PROTEIN PROFILES OF *NEUROSPORA CRASSA* AND THE EFFECTS OF *NIT-2* UNDER VARYING LEVELS OF NITROGEN AVAILABILITY

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Protein profiles of *Nerurospora crassa* and the effects of *nit-2* under varying levels of nitrogen availability

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<u>Abstract</u>

The fungus *Neurospora crassa*, like most fungi, is very metabolically flexible. *N. crassa* metabolizes ammonia as a preferred nitrogen source, but has the ability to metabolize other nitrogen sources when the need arises. Furthermore, the nitrogen metabolism of *N. crassa* is heavily controlled by the gene *nit-2*. This study analyzes the protein profiles of both wild type *N. crassa* and a *nit-2* mutant *N. crassa* grown on both Vogels minimal media, which contains ammonia, or Westergaards media, which contains nitrate. Protein was extracted from *N. crassa* tissue of each genetic composition from each growth medium and analyzed by two-dimensional gel eletrophoresis (2DGE) with the resulting gels being imaged by the PharosFX™ imaging system and analyzed by the use of PDQuest™ software.

The study showed that protein profiles change based on the nitrogen source and whether the *nit-2* gene was active. *N. crassa* grown under poor nitrogen had a significantly lower number of protein spots than when grown under a preferred nitrogen source for both the wild type and *nit-2* mutant strains. When grown in poor nitrogen over 100 proteins were produced by the wild type that did not match the Vogels grown counterpart and there were over 60 proteins that were produced by the wild type that were not produced by the *nit-2* mutant under the same condition. The identified proteins include a heat-shock protein, a translation Sh3-like protein, an enolase, an aldolase, and a mitochondrial aconitate. Spot differences also suggest a new, previously not discussed, role for *nit-2* as a possible repressor under normal growth conditions.

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Introduction

1. KINGDOM FUNGI

The Kingdom Fungi is one of four kingdoms in the domain eukaryia, and contains over 1.5 million members. These members affect almost every other form of life either directly or indirectly and have been evolving and increasing their diversity for over 400 million years. The affects Fungi can have on other organisms range from direct contact through pathological or symbiotic means, or through affecting the environment of organisms around them. Despite the importance of Fungi, it was not until 2000, when a consortium of mycologists gathered to accelerate the research of fungi that fungal genomes were examined (Galagan et al, 2005). This movement in 2000 was brought on by the emphasis placed on eukaryotic genetics when Goffaeu sequenced the entire genome of *Saccharomyces ceravisiae* in 1996 (Galagan et al, 2005).

A large number of fungi are saprobes, meaning they decompose organisms and obtain their nutrients from the organic compost. Many other are parasites that feed off of living organisms without killing them. Many of these fungi can be detrimental to the organism they are growing on. Some of these parasitic fungi can also live on dead organic material, and are not limited to simply living organisms. Most fungi are filamentous, containing hyphae, and are multicellular (Alexopoulos et al, 1962). Additionally, fungi use spores as a means to reproduce in both sexual and asexual reproduction.

2. Phylum Ascomycota

Ascomycetes is one of four major fungal groups (Galagan et al, 2005). It has a large importance of arthropods as symbiotes and parasites. A number of fungi in this

phylum, such as Neurospora *crassa*, are important to research as models for genetics. Additionally, a number of these fungi, referred to as pyrenomycetes, are important to a variety of plants as endophytes. Further members of this phylum function as mammalian and plant parasites and saprobes.

The primary structure that separates this phylum from the others is the production of asci. These asci contain spores, and are referred to as ascospores. Ascospores are haploid and produced by meiosis of the diploid asci. Due to the ascospore being the defining feature of the fungal group, all fungi within the group have them as part of their reproductive cycle. The members of this group also do not produce their ascospores in the same compartments as each other. There are a large number of fungi in this group that produce their ascospores in large fruiting bodies, known as ascocarps. Members that used this method of ascospore storage were given the sub-group name of Euascomycetes, to indicate that they were "true" ascospore producers. Some examples of fungi that use ascopores include *N. crassa*, *Pyronema omphalodes*, and truffles (Carlile et al, 2001).

It is also important to note that reproduction through the use of acsospores in not the only method that this fungal group can reproduce. Most members of this group are also capable of asexual reproduction through the use of conidiospores (conidia). These conidiospores are produced on aerial hyphae, called conidiophores, that rise out of the substratum.. Some examples of members of this group that utilize conidia are *N. crassa* and *Ophiostoma ulmi* (Carlile et al, 2001).

3. Neurospora crassa

Neurospora *crassa* is a fungus of the phylum Ascomycota, and was originally described as an orange mold that infested French bakeries in the 1800's. The fungus was used in the early 1900's by FAFC Went, a Dutch plant physiologist working in Indonesia and Surinam. This was because it was common to use fungus to prepare "ocham" and alcoholic beverages. This fungus was later studied by Shear and Dodge, and was named Neurospora by them due to the similarity of its ascospore wall formations to nerves (Raju, 2009). Carl C. Lindegren and Bernard O. Dodge would later domesticate it, making it a popular experimental organism. This organism would see extensive use in photobiology, the study of circadian rhythms, genetics, developmental biology, evolution and ecology (Borkovich et al, 2004). The field affected most by Neurospora would be genetics, with Beadle and Tatum establishing the field of modern molecular genetics in 1941. Using Neurospora they were able to link genes to proteins and develop the theory of "one gene, one enzyme", revolutionizing the world of genetics (Hynes, 2003).

Neurospora has a number of features that make it a desirable model organism. One of these features is its traceable genetics, while remaining relatively complex. The fungus also produces 28 morphologically distinct cell types and is a multicellular organism (Borkovich et al, 2004). Another feature of this organism that makes it attractive is that it can be grown on a defined media, using minimal nutrients. This allowed for the development of mutant isolations – the first of which was an auxotroph (Hynes, 2003). In addition to the simple nutrient requirements of Neurospora, the organism also has a very rapid vegetative growth cycle, lasting only 2-3 weeks, with two distinct mating types, allowing for genetic crosses. Even though the fungus has a rapid

development, it has very distinct phases of development that can be readily recognized. One stage of development is particularly important to some labs, and that is the formation of large asci, which are meiotic and allow for the studying of the meiotic process. These various features make Neurospora a common organism in the genetics lab to this day (Raju, 2009).

The genome of Neurospora has been sequenced (Galagan et al, 2005). It shows that the genome is 39.9Mb in total length, with 10,082 protein encoding genes. This genome is predicted to have an average of 1.7 introns on each gene. Approximately 41% of the predicted proteins have no matches to any known proteins. This indicates that the genome has a great deal of discovery potential in that almost half of the proteins are ones whose function is unknown. What is further interesting is that 1,421 genes have high matches to plant or animal genes, but very little significant matching to S. *cerevisiae* or S. *pome*. Additionally, a number of gene homologs for Neurospora genes are only found in prokaryotes. This suggests the possibility that these two groups inhabited the same ecological niches, resulting in the conservation of certain critical genes (Hynes, 2003).

4. Fungal Proteomics

Proteomics, especially in regards to fungi, have grown significantly within the past decade. A number of techniques that would never have been thought possible a decade ago are now readily available and used today. The techniques found in comparative proteomics, immunoproteomics, coculture proteomics, and protein localization are all used in modern fungal proteomic research. Even though all of these techniques have their own uses, not every technique is used in every experiment (Doyle, 2011).

Comparative proteomics is one of the most extensively used methods of testing various mutations in filamentous fungi. One form of this method exposure to various stressors and cell perturbants to force a change in the cell's phenotype before comparing it to previously done phenotypic analyses. More recently, however, the method of deleting genes directly, then comparing the proteins of the new mutant with an unaffected wild type of the organism has been used. The use of various vectors allow for specific gene replacement, protein expression, and a host of other phenotypic changes to the organism (Honda and Selker, 2009). This allows a direct comparison to changes within the cells without the need to take into account the effects of various chemicals on other parts of the genome of the organism (Doyle, 2011). This field of proteomics is the one being employed by this study to look at the differences in proteins between a wild type and mutant (*nit-2* knock out) of *N. crassa* to see what proteins are used for nitrogen in low nitrogen conditions and see which of these are under *nit-2* control.

Another school of techniques being utilized is that of pathogen-induced expression. This method allows us to explore the relationship and interactions between fungi and other organisms—particularly those with pathogenicity. A popular topic of research in recent history is that of *Fusarium graminearum*. This fungus is a pathogen that infects wheat, raising concerns for the production of crops. The initial research done involved the mycotoxins produced by *F. graminearum* during early plant infection. This research resulted in a context in which the toxin can be placed for future proteomic studies. Other organisms including *Aspegillus flavus* and *Aspergillus fumigatus* are also capable of pathogenicity, highlighting why knowing pathogen interactions is important (Doyle, 2011).

A much more recent school in immunoproteomics is emerging on the forefront of fungal proteomic research. This method uses techniques from mass spectroscopy and immunoblotting to pinpoint certain immunoreactive molecules from fungi. Some of these molecules can be highly potent allergens. Two fungi the method has been used on it *Cryptococcus gattii* and *Cryptococcus neoformans*. Both of these fungi can be fatal to humans and only infects immunocompromised patients. By mapping the entire "immunome" of these two organisms, insight can be gleaned into possible therapeutic agents and ways to inhibit the damage inflicted by the fungus once infection has already occurred (Doyle, 2011).

Proteomics of different types will continue to play a pivotal role in fungal protein and genetic research. The tools it offers vary enough to allow more insight than has been possible in previous decades. Some problems with proteomics, such as assigning roles to *in silico* genes, remain to be mastered. Other, newer, problems are arising that are being mastered to this day. One of these problems is now the sheer quantity of data we have collected about various fungal species. This problem is being resolved through the expanding field of bioinformatics and the development of online databases that house these vast quantities of information (Doyle, 2011).

5. Nitrogen Metabolism

Nitrogen is an element that is common in many organic molecules, from proteins to DNA. Having a way to break down complex macromolecules containing nitrogen is incredibly useful for an organism whose means of obtaining nitrogen is limited (Marzluf, 1981). Certain compounds, such as glutamine and ammonia, are preferred over most

other compounds as a source for nitrogen, but most fungi, being saprobes, are flexible enough to use a wide variety of molecules as a source of nitrogen (Marzluf, 1997).

An example of a fungus' flexibility towards nitrogen source can be seen in the breaking down of purines to the eventual product of ammonia. These purines are obtained through breaking down the bases adenine and guanine by their respective deaminases (adenine deaminase for adenine, and guanine deaminase for guanine). Adenine deaminase produces hypoxathine, which needs further broken down into xanthine by purine dehydrogenase. Guanine deaminase results in xanthine directly. Xanthine is then broken down into uric acid by purine dehydrogenase. The uric acid is broken down into allantoin by uricase, and allantoin is broken down into allantoic acid by allantoinase. Allantoinase is used again to break down allantoic acid, resulting in the two products ureidoglycollate and urea. Ureidoglycollate is converted into urea by ureidoglycollase. Finally, the urea products are broken down by urease to ammonia – a preferred nitrogen source. This pathway is for *Aspergillus nidulans* as described, but is entirely identical in *N. crassa* (Figure 1) (Marzluf, 1997).

Purines, however, are not the only source of nitrogen a fungus can use. A number of amino acids, including alanine, arginine, glutamate, glutamine, and glycine, can be used by *N, crassa* as a source of nitrogen. Five transporter systems have been recognized that are used in the uptake of amino acids. Each amino acid is taken up by a number of systems that varies from amino acid to amino acid. Each of these systems can be controlled individually. One such example of this regulation can be seen in "System IV", which can be controlled by starving the organism of certain vital molecules, like nitrogen, sulfur, and carbon. In this system, if a peptide is short enough, the nitrogen can be

removed without having to break down the entire peptide into its individual amino acids (Davis, 2000).

In *N. crassa*, proteins also can serve as nitrogen sources. Proteases required for this process can be seen to appear after starvation of nitrogen, or if the organism is grown in a particularly bad nitrogen source. The nature of what induces this effect is not presently known. One idea is that certain concentrations of growth agent in the media induces, but varying concentrations of media have never been tested (Davis, 2000).

Breaking down nitrogen products into ammonia is also not the final stage for nitrogen in *N. crassa*. Ammonia is also used to make glutamate and glutamine. The pathway for the production of glutamate uses NADPH as a reducer and the constitutive enzyme NADPH-GDH to reduce α-ketoglutarate and NH_4^+ to glutamate. This pathway is used as the main route of NH_4^+ assimilation when the molecule is plentiful. The pathway for the production of glutamine is 2-part in that it is used to make glutamine and break it back down into glutamate. Glutamine synthetase uses NH_4^+, ATP, and one molecule of glutamate to produce glutamine. Glutamine can then be broken down by NADPH and α-ketoglutarate, in what is called the GOGAT cycle, to produce two molecules of glutamate. Although this process uses ATP, it yields a net gain of one glutamate. This makes it much more useful at low concentrations of nitrogen (Figure 2) (Davis, 2000).

Figure 1: Catabolic pathway of purines into ammonia by *Aspergillus nidulans.* Adapted from "Regulation of Mitrogen Metabolism and Gene Expression in Fungi" By George A. Marzluf.
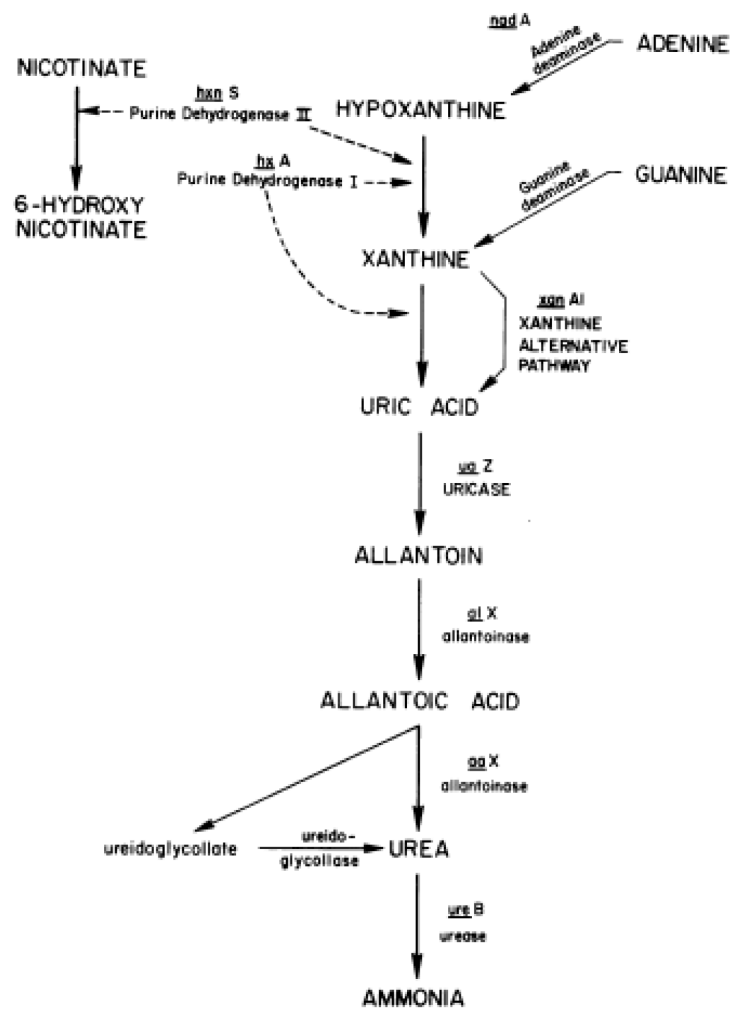
6. Nit-2 Mutant

As previously mentioned, *N* crassa breaks down proteins to use as a nitrogen source when the environment limits the amount that can be obtained externally. The question then that is raised is to know what proteins are needed to obtain more nitrogen. One way to determine this would be to look at the wild type protein profile and compare it to a mutant during times of low nitrogen availability. One of the best genes to knock out is the *nit-2* gene.

The *nit-2* gene is a regulatory gene that controls nitrogen catabolism. It regulates transcription of both nitrate reductase and nitrite reductase, and a number of other enzymes involved in metabolism of alternative nitrogen sources (Marzluf, 1997). To function properly, *nit-2* is dependent on a number of other genes. The *nit-4* gene product acts positively on *nit-2* at the level of transcription. In this pathway, nitrate is the inducer. This reliance of *nit-2* on *nit-4* is not one way, however. If there is a mutation in either gene, neither of the protein products required for nitrate assimilation will be expressed. A third protein, produced by the *nmr-1* gene, is also able to function on this pathway. This enzyme acts as a repressor to the other two regulators (Davis, 2000).

The *nit-2* gene also affects the *nit-3* gene transcription – the gene responsible for nitrate reductase. NIT-2, the protein product of the *nit-2* gene, binds at two copies of a GATA sequence upstream of the *nit-3* gene. These two upstream sites are found at over 1kb and at 180bp from the 5' start. These two sites are both required for full regulation, with a deletion of either site resulting in an inactive *nit-3* gene. The *nit-3* gene is also bound by NIT-4, the nit-4 gene product. The binding sites of the NIT-2 and NIT-4

proteins are very close, and their interaction is necessary for normal nit-3 function (Davis, 2000).

NMR-1, the protein product of the *nmr-1* gene, also affects the NIT-2 protein in addition to the gene *nit-2*. The NMR-1 protein represses *nit-2* function by binding the NIT-2 protein, causing the inhibition of DNA binding of NIT-2. This is known because when a mutation exists in the *nmr-1* gene, leading to a non-functioning or non-existent NMR-1 protein, *nit-2* is able to function normally, even if cellular indicators are present that should stop its function (Figure 3) (Davis, 2000).

Mutations in the *nit-2* gene lead to a number of problems with the fungal cell's capability of breaking down molecules to obtain and assimilate alternative nitrogen sources. As previously noted, the *nit-2* gene has a role in interactions with *nit-3*, *nit-4*, and *nmr-1*. If the *nit-2* gene is not functioning properly, the functions of certain gene complexes, such as NIT-2, NIT-4, and NIT-3, will not function properly. In some cases, such as *nit-3* gene activity, if *nit-2* is deleted or mutated, it will not function at all.

The *nit-2* mutant used in this study is a knock out of the *nit-2* gene. This knock out was achieved through means outlined by Colot (Colot, et al 2006). More specifically, the strategy used the construction of a deletion cassette DNA. This was done by creating a 5' and 3' flank that were made by using restriction enzymes at the ends of the ORF of the gene to be deleted. The 5' and 3' flank sequences would then bind to the *hph* cassette and a yeast shuttle vector that had been opened to allow the free sticky ends of the 5' and 3' end to bind (Figure 4). Amplification of the shuttle vector was then done by PCR and the vector was cotransformed into *Neurospora.* The inserted *hgh* gene would

Figure 2: Major routes of nitrogen assimilation yielding glutamate and glutamine.

As adapted from <u>Neurospora: Contributions of a Model Organism</u>. Davis, Rowland H.,

2000.

15
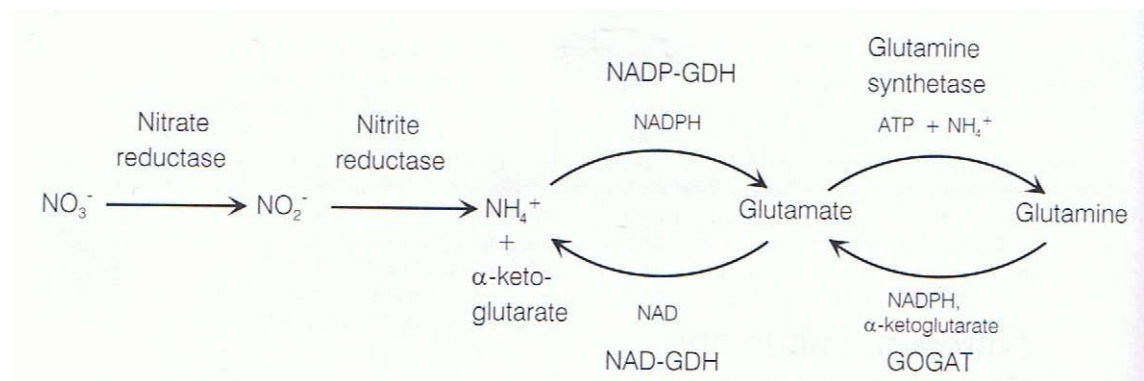
Figure 3: Construction of the *nit-2* mutant as adapted from "A high throughput gene knockout procedure for *Neurospora* reveals functions for multiple transcriptions factors" by Colot et al.
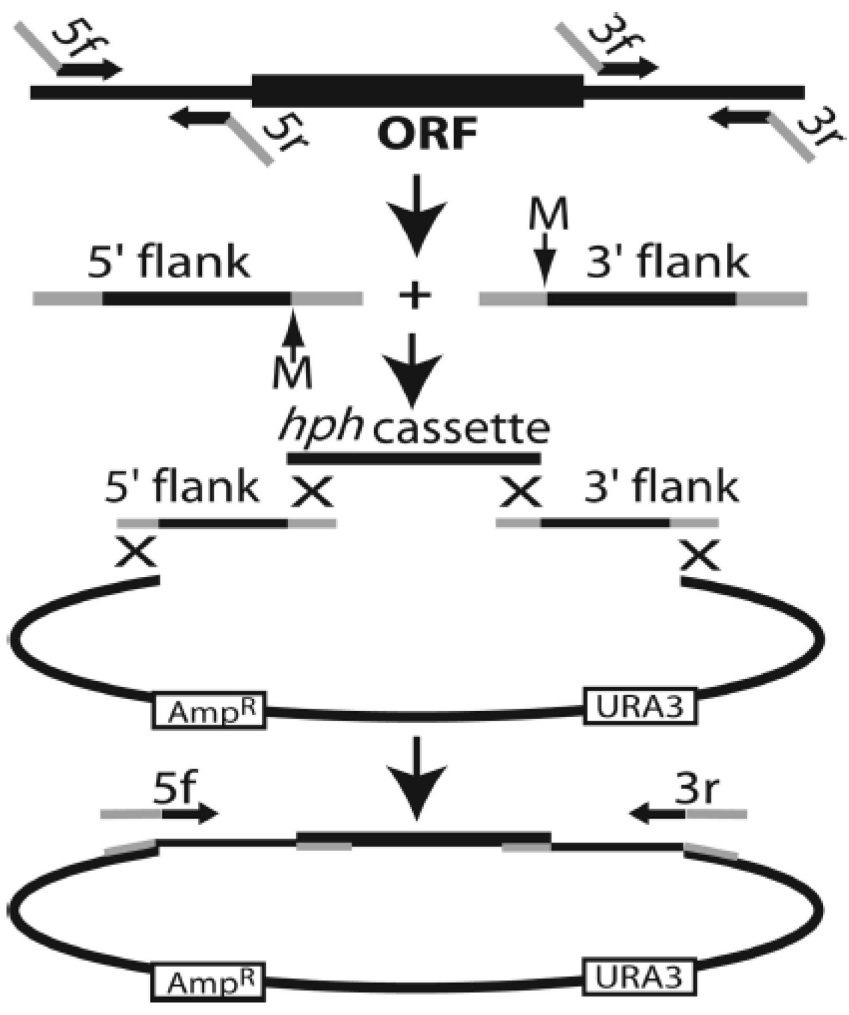
act as a selectable marker through conveying hygromycin resistance by allowing the production of hygromycin B phosphotransferase (Colot et al, 2006). This cleanly disrupts the *nit-2* gene without causing any other mutations.

7. Recent NIT-2 and Homolog Proteomics Work

Some work has been done previous on nit-2 in *Neurospora crassa* and its homologs, such as AreA in *Penicillium marneffei*. The first study to highlight involving proteomic work was done by Hayley E. Bugeja et. Al. In this study, AreA is looked at in the way it controls nitrogen source utilization during both growth forms of *Penicillium marneffei*. This was to get a deeper insight into the function of both AreA and how nitrogen source controls affect pathogenicity of the fungus. What was found, through the use of an AreA knockout mutant, was that pathogenicity as a whole is affected by the AreA gene. This pathogenicity is affected by the ability of the fungus to obtain nitrogen from non-preferred nitrogen sources during different growth phases. Additionally, it was found that AreA was not needed for the utilization of amino acids as a sole nitrogen source and that AreA was not needed for filamentous growth as a response from poor nitrogen availability (Bugeja et al, 2011).

The next study, done by Xiaokui Mo and George Marzluf , looked at the way NIT-2 and NIT-4 cooperate in gene expression in *N. crassa*. This study revealed that NIT2 and NIT4 are both required for the expression of a specific protein—cys-14. The reason cys-14 was looked at was because it is a gene that is normally controlled by one regulatory protein. Their results showed that neither NIT2 or NIT4 could induce expression of this gene by themselves, but together could get a strong response. The problem that arises

from this research is that it only allowed for the researchers to look at one gene control through electrophoretic mobility shifts, which only looked at DNA binding. This meant that the researchers could not look at entire protein profiles, but had to specify which proteins they were going to look at based on theoretical work leading to only one protein being looked at at a time, whereas now an entire protein profile can be looked at and analyzed, giving a broader spectrum of information (Mo et al, 2002).

The final study was done by Hongoo Pan, Bo Feng, and George Marzluf, and looked at the role of NIT2 an NMR in nitrogen metabolite repression. The primary focus of the stuady was to look at how certain parts of NIT2 are required for binding to certain genes. What was found was that most of the mutants of NIT2 made resulted in truncated proteins and were still capable of binding to their respective binding sites. This meant that proper nitrogen repression of nitrate reductase was still happening, despite these mutations as indicated by the DNA binding. To add to this, however, they found that there were certain regions that, when mutated, caused drastic changes in the functionality of nitrogen repression. Specifically, mutations in the zinc finger and carboxy-terminal tail resulted in a loss of NIT2 and NMR interactions, thus limiting nitrogen repression. Once again, this study was only able to look at one protein at a time, using limited techniques in enzyme activity assays (Pan et al, 1997).

Materials and Methods

1. Strains

Wild-type *Neurospora crassa* 74A (FGSC No. 2489) was obtained and used in conjunction with mutant *Neurospora crassa* un-16; mat A (FGSC No. 11392) throughout the course of this study.

2. Tissue Growth

The wild-type and mutant *Neurospora crassa* was inoculated into 50mL minimal media containing 1.5% agar and 2% sucrose in a 250 Erlenmeyer flask. The of *N. crassa* was then incubated at 30° C for 3 days, after which it was grown under fluorescent light at room temperature for 14 days. The conidia were then harvested by adding 25mL of Vogels media (Vogels, 1956) to the flask and swirling until all loose conidia and tissue were in the liquid media. The liquid was then passed through sterile cheesecloth into a new, sterile Erlenmeyer flask. The harvested liquid was then split into even portions and added to two new 50mL Vogels media containing 2% sucrose. These cultures were grown overnight at 30° C in a shaker at approximately 130rpm. The mycelia from the overnight culture were collected through suction filtration using Whatman® filter paper. Mycelial pads were then washed with sterile water and refiltered. One wild-type and one mutant mycelial pad were then transferred to a new flask of 50mL Vogels media containing 2% sucrose (one flask for each sample), while the other wild-type and mutant pads were then transferred to flasks of 50mL of Westergaard media (Westergaard and Mitchell, 1947) containing 2% sucrose (one flask per sample). The cultures were then grown at 30° C in the shaker at

approximately 130rpm for 3 hours. The mycelia obtained were filtered separately and stored separately at -80° C.

3. Protein Isolation

After freezing completed the mycelia pads were ground in liquid nitrogen and baked sand, using a mortar and pestle. The ground tissue was transferred to 1.5 mL Eppendorf tubes until the tube was roughly half full. 800 μL of lysis buffer (200mM Tris-HCL, 10mM NaCl, 0.5mM Deoxycholate) was added to each Eppendorf tube containing the ground mixture. The Eppendorf tubes were then vortexed for 1 minute then iced for 2 minutes. This vortex and icing was repeated three times. The tubes were then spun in the centrifuge for 10 minutes at 12,000 rpm at 4°C. After centrifugation, the supernatant was collected (700 μL) and transferred to new 1.5 mL Eppendorf tubes and stored at -80° C.

4. Protein Quantification

The collected protein samples were first ran on 1-dimensional, handmade 10% acrylamide gels to test for the presence of protein. The gels contained 58mL water, 15mL 15% acrylamide, 25mL 1.5M Tris (pH 8.8), 1mL SDS, 1mL ammonium persulfate, and 800μL TEMED. Loaded into each well was 10 μL of each protein sample added to 10 μL of 2x SDS-PAGE buffer (2.5% SDS, 25% glycerol, 100mM DTT, 125mM Tric-HCl, 0.01% Bromophenol Blue) from their own new Eppendorf tubes. One well contained 10 μL of ladder (Protein Precision Plus® by Bio-Rad ™). Five wells contained BSA standards (100μg Bovine Serum Albumin per 1mL water) in a 1:1 ratio with 2x SDS-PAGE buffer. The BSA standards were 200ng (2 μL),

Table 1: 50X Vogels Media:

Na3 citrate- 51/2 H2	150g
KH2PO4 anhydrous	250g
NH4NO3	100g
MgSO4- 7H2O	10g
CaCl2 anhydrous	5g
Trace element solution	5 mL
Biotin solution	5 mL
Water	750 mL

Table 2: 10X Westergaard Media

KNO3	20g
KH2PO4	20g
MgSO4 - 7H2O	10g
NaCl2	2g
CaCl2	2g
Trace element solution	2mL
Biotin	2mL
Water	to 1 liter

Table 3: Biotin Solution

Biotin	5.0 mg
50% Ethanol	50 ml

Table 4: Trace Elements Solution

Citric acid 1H2O	5.0 g
ZnSO4-7H2O	5.0 g
Fe(NH4) (SO4)2 – 6H2O	1.0 g
CuSO4 -5H2O	0.25 g
MnSO4 – 1H2O	0.25 g
H3BO3 anhydrous	0.05 g
Na2MoO4 – 2H2O	0.05 g
Chloroform	0.05 g
Water to make 100 ml	1.0 mL

400ng (4 µL), 600ng (6 µL), 800ng (8 µL), and 1000ng (10 µL). The gels were then run at 100V for approximately 1 hour.

After protein was confirmed, quantification gels (8-16% Criterion™ TGX™ Precast Gels) were ran. The same loading procedure as the test gels was used in that loaded into each well was 10 µL of each protein sample added to 10 µL of 2x SDS-PAGE buffer from their own new Eppendorf tubes. One well contained 10 µL of ladder (Protein Precision Plus®). Five wells contained BSA standards in a 1:1 ratio with 2x SDS-PAGE buffer. The BSA standards were 200ng (2 µL), 400ng (4 µL), 600ng (6 µL), 800ng (8 µL), and 1000ng (10 µL). The gels were then ran at 100V for approximately 1 hour. Once the gels finished, they were stained in Coomassie Blue stain for one hour. After the Coomassie blue stain, they were destained in high destain for one hour, then low destain overnight. The gels were then imaged using the PharosFX™ imaging system by Bio-Rad®, and concentrations of proteins were determined using ImageJ.

5. Two-Dimensional Gel Electrophoresis

Protein extract from *Neurospora crassa* was obtained from the -80° C freezer and thawed. The thawed protein was then added in a manner to obtain a total of 125ng of protein in the sample, and a volume of MSB (8-9.8M Urea, 0.5% CHAPS, 10mM DTT) was added to raise the total volume of the protein to 200 µL. The 200 µL of MSB/protein mixture was then added to the IEF focusing tray. Wicks were then added over the electrodes of the refocusing tray and water was added to the wicks to allow for a full contact to be made to the gel strip. An 11cm IGP strip (Bio-Rad ReadyStrip™ 11cm, 4-7pH) was then laid gel side down on the MSB/protein mixture in the IEF tray (positive

side to positive side and negative side to negative side) and allowed to absorb into the gel strip for one hour. After this one hour, 3mL of mineral oil was added to the top of each IPG strip to prevent dehydration. Active rehydration was then performed by allowing the machine to sit, inactive, for 12 hours before running the IEF refocusing procedure. For these 11cm gel strips, the program was set to the preset method at linear ramp mode for a total V-Hour of 35,000V. After running for the 35,000 V-Hour, the gels were held at 500V until removed.

Once refocusing was done, if the gel strips were not able to be run that day were wrapped in parafilm and frozen overnight at -80° C. The strips were run on 8-16% Criterion™ TGX™ Precast Gels. To do this, the gel strips were first drained of the mineral oil that covered them from the focusing tray, then blotted to remove excess oil. Once the excess oil was removed, the IPG strips were immersed in Equilibration Buffer I (6M urea, 2% SDS, 0.375M Tris-HCL, pH 8.8, 20% glycerol, 2% DTT) and placed on orbital shaker for 10 minutes. After the 10 minutes on the orbital shaker, the Equilibration Buffer I was drained, and the gel strips were immersed in Equilibration Buffer II (6M urea, 2% SDS, 0.375M Tris-HCL, pH 8.8, 20% glycerol, 2.5% iodoacetamide) and placed back on the orbital shaker for an additional 10 minutes. After the 10 minutes on the orbital shaker, the gel strips were drained of the Equilibration Buffer II and dipped into 1x TGS buffer to remove any excess buffer. Once the gels were dipped, the Precast Criterion Gels were obtained from the refrigerator and the gels were placed in the long opening at the top of the gel, with the positive side of the strip closest to the empty ladder well. The strips were then covered with overlay agarose (0.5g agarose, 100 mL 1 X TGS buffer, 1 gram of bromophenol blue) and the agarose was allowed to solidify. Once solidified, the gels

were placed into the electrophoresis cell and the cell was filled with 1x TGS buffer to run the gels in the second dimension. The ladder well was then loaded with 10 µL of ladder (Amresco® Wide-Range Protein Molecular Weight Marker™). The gels were ran at a constant 200V for approximately 2 hours or until the bottom of the ladder reaches the bottom of the gel. After electrophoresis was finished, the gels were stained with Bio-Rad™ SYPRO® Ruby Red stain.

To stain with SYPRO® Ruby Red, each gel was placed into its own container and was covered with SYPRO® Ruby Red stain. The gels were then placed on the orbital shaker overnight to complete the staining. After shaking overnight, the gels were drained of the SYPRO® Ruby Red stain and immersed in water for 1 hour on the orbital shaker. Once the gels were rehydrated, they were imaged. After imaging the gels were placed in a solution of 5% Acetic Acid (5% acetic acid to 95% water).

6. Imaging and Analysis

The 2 dimension gels were imaged using the PharosFX™ imager. After imaging, the gels were saved in original image and .jpeg format. These images were then analyzed through the use of Bio-Rad ® PDQuest™ version 7.3. The spot analysis was done to look at differences between no more than 3 gels at a time. The first set of gels compared was the Vogels grown wild-type against the Westergaard grown wild-type. The second set was the Westergaard grown wild-type measured against the Westergaard grown mutant. A third set was looked at containing all three and spots that existed in the Westergaard wild-type that were not in the Vogels wild-type were check for presence in the

Westergaard mutant. Spot differences were marked on gel images and referred to later for spot excision and mass spectroscopy analysis.

7. Spot Excision and Mass Spectrometry

The spots marked in each gel image were carefully analyzed by density of the spot and presence to see if the protein in the spot is controlled by *nit-2*. If a spot is deemed important, it was excised and shipped to the Ohio State University for mass spectroscopy analysis. Protein spots were carefully labeled with their condition and a number (ex: Westergaard Wild Type 5 was made into WWt-5) before excision for tracking. The following was then done by The Ohio State University Proteomics Lab as adapted from the Ohio State University Proteomics Lab protocol:

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 -80°C until

samples can be analyzed.

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 200P^{oP}C 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

We examined the protein profiles of *Neurospora crassa* that were grown in the presence of two different nitrogen sources in order to identify proteins that were not only induced by a poor nitrogen source, but possibly also controlled by the *nit-2* gene. The two media types used were Vogels media, which contains ammonia (a preferred source of nitrogen), and Westergaards media, which contains nitrate (a non-preferred source of nitrogen). In addition to the use of two nitrogen sources, two strains of *N. crassa* were used -- a wild type strain (74A) and a *nit-2* knockout mutant (FGSC #11392). These two strains were grown in a liquid Vogels media (2% sucrose) overnight and then shifted to either liquid Vogels media or liquid Westergaards media. This gave us a total of four conditions to examine the protein profiles of; Vogels wild type, Vogels nit-2 mutant, Westergaard wild-type, and Westergaard mutant. After the mycelia was grown in these conditions, it was collected via suction filtration and frozen for protein extraction as described earlier.

In order to know how much protein sample to load in μl for the 2-D gels we first had to run a 1-dimensional (1-D) gel to quantify our protein sample so as to prevent over loading the 2-D gels. An additional benefit of running a 1-D gel was that we could get a preliminary look at how the protein profiles were affected under each condition. The 1-D gel used was an 8%-16% gradient Criterion™ TGX gels from Bio-Rad®. The gel was loaded with each protein sample to be quantified, a ladder (Ameresco® Wide Protein Molecular Weight Marker), and a set of Bovine Serum Albumin (BSA) standards (made 100ng BSA to 1mL volume) to measure the density of our bands against. The Bovine Serum Albumin levels were 200ng, 400ng, 600ng, 800ng, and 1000ng and were loaded

with a 1:1 ratio of 2x SDS-PAGE buffer. Figure 4 shows the results of this quantification.

After running, the gel was stained with Coomassie Blue stain. What can be seen is that

there is a marked difference between both the protein quantity and the protein distribution

between the Westergaards shifted wild type and the rest of the conditions, and both of the

mutants from the other conditions. Most notably, the Westergaard wild type lane seems to

be missing a great deal of protein as compared to the other lanes. This indicates a

preliminary expectation of differences between the protein profiles to be generated in the

2-D gels and the quantity of protein expected to be seen on the 2-D gels. Figure 4 shows

the resulting 1-dimensional gel. The protein volumes per 10μL were 35.4μg for Vogels

wild type, 28.3μg for Vogels mutant, 27.2μg for Westergaards wild type, and 29.7μg for

Westergaards mutant.

All protein profiles that were generated by two-dimensional gel electrophoresis

(2DGE or 2-D) and used in this study were in the standard format of 11cm, had a pI range

of 4-7, and ran in triplicate to ensure consistency. In order to visualize the protein spots

on the gels, SYPRO® Ruby Red stain was employed. Due to the SYPRO® stain being a

fluorescent stain, we had to image the gels in an imaging program using a gel scanner (the

Pharos FX imaging system). These gel images were then ran through PDQuest™ by Bio-

Rad® to filter the images and make them cleaner. The resulting triplicate 11cm gels can

be seen in Figures 5, 6, 7, and 8. Figure 5 shows the filtered images obtained from

scanning the gel images from the wild type *N. crassa* tissue shifted into Vogels media.

Figure 6 shows the filtered images obtained from scanning the gel images from the wild

type *N. crassa* tissue shifted into Westergaards media. Figure 7 shows the filtered images

obtained from scanning the gel images from the *nit-2* mutant *N. crassa* tissue shifted into

Vogels media. Figure 8 shows the filtered images obtained from scanning the gel images

from the *nit-2* mutant *N. crassa* tissue shifted into Westergaards media. Once we felt we

obtained adequate 2-D gels in triplicate from each condition, we moved on to analysis of

the gel images in their respective groups, then in higher level matched sets.

       In order to adequately analyze the gels, we employed the use of PDQuest™ gel

analysis software by Bio-Rad®. Using PDQuest™, we first made lower level matched

sets for each condition. These matched sets were turned into normalized, Gaussian images

through PDQuest™. Our experiment utilized 4 conditions spanning 2 nitrogen sources

and 2 genetically different *N. crassa*; wild type in Vogels, wild type in Westergaard, *nit-2*

mutant in Vogels, and *nit-2* mutant in Westergaard. Thus, the 4 conditions used were each

made into their own matched set and included a master image for each set that was a

Gaussian image. These Gaussian lower level matched sets were then used to make higher

level matched sets that allowed us to look at this differences between the various

conditions. Figures 9-16 show the results of the higher level matched sets in their

respective image sets, including unique spots, and through venn diagram comparisons.

Figure 9 shows the higher level set comparing wild type in Vogels and wild type in

Westergaard. The wild type in Vogels has 203 total spots with 166 unique spots. The

wild type in Westergaard has 109 total spots, with 72 unique spots. These unique spots

can be seen highlighted in Figure 10. Figure 11 shows a venn diagram that is

representative of spots unique to each of the 2 aforementioned conditions (unique to

Vogels wild type or unique to Westergaard wild type) and the number of spots

Figure 4: 1-dimensional electrophoretic gel (Criterion® 8-16% gradient by Bio-Rad) was run to determine protein concentrations. BSA standards were used for measurement and loaded with a one-to-one ratio of 2x SDS buffer. Ladder was loaded on each side of the BSA standards. Lanes labeled with contents. Unlabeled lanes contained protein ran for quantification from a separate experiment.
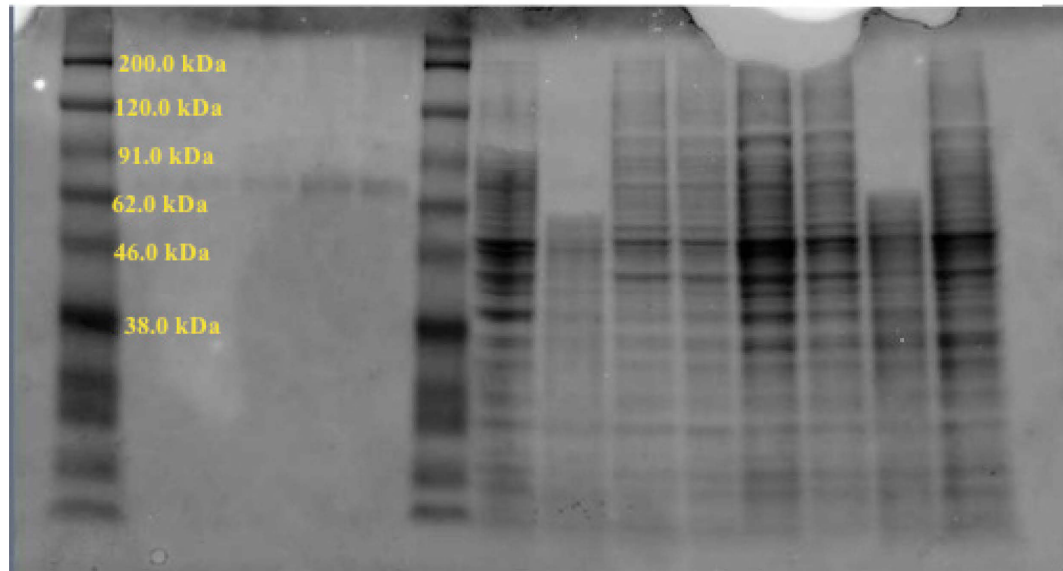
Blue Step Protein Marker
BSA Marker 200 ng Protein
BSA Marker 400 ng Protein
BSA Marker 600 ng Protein
BSA Marker 800 ng Protein
BSA Marker 1000 ng Protein
Blue Step Protein Marker
Wild Type *N. crassa* grown on Vogels Media
Wild Type *N. crassa* grown on Westergaard Media
N. crassa Mutant *Nit-2* knockout grown on Vogels Media
N. crassa Mutant *Nit-2* knockout grown on Westergaard Media

200.0 kDa
120.0 kDa
91.0 kDa
62.0 kDa
46.0 kDa
38.0 kDa

Figure 5: SYPRO® Ruby Red stained gels (11cm, pI range of 4-7) showing wild type *N. crassa* proteins from tissue grown in Vogels minimal media, shifted into Vogels minimal media and resolved by two-dimensional gel electrophoresis. Proteins were extracted as described earlier in the Materials and Methods. Gels were done in triplicate.

Figure 6: SYPRO® Ruby Red stained gels (11cm, pI range of 4-7) showing wild type *N. crassa* proteins from tissue grown in Vogels minimal media, shifted into Westergaards media and resolved by two-dimensional gel electrophoresis. Proteins were extracted as described earlier in the Materials and Methods. Gels were done in triplicate.
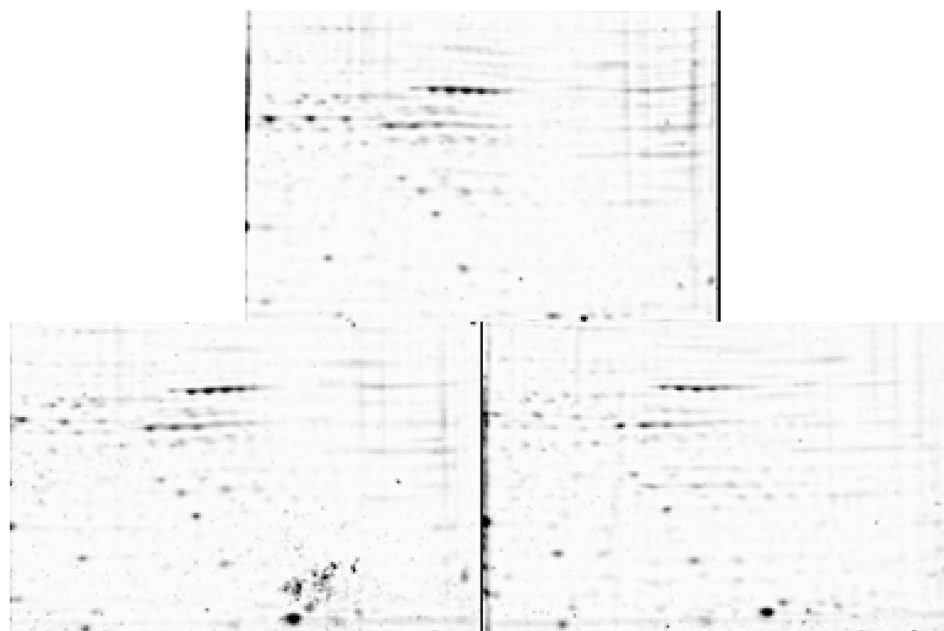
Figure 7: SYPRO® Ruby Red stained gels (11cm, pI range of 4-7) showing *nit-2* mutant *N. crassa* proteins from tissue grown in Vogels minimal media, shifted into Vogels media and resolved by two-dimensional gel electrophoresis. Proteins were extracted as described earlier in the Materials and Methods. Gels were done in triplicate.
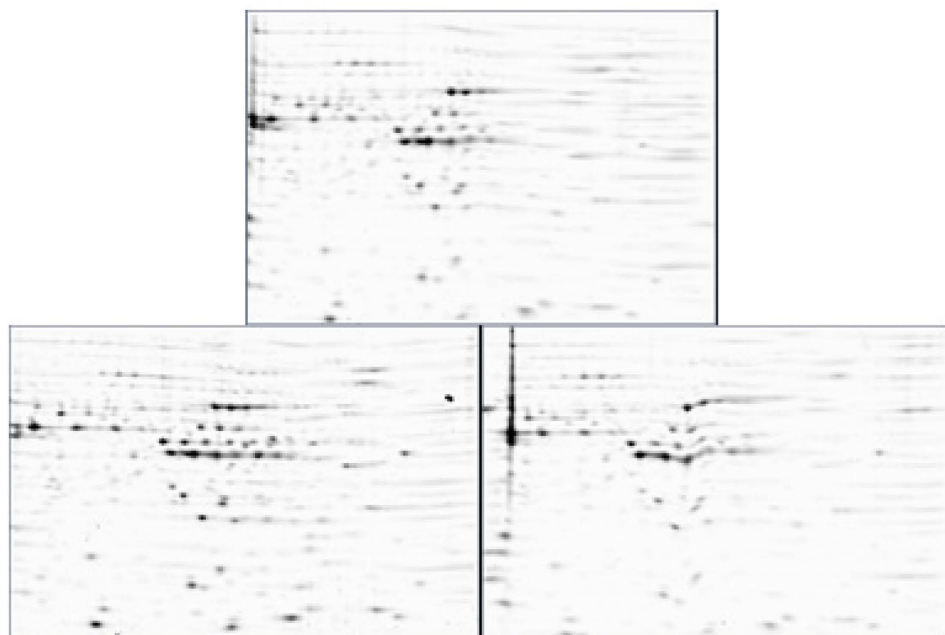
Figure 8: SYPRO® Ruby Red stained gels (11cm, pI range of 4-7) showing *nit-2* mutant *N. crassa* proteins from tissue grown in Vogels minimal media, shifted into Westergaards media and resolved by two-dimensional gel electrophoresis. Proteins were extracted as described earlier in the Materials and Methods. Gels were done in triplicate.
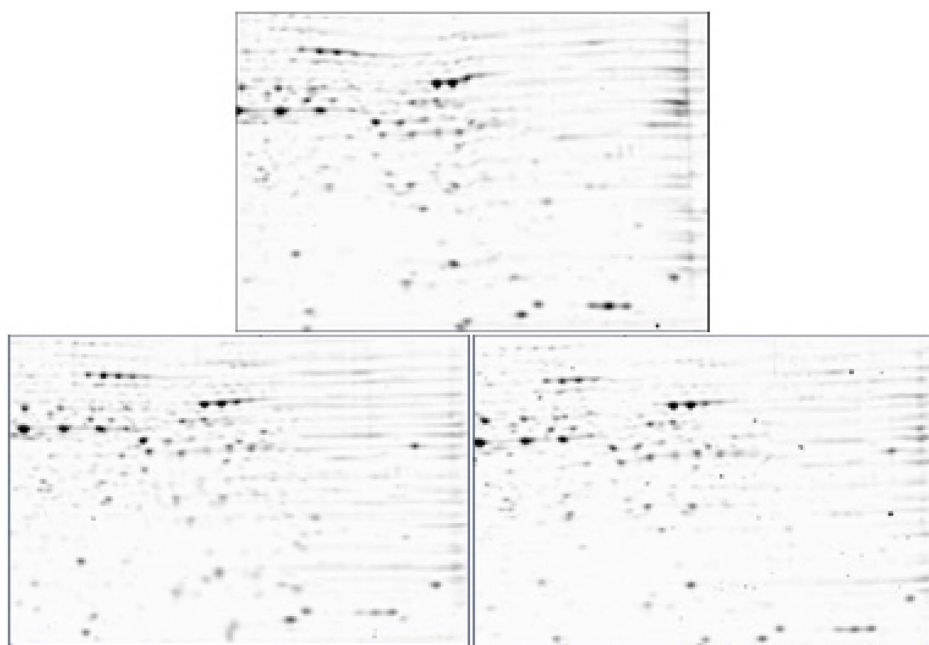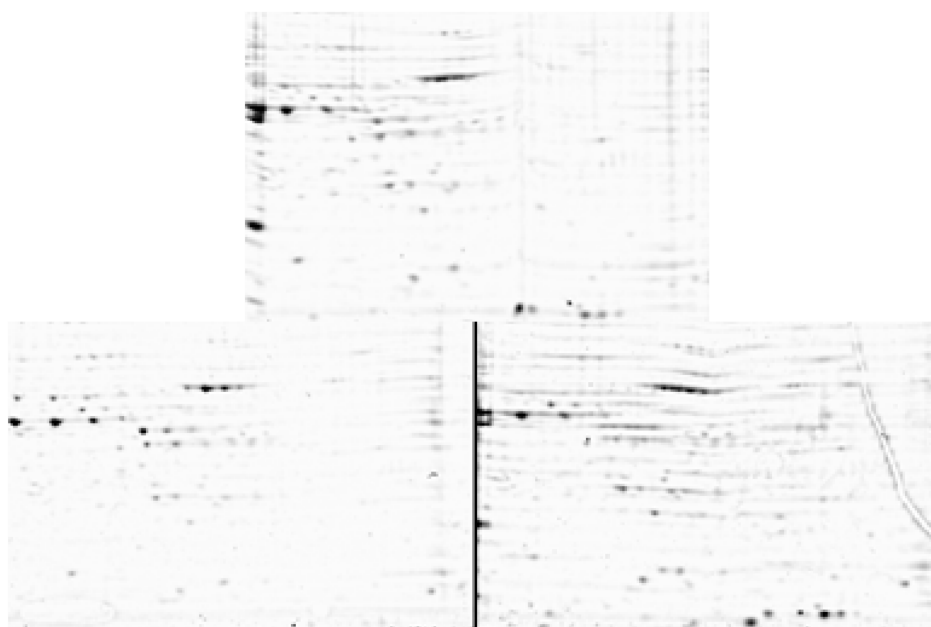
shared between them.   Figure 12 shows the higher level matched set comparing wild type

in Westergaard to mutant in Westergaard.In this comparison the wild type in Westergaard

has 109 total spots, but only 66 unique to it. The mutant in Westergaard has 108 total

spots, with 65 unique spots. These unique spots can be seen highlighted in Figure 13.

Figure 14 shows a Venn diagram that is representative of spots unique to each of the 2

aforementioned conditions (spots unique to Westergaard wild type or spots unique to

Westergaard mutant) and the number of spots shared between them. Figure 15 shows a

higher level matched set comparing all 3 aforementioned conditions (Vogels grown wild

type, Westergaard grown wild type, and Westergaard grown mutant). Figure 16 shows a

Venn diagram that is representative of the total number of spots on each condition, the

spots shared between 2 conditions, and the spots that are found in all conditions. The total

number of spots shared between all conditions is 17. The final matched set looked at was

between the masters of the Vogels grown Wild Type and the Vogels grown *nit-2* mutant.

Figure 18 shows a higher level matched est comparing the Vogels grown wild type and the

Vogels grown *nit-2* mutant. Figure 19 shows the spots unique to each condition when

compared. The *nit-2* mutant in Westergaard had a total of 330 spots, with 229 unique and

101 shared. The wild type in Westergaard had 102 unique spots, giving a total of 203

spots. A Venn diagram highlighting the number of unique and shared spots can be seen in

Figure 20.

    After comparing higher level matched sets, we identified proteins that would be

valuable for excision and mass spectrometry analysis. These proteins had to fit certain

criteria to be

Figure 9: Higher Level matched set comparing master gels of wild type *N. crassa* grown on Vogels minimal media and wild type *N. crassa* grown on Westergaard media.
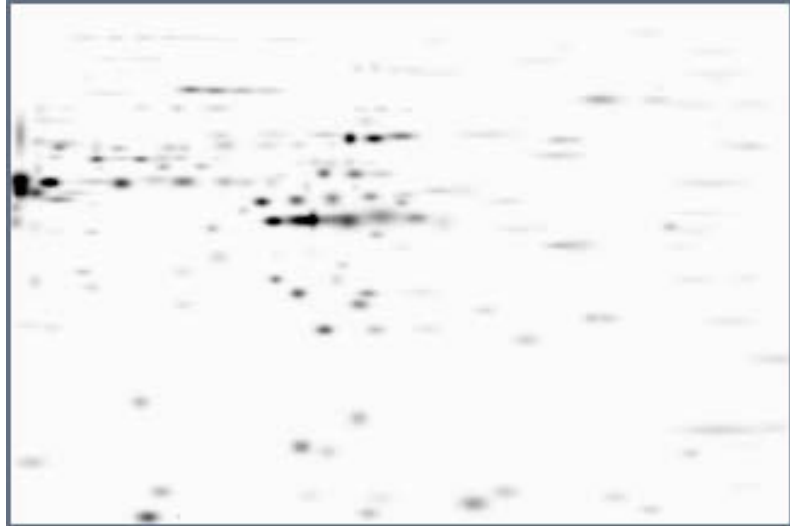
Vogels Wild Type Master
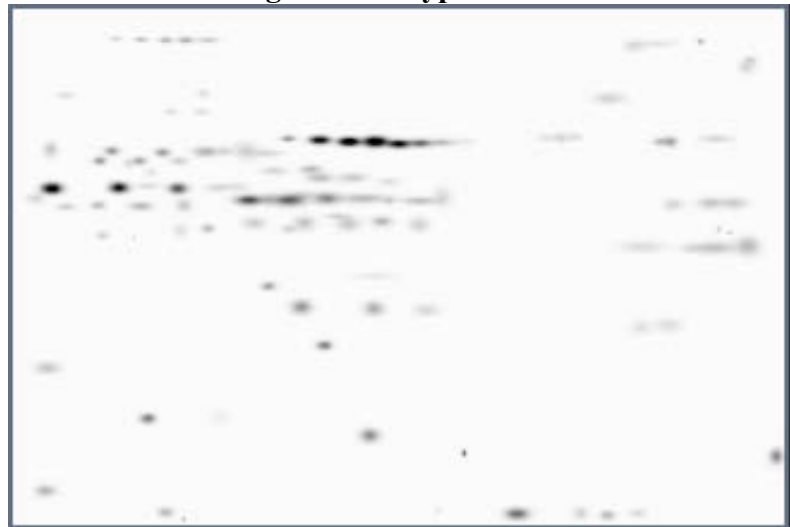
Westergaard Wild Type Master
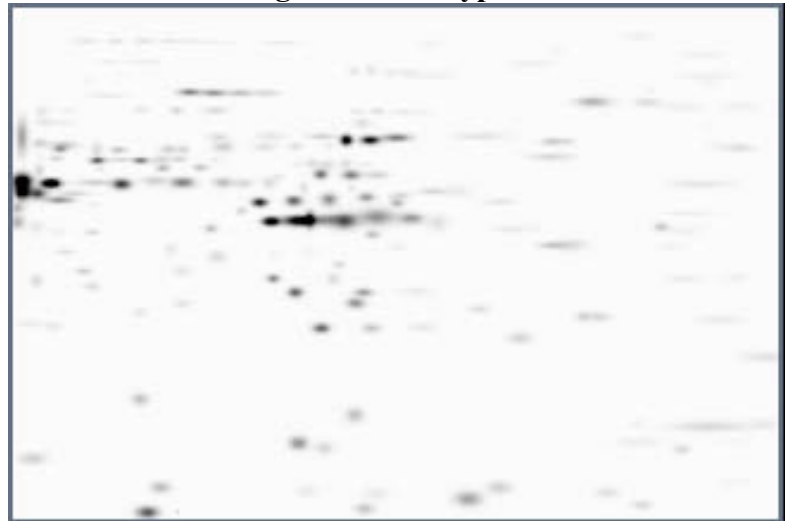
Wild Type Higher Level Master

Figure 10: Spot comparison between wild type *N. crassa* grown on Vogels minimal media and wild type *N. crassa* grown on Westergaard media. Unique spots to each respective gel are circled in red.

Vogels Wild Type Master
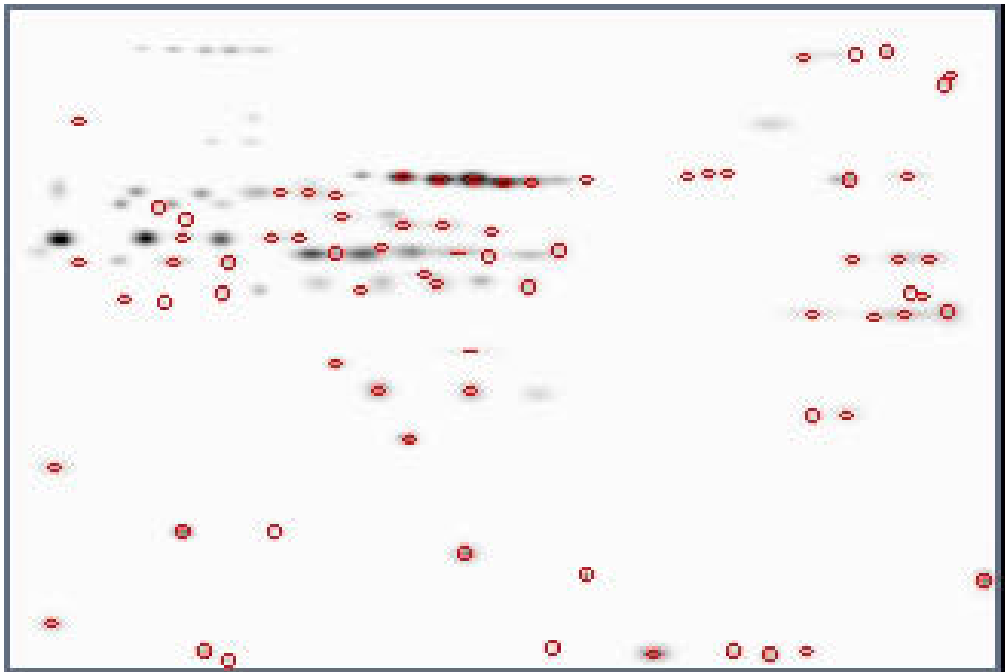
Westergaard Wild Type Master

Figure 11: Venn diagram comparing spot numbers between wild type *N. crassa* grown on Vogels minimal media and wild type *N. crassa* grown on Westergaard media. The number where the circles overlap is the total number of spots found to be shared in both gels. The side circle numbers are the unique number of spots for each respective gel.

Vogels Wild Type Unique   Westergaard Wild Type Unique

166   37   72

Figure 12: Higher Level matched set comparing master gels of wild type *N. crassa* grown on Westergaard media and *nit-2* mutant *N. crassa* grown on Westergaard media.

Westergaard Wild Type Master
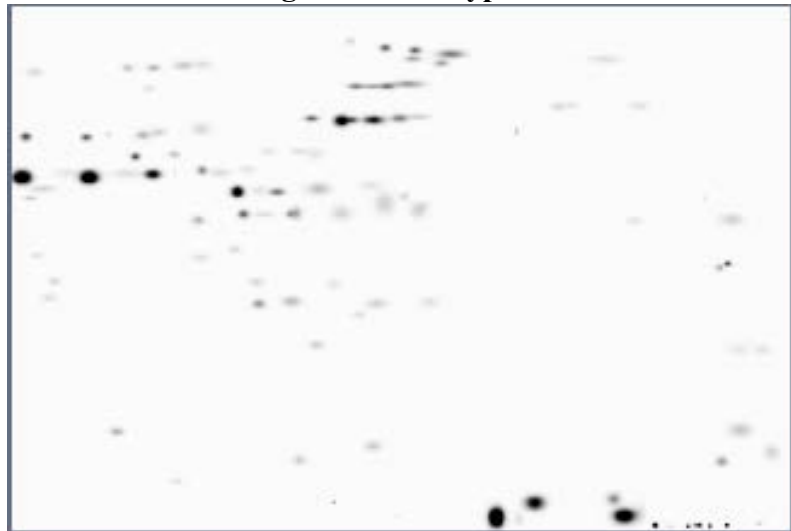
WEstergaard Mutant Master
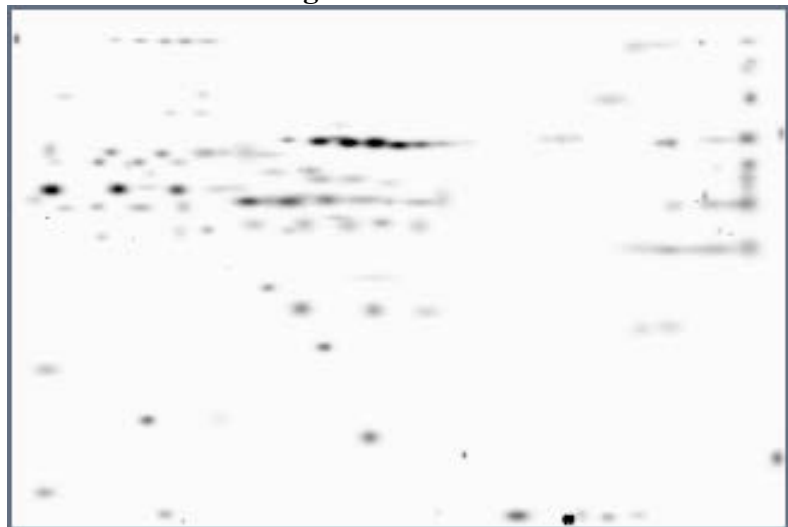
Westergaard Higher Level Master

Figure 13: Spot comparison between wild type *N. crassa* grown on Westergaard minimal media and *nit-2* mutant *N. crassa* grown on Westergaard media. Unique spots to each respective gel are circled in red.
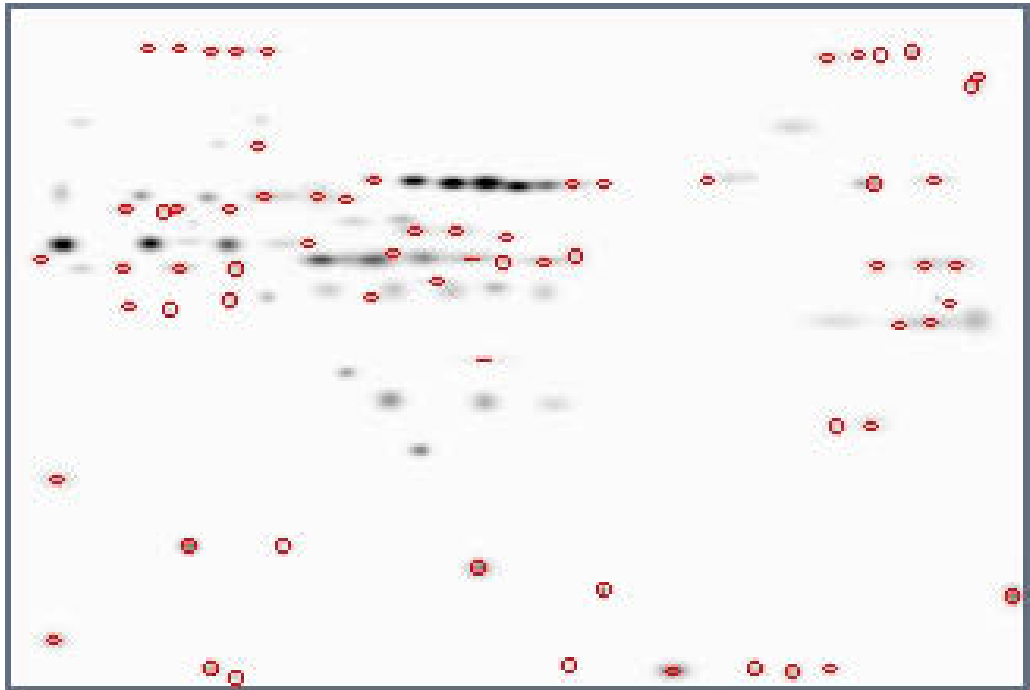
Westergaard Wild Type Master

Westergaard Mutant Master

Figure 14: Venn diagram comparing spot numbers between wild type *N. crassa* grown on Westergaard media and *nit-2* mutant *N. crassa* grown on Westergaard media. The number where the circles overlap is the total number of spots found to be shared in both gels. The side circle numbers are the unique number of spots for each respective gel.

Westergaard Wild Type Unique Spots   Westergaard Mutant Unique Spots

66   43   65
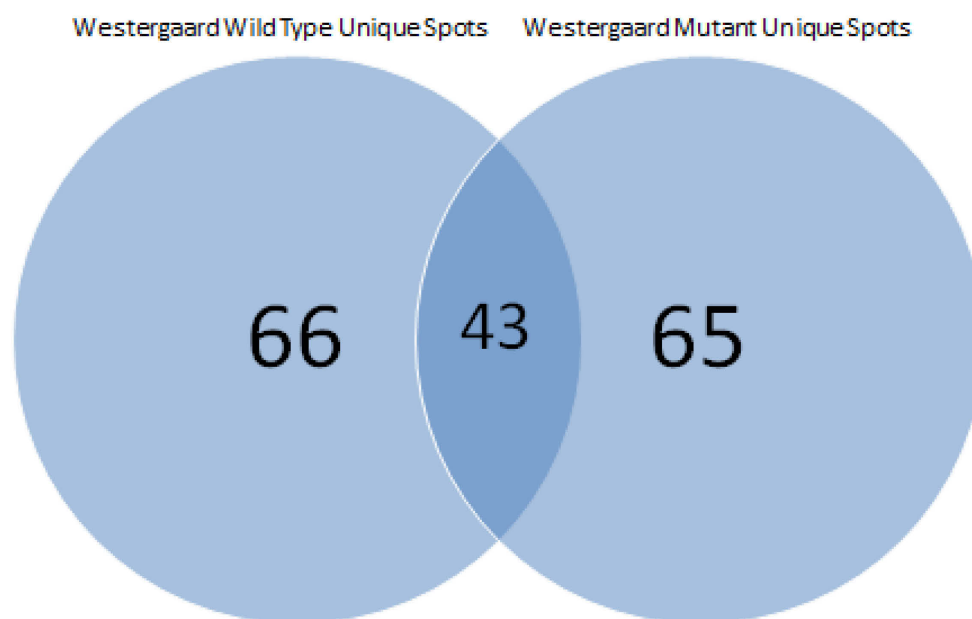
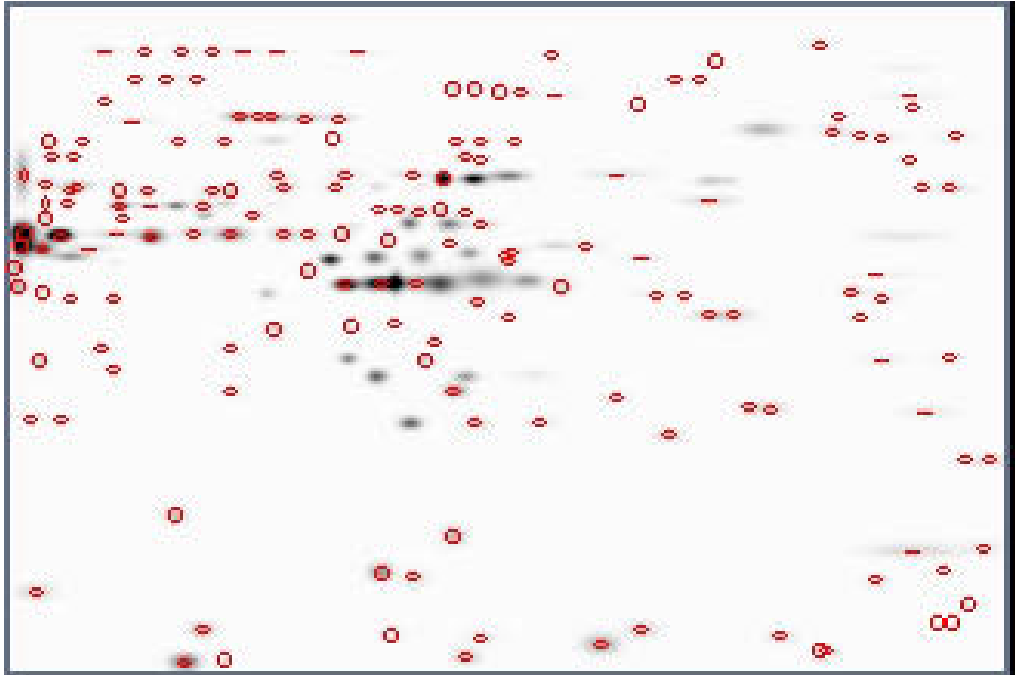**Figure 15:** Higher Level matched set comparing master gels of wild type *N. crassa* grown on Vogels minimal media, wild type *N. crassa* grown on Westergaard media, and *nit-2* mutant   *N. crassa* grown on Westergaard media.

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Vogels Wild Type Master

Westergaard Wild Type Master

Westergaard Mutant Master

Higher Level Master

Figure 16: Venn diagram comparing master gels of wild type *N. crassa* grown on Vogels minimal media, wild type *N. crassa* grown on Westergaard media, and *nit-2* mutant *N. crassa* grown on Westergaard media. The numbers in the outer circles are the number of spots found in each respective gel. The number where two circles overlap is the number of spots shared between the two conditions labeling the circles. The central number, where all three circles overlap, is the total number of spots shared between each condition.
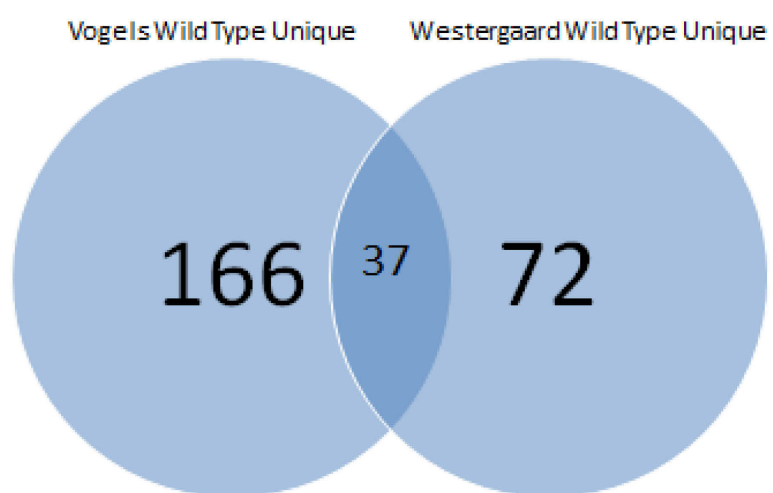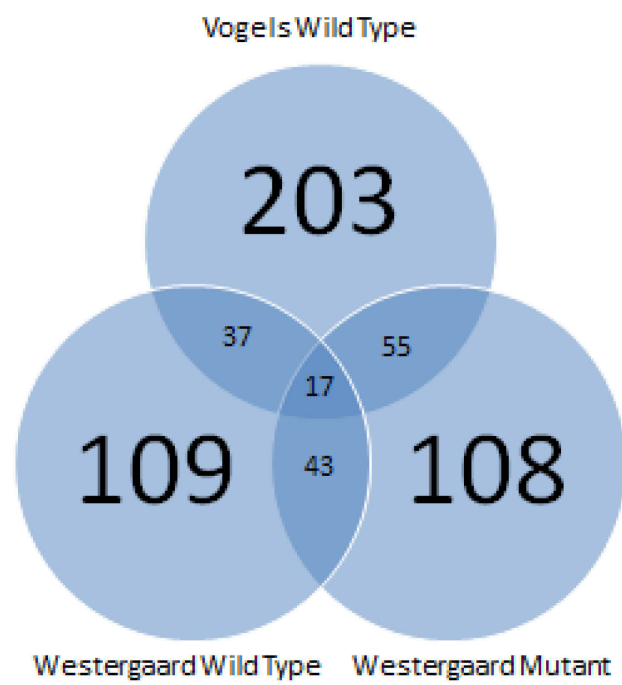
Figure 17: Higher Level matched set comparing master gels of wild type *N. crassa* grown on Vogels minimal media and *nit-2* mutant *N. crassa* grown on Vogels minimal media.

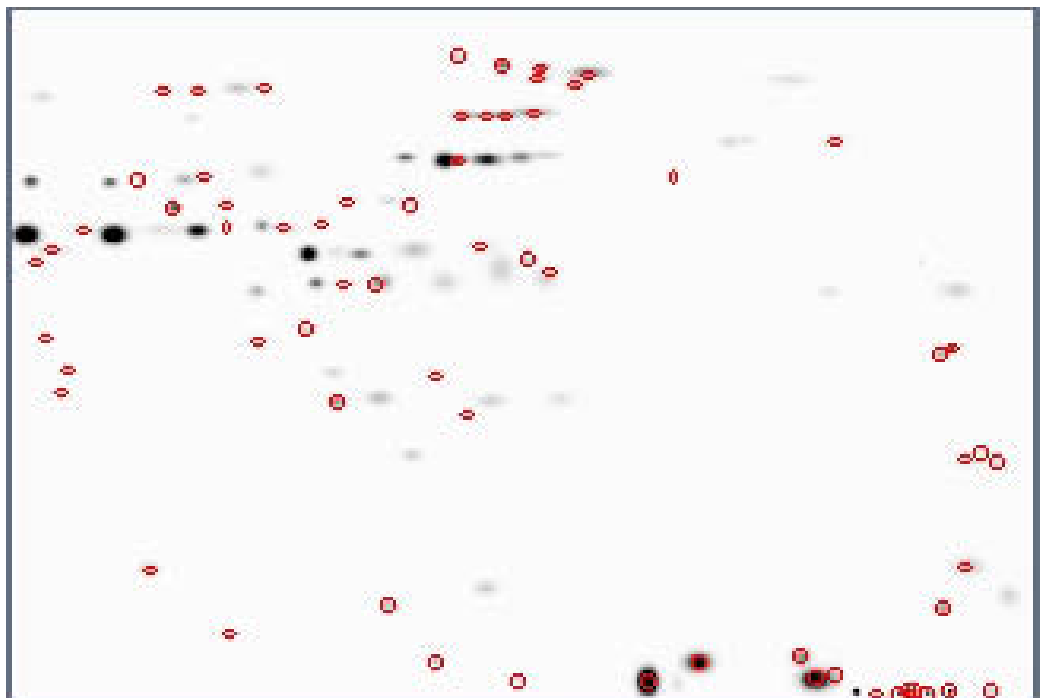**Vogels Wild Type Master**

Vogels Mutant Master
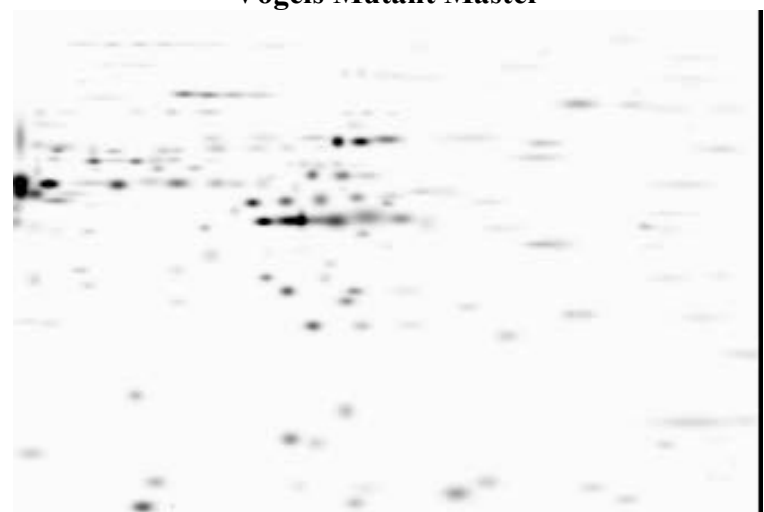
Vogels Higher Level Master

64

Figure 18: Spot comparison between wild type *N. crassa* grown on Vogels minimal media and *nit-2* mutant *N. crassa* grown on Vogels minimal media. Unique spots to each respective gel are circled in red.

Vogels Wild Type Master
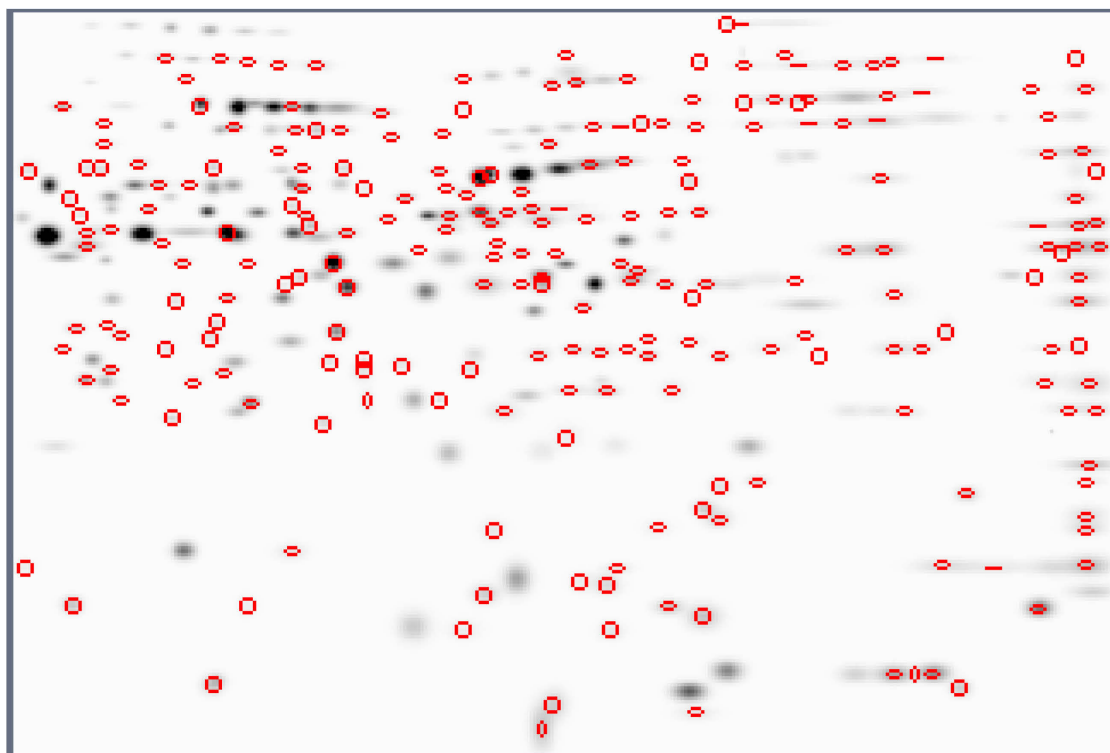
Vogels Mutant Master

Figure 19: Venn diagram comparing master gels of both genetic variants of *N. crassa* grown on Vogels minimal media; wild type *N. crassa* grown on Vogels media and *nit-2* mutant *N. crassa* grown on Vogels media. The numbers in the outer circles are the number of spots found in each respective gel. The number where two circles overlap is the number of spots shared between the two conditions labeling the circles. The central number, where all three circles overlap, is the total number of spots shared between each condition.
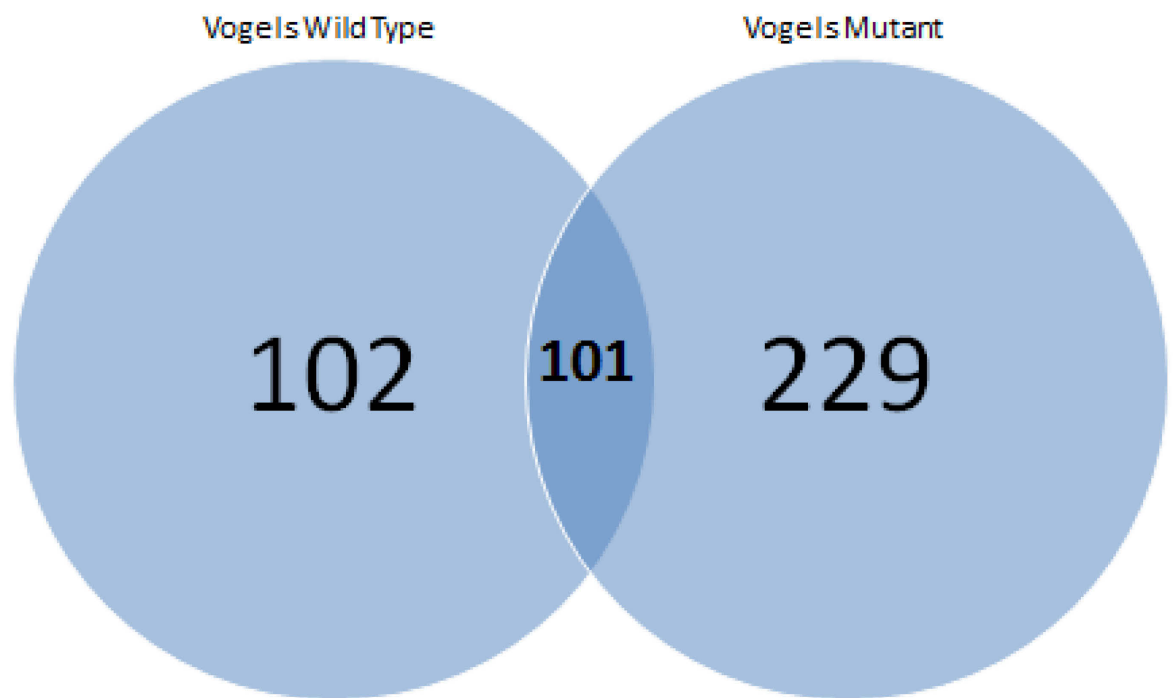
useful in the study. First we looked for a protein spot that was present in each condition to use as a control. Then we looked specifically for proteins that were found in the Vogels grown wild type *N. crassa*, but not in the Westergaard grown wild type *N. crassa*. Spots that appeared in the Westergaard grown wild type that fit this condition would be turned on by the presence of a poor nitrogen source (nitrate). Next we looked for proteins that fit the previous description (turned on by a poor nitrogen source) in the Westergaard grown wild type and looked to see if any of these spots were present in the Westergaard grown *nit-2* mutant to see if these spots of proteins were also reliant on the *nit-2* gene. Spots that were found in the Westergaard grown wild type that did not appear in the Westergaard grown *nit-2* mutant were reliant on the *nit-2* gene for expression, as the *nit-2* mutant does not have a functioning *nit*-2 gene. Spots that fit these descriptions were selected and excised to be sent for mass spectrometry at The Ohio State University Mass Spectrometry and Proteomics Facility for partial sequence determination and indentification. Figures 20, 21, and 22 show the spots selected for excision from Westergaard grown wild type *N. crassa*. Figure 23 shows the spots excised from Westergaard grown *nit-2* mutant *N. crassa*.

Once the excised spots were received by The Ohio State University, apillary-liquid chromatography tandem mass spectrometry was performed in order to obtain the protein material and sequence information from the samples. The identifications and functions of these unknown proteins were then determined by searching the NCBI database of all known fungal proteins. The results of the mass spectrometry and analysis are found in Table 5. In Table 5 the protein spots that came back with what is considered an acceptable Mascot Score (greater than 75) are shown. Of the 13 spots excised, only 9

Figure 20: Gaussian gel image produced from a 2-dimensional SDS-PAGE gel run with protein extracted from wild type *N. crassa* shifted into Westergaard media. The set of gels was run in triplicate, with this being gel 1 of 3. Proteins that were excised are circled and labeled with their condition and spot number used in shipping to The Ohio State University for mass spectrometry analysis.

Westergaard Wild Type Gel 1

Figure 21: Gaussian gel image produced from a 2-dimensional SDS-PAGE gel run with protein extracted from wild type *N. crassa* shifted into Westergaardmedia. The set of gels was run in triplicate, with this being gel 2 of 3. Proteins that were excised are circled and labeled with their condition and spot number used in shipping to The Ohio State University for mass spectrometry analysis.

Westergaard Wild Type Gel 2

Figure 22: Gaussian gel image produced from a 2-dimensional SDS-PAGE gel run with protein extracted from wild type *N. crassa* shifted into Westergaard media. The set of gels was run in triplicate, with this being gel 3 of 3. Proteins that were excised are circled and labeled with their condition and spot number used in shipping to The Ohio State University for mass spectrometry analysis.
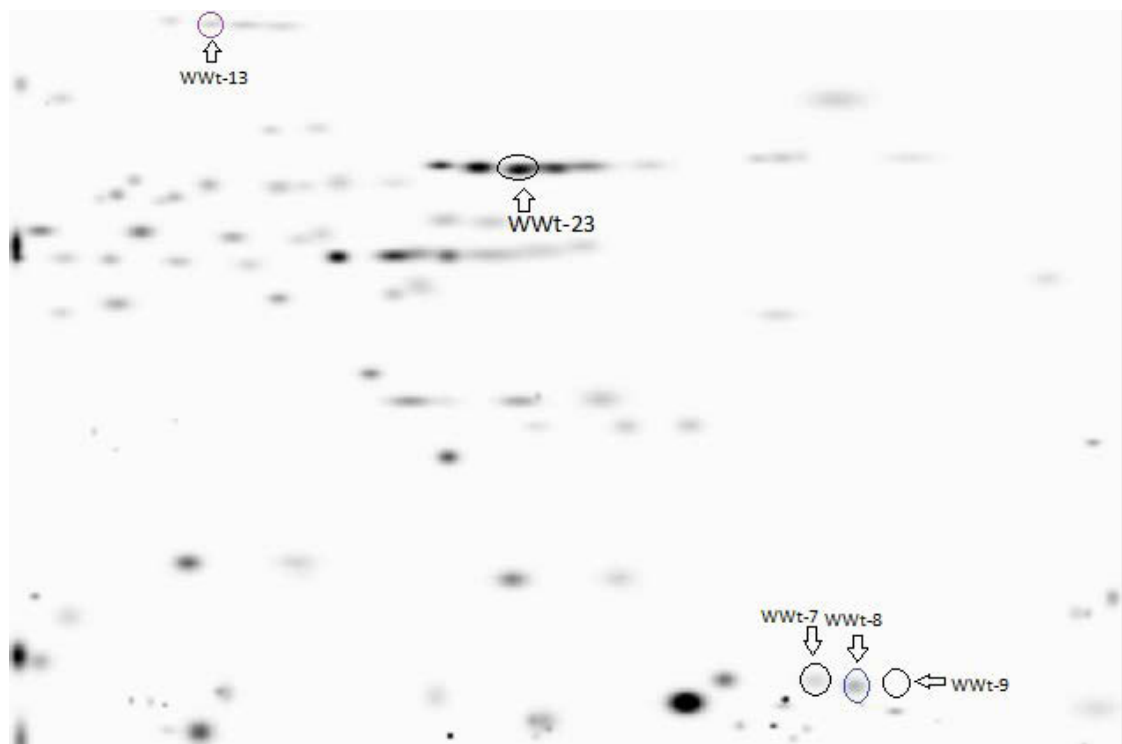
Westergaard Wild Type Gel 3

Figure 23: Gaussian gel image produced from a 2-dimensional SDS-PAGE gel run with protein extracted from *nit-2 mutant N. crassa* shifted into Westergaard media. The set of gels was run in triplicate, with this being gel 1 of 3. Only 1 gel of the triplicate set had spots suitable for excision. Proteins that were excised are circled and labeled with their condition and spot number used in shipping to The Ohio State University for mass spectrometry analysis.

Westergaard Mutant Gel 1

Table 5: Mass Spectrometry results from The Ohio State University. The results are shown only from protein spots that have a Mascot Score of above 75. All other spots with Mascot Scores below 75 have been omitted due to inaccuracy of resulting data and low confidence.

Spot (Fig.)	Condition	NCBI Acession	Mascot Score	Percent Coverage	Nominal Mass	Calculated pI	Protein Name
WWt-5	N. crassa Wild Type grown in Vogels media and shifted into Westergaards media	gi|85110952	315	22%	19229	6.43	hypothetical protein NCU08332 [Neurospora crassa OR74A]
WWt-7	N. crassa Wild Type grown in Vogels media and shifted into Westergaards media	gi|85107476	258	30%	11057	5.17	hypothetical protein NCU06346 [Neurospora crassa OR74A]
WWt-9	N. crassa Wild Type grown in Vogels media and shifted into Westergaards media	gi|85107476	175	22%	11057	5.17	hypothetical protein NCU06346 [Neurospora crassa OR74A]
WWt-10	N. crassa Wild Type grown in Vogels media and shifted into Westergaards media	gi|85093919	138	5%	85471	6.22	hypothetical protein NCU02366 [Neurospora crassa OR74A]
WWt-12	N. crassa Wild Type grown in Vogels media and shifted into Westergaards media	gi|85093919	168	4%	85471	6.22	hypothetical protein NCU02366 [Neurospora crassa OR74A]
WWt-16	N. crassa Wild Type grown in Vogels media and shifted into Westergaards media	gi|85089455	272	25%	47679	5.21	enolase [Neurospora crassa OR74A]
WWt-23	N. crassa Wild Type grown in Vogels media and shifted into Westergaards media	gi|85090389	187	14%	40035	5.42	fructose-bisphosphate aldolase [Neurospora crassa OR74A]
WMut-5	nit-2 mutant N. crassa grown in Vogels media and shifted into Westergaards media	gi|336463434	90	6%	67081	5.25	hypothetical protein NEUTE1DRAFT_132552 [Neurospora tetrasperma FGSC2508]

Figure 21: Peptide sequences shown with the areas that match from the analyzed sample to the resulting hypothetical protein sequence are highlighted in bold red. A is WWt-5, which is from wild type *N. crassa* that was grown in Vogels media then shifted in Westergaard media and thought to be hypothetical protein NCU08332 [Neurospora crassa OR74A]. B is WWT-7, which is from wild type *N. crassa* that was grown in Vogels media then shifted in Westergaard media and thought to be hypothetical protein NCU06346 [Neurospora crassa OR74A]. C is WWt-9, which is from wild type *N. crassa* that was grown in Vogels media then shifted in Westergaard media and thought to be hypothetical protein NCU06346 [Neurospora crassa OR74A]. D is from wild type *N. crassa* that is WWt-10, which was grown in Vogels media then shifted in Westergaard media and thought to be hypothetical protein NCU02366 [Neurospora crassa OR74A]. E is WWt-12, which is from wild type *N. crassa* that was grown in Vogels media then shifted in Westergaard media and thought to be hypothetical protein NCU02366 [Neurospora crassa OR74A]. F is WWt-16, which is from wild type *N. crassa* that was grown in Vogels media then shifted in Westergaard media and thought to be enolase [Neurospora crassa OR74A]. G is WWt-23, which is from wild type *N. crassa* that was grown in Vogels media then shifted in Westergaard media and thought to be fructose-bisphosphate aldolase [Neurospora crassa OR74A]. H is WMut-5, which is from *nit-2* mutant *N. crassa* that was grown in Vogels media then shifted in Westergaard media and thought to be hypothetical protein NEUTE1DRAFT_132552 [Neurospora tetrasperma FGSC 2508].

A)

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

B)

```
  1  MSAAFEKAVA DSKKLTSKPS NEDLLEIYAL YKVATGADFS KADKPGMFDL
 51  KGKAKYNAWQ KVVDDGLSAE EAQTKYVAKV EEMKTQYGYD ENKAPEAVGS
101  N
```

C)

```
  1  MSAAFEKAVA DSKKLTSKPS NEDLLEIYAL YKVATGADFS KADKPGMFDL
 51  KGKAKYNAWQ KVVDDGLSAE EAQTKYVAKV EEMKTQYGYD ENKAPEAVGS
101  N
```

D)

```
  1  MLASRQLLGV ARSRAAAGLG RRCMATATEN PLDRKVKQNL LEEGNFINYK
 51  KMSENLAVVR SRLNRPLTYA EKILYSHLDN PHEQDIERGV SYLRLRPDRV
101  ACQDATAQMA ILQFMSAGMP SVANPTTVHC DHLIEAQVGG EKDLERAINI
151  NKEVYDFLAS ACAKYNIGFW KPGSGIIHQI ILENYAFPGG LLIGTDSHTP
201  NAAGLGMAAI GVGGADAVDV MANLPWELKA PKVIGVKLTG ALNGWTTPKD
251  VILKLAGILT VKGGTGSIVE YYGEGASSMS ATGKATCGNM GAEIGATTSV
301  FPYDKAMFDY LVATKRSDIA EFAKTYQKEL QADEGAEYDQ HIEINLNELE
351  PHINGPFTPD LATPISKFAQ AAKENNWPEE LKVGLIGSCT NSSYEDMTRA
401  ASIARDALDH GLKAKAAFVV TPGSEQIRAT IARDGQLQTF EEFGGTVLAN
451  ACGPCIGQWD RRDTPKGTPN SILSSYNRNF TGRNDGNPAT HSFVTSPDLV
501  VAMSIAGSLY FNPLTDSLKD KDGKEFKLQA PTGAGLPDRG YDPGQNTYQA
551  PPADRATVQV QVSPTSDRLQ VLSPFNAWNG KDATDMPILI KAQGKTTTDH
601  ISMAGPWLKY RGHLDNISNN MLIGAINAAN GEANKVQNFT TGEWDAVPAV
651  ARDYKAKNIP WVVIGDWNYG EGSSREHAAL EPRHLGGLAI ITRSFARIHE
701  TNLKKQGMLP LTFADPADYD KISPEDKVDI LCTELAVGKP ITMRVHPKNG
751  KDFEVKLNHT FNDAQIEWFK NGSALNTMAK NAKN
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E)

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  1    MLASRQLLGV ARSRAAAGLG RRCMATATEN PLDRKVKQNL LEEGNFINYK
 51    KMSENLAVVR SRLNRPLTYA EKILYSHLDN PHEQDIERGV SYLRLRPDRV
101    ACQDATAQMA ILQFMSAGMP SVANPTTVHC DHLIEAQVGG EKDLERAINI
151    NKEVYDFLAS ACAKYNIGFW KPGSGIIHQI ILENYAFPGG LLIGTDSHTP
201    NAAGLGMAAI GVGGADAVDV MANLPWELKA PKVIGVKLTG ALNGWTTPKD
251    VILKLAGILT VKGGTGSIVE YYGEGASSMS ATGKATCGNM GAEIGATTSV
301    FPYDKAMFDY LVATKRSDIA EFAKTYQKEL QADEGAEYDQ HIEINLNELE
351    PHINGPFTPD LATPISKFAQ AAKENNWPEE LKVGLIGSCT NSSYEDMTRA
401    ASIARDALDH GLKAKAAFVV TPGSEQIRAT IARDGQLQTF EEFGGTVLAN
451    ACGPCIGQWD RRDTPKGTPN SILSSYNRNF TGRNDGNPAT HSFVTSPDLV
501    VAMSIAGSLY FNPLTDSLKD KDGKEFKLQA PTGAGLPDRG YDPGQNTYQA
551    PPADRATVQV QVSPTSDRLQ VLSPFNAWNG KDATDMPILI KAQGKTTTDH
601    ISMAGPWLKY RGHLDNISNN MLIGAINAAN GEANKVQNFT TGEWDAVPAV
651    ARDYKAKNIP WVVIGDWNYG EGSSREHAAL EPRHLGGLAI ITRSFARIHE
701    TNLKKQGMLP LTFADPADYD KISPEDKVDI LCTELAVGKP ITMRVHPKNG
751    KDFEVKLNHT FNDAQIEWFK NGSALNTMAK NAKN
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F)

```
  1    MPISKIHARY VYDSRGNPTV EVDVVTELGL HRAIVPSGAS TGQHEACELR
 51    DGDKTKWGGK GVLKAVQNVN EVIGPALIKE NIDVKDQSKV DKFLIDLDGT
101    PNKTKLGANA ILGVSLAVAK AGAAEKGVPL YAHISDLAGT KKPYVLPVPF
151    MNVLNGGSHA GGRLAFQEFM IVPSAAPTFS EALRQGAEVY QILKSLAKKK
201    YGQSAGNVGD EGGVAPDIQN PEEALDLITE AIEKAGYTGQ VKIAMDVASS
251    EFYKEDVKKY DLDFKNPESD PSKWLTYEEL ANLYSELCKK YPIVSIEDPF
301    AEDDWEAWSY FYKTQDIQIV ADDLTVTNPL RIKKAIELKA ANALLLKVNQ
351    IGTLTESIQA AKDSYADGWG VMVSHRSGET EDVTIADIVV GIRSGQIKTG
401    APARSERLAK LNQILRIEEE LADNAIFAGE KFRKAVEL
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G)

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  1    MGIFDELNLP AGVLYGDDVL KLFQYAREKQ FAIPACNVTS SSTAVAALEA
 51    ARDQKAPIIL QTSQGGAAFF AGKGIKDSAE KREASVAGAI AAAHYIRSIA
101    PIYGIPVVLH TDHCAKKLLP WLDGMLEEDE KFFKANGVPL FSSHMIDLSE
151    EPVEENISTC VKYLKRMAPM KQWLEMEIGI TGGEEDGVDN SEVDNASLYT
201    QPEDIWQIEE AFRPISPYFS IAAGFGNVHG VYAPGNVKLH PELLGKHQAY
251    VSEKLGGKDK KPVFFVFHGG SGSSKEEYRE AISNGVVKVN VDTDLQWSYL
301    VGIRDYILNN IDYLRSQVGN PEGPNKPNKK KYDPRVWIRE GEKTMKARVE
351    EALKDFNAAG TV
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H)

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  1   MADEVYDGAI GIDLGTTYSC VAIYEGTNVE IIANEQGSFT TPSFVSFTPE
 51   ERLIGEAAKN QAAMNPKNTV FDVKRLIGRR IDDPTVKKDQ ESWPFKVVDD
101   GAGNPKVEVD YLNGVHTFSP QEISAMVLTK MKEIAEVKLG KKVEKAVITV
151   PAYFNDNQRQ ATKDAGAIAG LNVLRIINEP TAAAIAYGLG ANKSNKERNV
201   LIYDLGGGTF DVSLLNIQGG VFTVKATAGD THLGGQDFDT NLLDYCKKEF
251   TRKTKKDLSG DARALRRLRT ACERAKRTLS SGAQTTIEID SLFDGEDFNI
301   NVTRARFEDL NAKAFSGTLE PVAQVLKDAS IEKSAVDEIV LVGGSTRIPK
351   VQKLLSEFFD GKKLEKSINP DEAVAYGAAV QAGILSGKAT SAETSDLLLL
401   DVVPLSLGVA MEGNIFAPVV PRGQTVPTIK KRTFTTVADN QQTVQFPVYQ
451   GERVNCEDNT SLGEFTLAPI PPMKAGEPVL EVVFEVDVNG ILKVTATEKT
501   SGRSANITIS NSVGKLSSSE IEKMVEEAEK FKSNDEAFSK RFEAKQQLES
551   YISRVEEIVS DPTLSLKLKR GQKEKIEQSL SEAMSQLEIE DSSAEDLKKK
601   ELALKRLVTK AMSSR
```

spots are shown with their conditions, Mascot Score, NCBI accession number, nominal

mass, calculated pI, percent coverage of the hypothetical protein matched to, and given

name. These 9 spots are the samples that came back with results higher than the minimal

required Mascot Score of 75.

The percent coverage shows the percent of the peptides analyzed that matches to

the peptides in a known protein or a hypothetical protein. The overlap from that gave each

sample's percent coverage can be seen in Figure 21. The letters highlighted in red

indicate that that is an area where an overlap between the sample protein and the

hypothetical protein.

The resulting hypothetical proteins that correlate to each individual protein sample

were then ran in BLAST to determine their possible functions. These BLAST results can

be seen in Table 6 with the corresponding spot, hypothetical protein, and hypothetical

protein NCBI accession number. The spot WWt-5 was excised from a 2-dimensional gel

that was ran with protein extracted from wild type (74A) *N. crassa* that was shifted into

Westergaard media. The mass spectrometry analysis from Ohio State University showed

the spot to be "hypothetical protein NCU08332 [Neurospora crassa OR74A]". Running

the hypothetical protein in BLAST showed that WWt-5 is similar to a "translation protein

SH3-like protein" found in *Neurospora tetrasperma*. The spots WWt-7 and WWt-9 were

the same spots in different gels with the same conditions of wild type (74A) *N. crassa* that

was shifted into Westergaard media. The mass spectrometry analysis showed the spot to

be "hypothetical protein NCU06346 [Neurospora crassa OR74A]". Running the

hypothetical protein in BLAST showed that WWt-7 and WWt-9 are similar to another

hypothetical protein, "hypothetical protein SMAC_01001 [Sordaria macrospora k-hell]",

Table 6: Each sample that had a Mascot Score above the minimum required for confidence (75) was ran in BLAST to compare to other known and hypothetical proteins. The results of the BLAST can be found below. Each BLAS result had a minimum Query Coverage of 90% and had a Maximum Identity percent of above 90%.

Spot Name	Hypothetical Name	NCBI Accession	BLAST Result(s)
WWt-5	hypothetical protein NCU08332 [Neurospora crassa OR74A]	gi|85110952	translation protein SH3-like protein [Neurospora tetrasperma FGSC 2509] hypothetical protein NCU08332 [Neurospora crassa OR74A]
WWt-7	hypothetical protein NCU06346 [Neurospora crassa OR74A]	gi|85107476	hypothetical protein NCU06346 [Neurospora crassa OR74A] hypothetical protein SMAC_01001 [Sordaria macrospora k-hell] putative fatty acid binding protein [Chaetomium thermophilum var. thermophilum DSM 1495]
WWt-9	hypothetical protein NCU06346 [Neurospora crassa OR74A]	gi|85107476	hypothetical protein NCU06346 [Neurospora crassa OR74A] hypothetical protein SMAC_01001 [Sordaria macrospora k-hell] putative fatty acid binding protein [Chaetomium thermophilum var. thermophilum DSM 1495]
WWt-10	hypothetical protein NCU02366 [Neurospora crassa OR74A]	gi|85093919	hypothetical protein NCU02366 [Neurospora crassa OR74A] mitochondrial aconitate [Colletotrichum orbiculare MAFF 240422] aconitate hydratase [Verticillium dahliae VdLs.17]
WWt-12	hypothetical protein NCU02366 [Neurospora crassa OR74A]	gi|85093919	hypothetical protein NCU02366 [Neurospora crassa OR74A] mitochondrial aconitate [Colletotrichum orbiculare MAFF 240422] aconitate hydratase [Verticillium dahliae VdLs.17]
Wmut-5	hypothetical protein NEUTE1DRAFT_132552 [Neurospora tetrasperma FGSC 2508]	gi|336463434	hypothetical protein NEUTE1DRAFT_132552 [Neurospora tetrasperma FGSC 2508] hypothetical protein SMAC_04972 [Sordaria macrospora k-hell] heat shock protein 70 [Chaetomium globosum CBS 148.51]

and a "putative fatty acid binding protein" found in *Chaetomium thermophilum*. The spots WWt-10 and WWt-12 were also the same spot in different gels with the same conditions of wild type (74A) *N. crassa* that was shifted into Westergaard media. The mass spectrometry analysis showed the spot to be "hypothetical protein NCU02366 [Neurospora crassa OR74A]". This hypothetical protein, when run in BLAST, was shown to be similar to a "mitochondrial aconitate" found in *Colletotrichum orbiculare* and an "aconitate hydratase" found in *Verticillium dahliae*. WMutt-5, he only successfully obtained and analyzed spots from the *nit-2* mutant *N. crassa* that was shifted into Westergaard, was found to be "hypothetical protein NEUTE1DRAFT_132552 [Neurospora tetrasperma FGSC 2508]". Running this hypothetical protein in BLAST revealed that this protein is similar to "heat shock protein 70", which is found in *Chaetomium globosum*. Furthermore, for the spot WMut-5, there were a large number of BLAST hits that yielded high confidence of which all were heat shock protein 70 from multiple fungal organisms.

Discussion

Very little research has been done in the field of proteomics that concerns limiting the nitrogen availability or looking at the *nit*-2 gene of *Neurospora crassa*. The research that has been done thus far has focused on protein binding to DNA, and never on 2-dimensional gels. The current study is the first that used the modern proteomic technique of 2-dimensional gel electrophoresis on *N. crassa* when nitrogen is made less available. Furthermore, it is also the first study to look at the proteins produced under these conditions using mass spectrometry and bioinformatics tools. To look at the *nit-2* gene and nitrogen availability we used a *nit-2* knockout in combination with Westergaards media in comparison to a wild type *N. crassa* grown on Westergaard media. In addition to these conditions both the wild type and *nit-2* mutant *N. crassa* were grown on Vogels media (containing ammonia) for comparison to a normal growth condition. Our goal in this study was to identify protein expression that is not only turned on by poor nitrogen availability, but to also see what role the *nit-2* gene played in expression under poor nitrogen availability. These proteins can, for the first time, be identified using modern proteomics and mass spectrometry tools.

In order to generate 2-dimensional gels, we first had to determine the amount of protein in each sample. This was done through the use of 1-dimensional gel electrophoresis, using a Criterion® 11cm 8%-16% gradient gel. Once the gel was run with bovine serum albumin as a standard, we were able to measure the amount of protein in each sample. In addition to giving us the protein quantities, this also gave us a rough idea on what to expect from the 2-dimensional gel results. The only noticeable piece of information we gleaned from the 1-dimensional gel, however, was that the wild type *N.*

crassa shifted onto Westergaards media had the lowest protein concentration, while all of the other conditions were approximately the same. This low level or protein concentration in the wild type sample shifted into Westergaards also came with significant reduction in protein bands that were clearly visible in the other samples. Both the protein concentration being low and the bands being reduced or absent lead us to believe that this sample would, more than likely, have a significantly reduced protein profile in both spots and intensity. At the time it was theorized that the fungus was consuming its own proteins for nitrogen, but was never pursued because I was not a key piece of data we were looking for.

After the 1-dimensional gels were run, we were able to go forward with our 2-dimensional gel electrophoresis. These gels were run on Criterion® 11cm 8%-16% gradient gels using IPG test strips (all from Bio-Rad™) with a pH range of 4-7. The gels were run in triplicate to get a consistent result of what spots were showing on the gels. After running the gels in triplicate they were scanned using the Pharos FX™ imaging system and uploaded into PDQuest™ by Bio-Rad™. Once the images were uploaded into PDQuest™ an analysis was done on them to make matched sets for each condition. The conditions were then compared to each other using higher level matched sets, which uses the master gel image produced from each lower level matched set. Each higher level matched set was then looked at individually for spots unique to each gel and spots that were shared between gels.

The first set looked at compared the protein extracted from wild type *N. crassa* shifted into Vogels media to that of the wild type *N. crassa* shifted into Westergaards media. The resulting gels showed 166 spots were unique to the wild type shifted into

Vogels, while the wild type shifted into Westergaards had 72 unique spots. What this means is that there were 166 proteins seen to be produced while the nitrogen source was good (Vogels media) that were not found when the nitrogen source was bad (Westergaards media). We expected there to be a number of proteins present that would be involved in normal nitrogen uptake and use in the cell. The 72 unique spots to the Westergaards shifted wild type indicate that there were 72 proteins present when the organism was subjected to a poor nitrogen source (Westergaards media). There were also 37 spots that were shared between the 2 conditions, indicating that 37 proteins were present under both conditions. These 37 proteins were also expected due to the requirement of cells to have certain housekeeping genes active and functioning under any condition.

The next comparison that was made was between the wild type *N. crassa* shifted into Westergaards media and the *nit-2* mutant *N. crassa* shifted into Westergaards media. The resulting gel showed that 66 spots were unique to the wild type *N. crassa* shifted into Westergaards media, while the *nit-2* mutant *N. crassa* shifted into Westergaards media had 65 unique spots. A total of 43 spots were shared between the two conditions. The 66 spots in the wild type sample indicated that there were 66 proteins found to only occur under poor nitrogen when the *nit-2* gene is inactive. This showed the *nit-2* gene plays a role in gene expression under poor nitrogen. Once again, there were a nominal number of spots (43) that were shared, indicating the housekeeping proteins that the cells needs and that would not be controlled by nitrogen availability. What is interesting, however, was that the *nit-2* mutant had 65 proteins (spots) that were unique to it. The reason that this is interesting is that our current understanding of *nit-2* is that it only actives genes by binding, while this result shows that *nit-2* might be responsible for turning genes off.

The final set of gels that was compared was the wild type *N. crassa* shifted into Vogels media and the *nit-2* mutant *N. crassa* shifted into Vogels media. These gels showed that there were 102 proteins (spots) unique to the wild type shifted into Vogels media, while the *nit-2* mutant shifted into Vogels media had 229 unique proteins (spots). The 102 unique proteins were expected, as the conditions for them to grow are the same except that the mutant has a non-functioning *nit-2* gene. This non-functioning gene is would result in proteins to not be expressed that would normally be expressed, even under normal nitrogen levels. The result of 229 unique proteins in the *nit-2* mutant, however, was not expected. With current knowledge of *nit-2*, we expected there to be minimal proteins that were unique to the mutant. This is because *nit-2* is seen as an activator, not a repressor. The sheer number of proteins that were shown as unique from the *nit-2* mutant suggests that *nit-2* may also play a role in repressing certain genes during normal nitrogen availability or simply in general.

After the gel images were analyzed we were then able to move forward with spots excision and mass spectrometry. The spots were selected largely from the wild type *N. crassa* shifted into Westergaards media. By using this sample we were able to select proteins that were both a result of the poor nitrogen source and were also controlled by *nit-2*. To do this, we selected spots that were not found in the wild type *N. crassa* shifted into Vogels gel or the *nit-2* mutant *N. crassa* shifted into Westergaards gel. In addition to selecting spots that fit this criteria, we also selected spots that were found in every gel type, and selected two spots that were found in the Westergaards shifted *nit-2* mutant and nowhere else. The selected spots were then shipped to The Ohio State University Department of Proteomics and Mass Spectrometry for mass spectrometry and

bioinformatics analysis. Once received, the mass spectrometry results were analyzed through the use of BLAST.

The two spots that were selected that were found in every condition did come back as proteins that would be expected to exist in every condition. The spot labeled WWt-16 was shown to be enolase, which is used in glycolysis (Diaz-Ramos et al., 2012). The spot labeled WWt-23 was fructose-bisphosphate aldolase. It would stand to reason that these two proteins would be necessary under a large number of conditions, and thus would not be affected by the presence or lack of nitrogen.

The spots labeled WWt-7 and WWt-9 are the same spot from different gels of the same condition. These two spots came back as the same thing—hypothetical protein NCU06346 from *N. crassa.* Furthermore, the mascot score for both of these spots was above 300, indicating a strong likelihood that there was a correct match. When run through BLAST, the resulting comparison yielded a putative fatty acid binding protein from *Chaetomium thermophilum.* Unfortunately, the term "fatty acid binding protein" is incredibly broad and covers a very large range of proteins from proteins that bind to the membrane to proteins that aid in the transportation of fatty acid.

The spots labeled WWt-10 and WWt-12 were spots from the same gel and the same band. We theorized that they may be different phosphorylations of the same protein. The results from the mass spectrometry analysis yielded that they were the same protein, which was the hypothetical protein NCU02366. This confirmed our previous thought that they were the same protein of a different phosphorylation. Furthermore, the mass spectrometry results showed that they were the same protein with hits on different areas of

the peptide sequence, while sharing one hit between them. The percent coverage for these spots was 5% for WWT-10, with a Mascot score of 138, and 4% for WWt-12, with a Mascot score of 168. When ran through BLAST the hypothetical protein was shown to be similar to mitochondrial aconitate from *Colletotrichum orbiculare*. Mitochondrial aconitate is a member of the aconitase superfamily of proteins.

The spot labeled WWt-5 was shown to be similar to the hypothetical protein NCU08332. This result came with a Mascot score of 315 and percent coverage of 22%. When ran through BLAST, it was shown to be similar to a translation protein SH3-like protein from *Neurospora tetrasperma*. The SH3-like protein is part of the S1 superfamily of proteins.

Finally, the only usable spots from the *nit-2* mutant gels, WMut-5, was shown to be similar to hypothetical protein NEUTE1DRAFT_132552 from *Neurospora tetrasperma*. Unlike the other spots excised, this spot did not have a mascot score over 100. Its mascot score was only 90 with a 6% coverage in the suggested hypothetical protein. What was more interesting was that this spot did not come back as a protein from *Neurospora crassa*. We theorize that the proteins between the two organisms might be similar enough that we are seeing a protein that is discovered in *N. tetrasperma*, but not in *N. crassa*. The other hypothesis as to why this spot was shown to be from a different organism was simply contamination. When ran through BLAST, this spot was shown to be similar to heat shock protein 70 from a number of various fungal species.

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