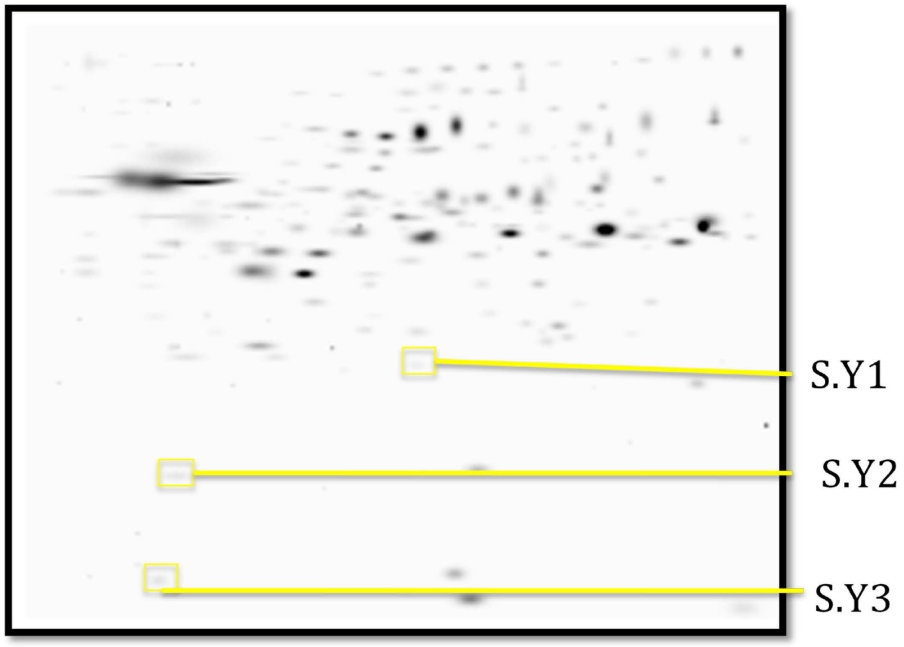

S.Y1
S.Y2
S.Y3

Table 5: Identification of proteins excised from wild-type *Neurospora crassa* grown on quinic acid. All proteins identified were correctly of the organism *N. crassa*. The spot name corresponds to figure 38. The protein name and accession number are from the National Center for Biotechnology Information (NCBI) database. The percent coverage indicates how much of the result protein does the query sequence cover. The Mascot score is a probability score obtained from the Mascot search.

Spot Name	Condition	Protein Name	Accession Number	Percent Coverage	Mascot Score
SB1A	Unique to wild type *N. crassa* growth on quinic acid	Hypothetical Protein NCU04072	gi|85086358	18%	148
SB1B	Unique to wild type *N. crassa* growth on quinic acid	Hypothetical Protein NCU04072	gi|85086358	16%	264
SB2A	Unique to wild type *N. crassa* growth on quinic acid	Hypothetical Protein NCU08332	gi|85110952	27%	211
SG2C	Absent only on *N. crassa qa-1F* knockout growth on quinic acid	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	gi|85083586	10%	231
SO1A	Present in all conditions	glyceraldehyde-3-phosphate dehydrogenase	gi|1326237	18%	110
SO1B	Present in all conditions	glyceraldehyde-3-phosphate dehydrogenase	gi|1326237	16%	172

Figure 39: Peptide sequences of hypothetical proteins identified as up-regulated due to the presence of quinic acid in the cell and affected by the *qa-1F* activator. The peptides that match the query from the sequenced proteins are shown in bold, red print. a) Spot SB1A; Hypothetical protein NCU 04072. b) Spot SB1B; Hypothetical protein NCU 04072. c) Spot SB2A; Hypothetical protein NCU08332.

a)
Spot SB1A. Hypothetical Protein NCU04072

1 MASHRFDPNF TQNVIDGMGP NTTPRNRQVL GALIRHIHDF AREVELTMDE
51 WMAGVKYINE VGRVYSESGQ TRNEAHRLSD ILGLETLVDE IAHKIVVEEG
101 LEPTSSSILG PFWSPNAPFR ENGGSIIQDG VPPKGRVTKM HGVIRDITTG
151 KPIPNAVFDI WQASANGKYD FQDPQNQTPN NLRGKFKANE KGEYWFYCLH
201 PTAYSLPTDG PSYALLQLMD RHPMRPAHIH IMVTHPEYQG CTTQLYPSDD
251 PWITNDTVFA VKDDLVVTFK PIEGDEKAVL ELEYNVNLAP KGFKGKV

b)
Spot SB1B. Hypothetical Protein NCU04072

1 MASHRFDPNF TQNVIDGMGP NTTPRNRQVL GALIRHIHDF AREVELTMDE
51 WMAGVKYINE VGRVYSESGQ TRNEAHRLSD ILGLETLVDE IAHKIVVEEG
101 LEPTSSSILG PFWSPNAPFR ENGGSIIQDG VPPKGRVTKM HGVIRDITTG
151 KPIPNAVFDI WQASANGKYD FQDPQNQTPN NLRGKFKANE KGEYWFYCLH
201 PTAYSLPTDG PSYALLQLMD RHPMRPAHIH IMVTHPEYQG CTTQLYPSDD
251 PWITNDTVFA VKDDLVVTFK PIEGDEKAVL ELEYNVNLAP KGFKGKV

c)
Spot SB2A. Hypothetical Protein NCU08332

1 MGYYDDDAHG HVEADAAPRA TTGTGTGSAS QTVTIPCHHI RLGDILILQG
51 RPCQVIRIST SAATGQHRYL GVDLFTKQLH EESSFVSNPA PSVVVQTMLG
101 PVFKQYRVLD MQDGSIVAMT ETGDVKQNLP VIDQSSLWNR LQKAFESGRG
151 SVRVLVVSDH GREMAVDMKV VHGSRL

Figure 40: Peptide sequence of 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase. The peptides that match the query from the sequenced proteins are shown in bold, red print.

Spot SG2C. 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase

1 MVQSSVLGFP RMGVLRDLKK ANEAYWADKI SQEALLAEGK RLRLAHWKIQ
51 KDAGVDIIPS NDFAHYDHVL DHIQLFNAVP ERYTSQKLSP LDEYFAMGRG
101 HQKGGVDVPA LEMVKWFDSN YHYVKPTLQD NQTFSLAKDP KPVREFLEAK
151 EAGFQTRPVL VGPVSFLALG KADRGSSVDP ITLLDKLVPV YVELLKQLKA
201 AGAESVQIDE PVLVFDLRPE VKAAFKPAYE AIAAAGDAVP KVVVATYFGD
251 IVHNFDVLPA FSGAAGLHVD LVRNPEQLEP VLKQLGPNQI LSAGVVDGRN
301 IWKNDFAKSL EILQTAVKAL GSERVIVATS SSLIHTPHTL ASEKKLPSDV
351 YEWFSFAVEK VKEVATLAKA VTEPEAVKAE LEANAAAIKA RTDSKRTNDP
401 AVKERQAQVT PEQHNRKAPF NTRYAEQKKH LSLPLFPTTT IGSFPQTSEI
451 RVQRNKFTKG EISAEEYERF IEKEIELAVK IQDELDLDVY VHGEPERNDM
501 VQYFGERLNG YVFTTHAWVQ SYGSRCVRPP IIVGDISRPA PMTVKESKYA
551 ASISKKPMKG MLTGPVTCLR WSFPRVDVHQ SVQCQQLALA LRDEVVDLEK
601 NGIYVIQVDE PALREGLPLR KGQEREAYLK WAVDSFKLAT AGVENSTQIH
651 SHFCYSEFQD FFHAIAALDA DVLSIENSKS DAKLLKVFID EEYPRHIGPG
701 VYDIHSPRVP TLEEFKQRIE EMLAYLKPEQ LWINPDCGLK TRKWDEVKGA
751 LSHMVEAAKY FREKYANKA

Figure 41: Peptide sequence of glyceraldehyde-3-phosphate dehydrogenase. The peptides that match the query from the sequenced proteins are shown in bold, red print. a) Spot SO1A; glyceraldehyde-3-phosphate dehydrogenase. b) Spot SO1B; glyceraldehyde-3-phosphate dehydrogenase.

a)
Spot SO1A. glyceraldehyde-3-phosphate dehydrogenase

1 MVVKVGINGF GRIGRIVFRN AIEHDDIHIV AVNDPFIEPK YAAYMLRYDT
51 THGNFKGTIE VDGADLVVNG KKVKFYTDAD PAAIPWSETG ADYIVESTGV
101 FTTTEKASAH LKGGAKKVII SAPSADAPMY VMGVNNETYD GSADVISNAS
151 CTTNCLAPLA KVIHDNFTIV EGLMTTVHSY TATQKTVDGP SAKDWRGGRT
201 AAQNIIPSST GAAKAVGKVI PDLNGKLTGM AMRVPTANVS VVDLTARIEK
251 GATYDEIKEV IKKASEGPLA GILAYTEDEV VSSDMNGNPA SSIFDAKAGI
301 SLNKNFVKLV SWYDNEWGYS RRVLDLISYI SKVDAKKA

b)
Spot SO1B. glyceraldehyde-3-phosphate dehydrogenase

1 MVVKVGINGF GRIGRIVFRN AIEHDDIHIV AVNDPFIEPK YAAYMLRYDT
51 THGNFKGTIE VDGADLVVNG KKVKFYTDAD PAAIPWSETG ADYIVESTGV
101 FTTTEKASAH LKGGAKKVII SAPSADAPMY VMGVNNETYD GSADVISNAS
151 CTTNCLAPLA KVIHDNFTIV EGLMTTVHSY TATQKTVDGP SAKDWRGGRT
201 AAQNIIPSST GAAKAVGKVI PDLNGKLTGM AMRVPTANVS VVDLTARIEK
251 GATYDEIKEV IKKASEGPLA GILAYTEDEV VSSDMNGNPA SSIFDAKAGI
301 SLNKNFVKLV SWYDNEWGYS RRVLDLISYI SKVDAKKA

Discussion

Neurospora crassa uses sugar as a preferred carbon source as they are easily metabolized. If these are absent, *N. crassa* is forced to use other carbon sources such as the organic acid, quinic acid. The fungus is able to metabolize quinic acid via the quinate pathway. The enzymes necessary for this pathway are encoded in the quinic acid gene cluster. *Qa-1F* is the activator for this gene cluster. In addition to these seven proteins, recent studies have found that many other proteins are also up-regulated in response to the presence of quinic acid in the cell. These studies also reveal over 800 genes to have a *qa-1F* binding site (Logan, et al. 2007). The purpose of this study is to identify some of the proteins up-regulated by *qa-1F* in the presence of quinic acid outside of the quinic acid gene cluster. Also, we sought to note significant differences in the protein profiles of wild-type and *qa-1F* knockout *N. crassa* when grown on dextrose or quinic acid.

There are fewer proteins up-regulated in wild-type *N. crassa* when grown on quinic acid then when grown on dextrose. On both carbon sources, the *qa-1F* knockout showed a noticeable decrease in protein when compared to their wild-type counterparts. In fact, this study reveals over 100 protein spots seen in the protein profile of wild-type *N. crassa* grown on quinic acid are absent from the protein profile of *qa-1F* knockout *N. crassa* grown on quinic acid. This confirms that *qa-1F* controls several genes outside the quinic acid gene cluster. One would expect the absence of *qa-1F* to have no effect when *N. crassa* is grown on a preferred carbon source. The 2DGE protein profiles of wild-type *N. crassa* grown on dextrose and *qa-1F* knockout *N. crassa* grown on dextrose were compared with the intention of being a control group. Oddly, the two images were noticeably different. The *qa-1F* knockout protein profile has 95 unique protein spots and

shares less than 100 spots with the wild-type. It would seem possible that *qa-1F* regulates far more than just the expression of quinic acid induced genes.

Proteins up-regulated by *QA-1F* in response to quinic acid were identified and the protein spots were excised from the 2DGE acrylamide gel. These spots were studied using mass spectrometry and bioinformatic analysis. The two proteins investigated were identified as hypothetical *Neurospora crassa* proteins. A BLAST search revealed high similarities to a catechol dioxygenase and a translation protein SH3-like protein. The catechol dioxygenase-like protein could be used to cleave the aromatic ring structure a few steps after the protocatechuic acid during quinic acid metabolism. The hypothetical protein similar to the translation protein SH3-like protein is likely part of a Woronin body.

A group of proteins were present in all conditions except *qa-1F* knockout *N. crassa* grown on quinic acid (present in wild-type *N. crassa* grown on dextrose, *qa-1F* knockout *N. crassa* grown on dextrose, and wild-type *N. crassa* grown on quinic acid). The results revealed this protein to be 5-methlytetrahydropteroyltriglutamate-homocysteine methyltransferase. This protein is a catalyst for the creation of methionine. The protein's regulation is not strictly responsive to carbon source or to the functionality of the *qa-1F* activator. It is uncertain what would cause a down-regulation of this protein only when *qa-1F* knockout is grown on quinic acid.

This data confirms previous studies' findings that quinic acid as a sole carbon source impacts the regulation of multiple proteins, not just the quinic acid gene cluster. Additionally, this study gives evidence for the existence of two hypothetical proteins. Further study of the role of *qa-1F* in cells growing on ideal carbon sources may explain

the differences seen between the wild-type and *qa-1F* knockout *Neurospora crassa* grown on dextrose. Additionally, further research to verify the identity of the *qa-1F*-dependant genes and the existence of the hypothetical proteins would confirm these findings.

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