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Investigations into the gene expression of Neurospora crassa during mycelial contact with fungi of increasing phylogenetic distance

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Investigations into the gene expression of *Neurospora crassa* during mycelial contact with fungi of increasing phylogenetic distance

By

Christopher Francisco Villalta

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Committee in charge:

Professor John W. Taylor, Chair
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Abstract

Investigations into the gene expression of *Neurospora crassa* during mycelial contact with fungi of increasing phylogenetic distance

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I was interested in studying how contact between the mycelia of *Neurospora crassa* and fungi of increasing genetic distance affected gene expression in *N. crassa*. The first chapter of my dissertation was a retesting of the phylogenetic species recognition concept in *Neurospora* and validation that previously discovered phylogenetic species (PS) were genuine. The second chapter was a comparison of *N. crassa* gene expression during encounters with *Neurospora* of decreasing genetic relatedness. The third chapter was a characterization of *N. crassa* gene expression during growth inhibition caused by the anti fungal peptide, PAF, produced by *Penicillium chrysogenum*.

In the first chapter, using phylogenetic information about *Neurospora*, I designed a successful two restriction enzyme digest assay that separated PS 1-3 from the other known *Neurospora* species and found one new PS1, nine new PS2, and one new PS3. The topography of the phylogenetic tree did not change with the addition of the new PS isolate data and neither did the interspecific mating patterns between *Neurospora*. As a result of finding new PS isolates and successfully retesting the PSR concept, the PS 1-3 were properly named and described as *N. hispaniola, N. metzenbergii, and N. perkinsii*.

In the second chapter I observed changes in *N. crassa* gene expression during contact with the mycelia of other *Neurospora*. I framed the question from a phylogenetic perspective and collected mycelia from *N. crassa* during a self-self interaction, a intrapopulation interaction, a interpopulation interaction, and a intragenus interaction. After comparing RNAseq profiles of *N. crassa* interacting with the different fungi I found that the smallest change in gene expression occurred between the self-self interaction and the largest difference occurred in the interpopulation interaction. The intrapopulation and intragenus interactions shared the most in common. There was a large downregulation of metabolism in *N. crassa* when comparing *N. crassa* growing alone to *N. crassa* growing on a plate with another *Neurospora*, but before contact between mycelia. During contact with the mycelia of the other nonself *Neurospora* there was an

upregulation of genes related to reactive oxygen species metabolism and melanin synthesis in *N. crassa*. In the interpopulation interaction there was visible production of melanin after mycelial contact between both *N. crassa* specimens. In interactions between nonself *Neurospora* there was a downregulation of genes involved in cell signaling and polar cell growth. Two genes, NCU01219 and NCU01074, were significantly upregulated in wild type *N. crassa* after contact between mycelia. Deletion mutants of both genes displayed reduced aerial mycelia in comparison to wild type *N. crassa* after contact. NCU01074 is an undescribed bzip transcription factor we found is closely related YAP bzip transcription factor family in *S. cereviseae* and NCU01219 is a glutaredoxin.

In the third chapter I characterized *N. crassa* gene expression during growth with a young 24 hour *P. chrysogenum* colony that did not inhibit mycelial growth and an old 72 hour *P. chrysogenum* colony that inhibited mycelia growth with the anti fungal protein, PAF. I wanted to find the genes, functional categories, and pathways that were affected by PAF induced growth inhibition in *N. crassa* and gain better insight into how fungi behave in the wild. I compared expression data of *N. crassa* interacting with *P. chrysogenum* to the *Neurospora* interaction data from Chapter 2. *N. crassa* interacting with *P. chrysogenum* had very different expression profiles from the other interactions, but genes related to melanin synthesis were upregulated similar to interactions between nonself *Neurospora*. A large amount of gene upregulation occurred in *N. crassa* when grown with the 72 hour old *P. chrysogenum* colony while the smallest change in gene expression occurred in *N. crassa* when interacting with the 24 hour old *P. chrysogenum* colony in comparison to all interactions from Chapter 2 and 3. I found 19 genes that were significantly differentially expressed in *N. crassa* during PAF induced growth inhibition caused by the 72 hour old *P. chrysogenum* colony that would be interesting candidates for further study with gene deletion and over expression mutants. The genes were related to the cell wall, cell membrane, cross membrane transporters, Ca^{2+} dependent signaling, virulence, and transcriptional regulation.

I want to dedicate my dissertation to my parents Carmen and Rodolfo, my grandparents Priscilla and Francisco, my sister Jacqueline, and my aunt Priscilla whom have all played a large role in my education and life. Growing up my family nurtured my enthusiasm for biology, learning, taught me patience, and to work hard. A part of this dedication also goes to Herbert Silber head of the MARC program at San Jose State University and Robert Fowler my undergraduate research advisor. Both have played an important role in helping me decide a PhD was for me and their guidance was instrumental in helping me apply and earn acceptance into University of California, Berkeley and is helpful to this day. Last I dedicate this thesis to the love of my life, my future wife Mouna, everyday I am glad to be with you and look forward to what the future holds for us.

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Acknowledgements

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Introduction

In nature the most important event for a fungus is when it comes into contact with the mycelium of another fungus (Rayner 1991). The type of fungi present and when they interact with each other will have an effect on the interaction and gene expression of the fungi. Whether the fungi involved are closely related or distant relatives plays a role in determining the outcome of the interaction and whether the fungi will mate, one fungus will overgrow the other fungus (Boddy 2000), they will ignore each other, undergo a heterokaryon incompatibility response (Glass et al. 2000), or be negatively affected by the production of toxins (Marx 2004). Studying interactions between fungi is important from an ecological standpoint because it helps us understand how fungi behave in the wild. Interactions between fungi can have important implications for humans because understanding how fungi interact can be used as a method of biological control of harmful fungi, such as plant pathogens (Bae Y.S. 2005). Understanding how fungi inhibit the growth of other fungi will help find new anti-fungals to treat mycoses in animals, including humans (Binder et al. 2010; Galgoczy et al. 2008; Kaiserer et al. 2003; Marx et al. 2007). Studying how non-pathogenic fungi interact with each other in the wild is also important from a human perspective because many virulence factors in pathogenic fungi are also present in fungi that do not cause disease, and are probably just genes expressed in fungi when interacting with another organism (Casadevall 2006; Madhani and Fink 1998; Veneault-Fourrey and Martin 2011). By studying interactions between fungi we will gain a better understanding of important biological pathways involved in these interactions and be able to assign function to the significant number of genes in fungi that have no known function (Kasuga and Glass 2008). To this point the majority of studies looking at interactions between fungi have involved studying gross phenotypic differences (Boddy 2000). A few microarray studies have been completed, looking at how interactions between fungi affect gene expression, but the majority involves fungi with no fully sequenced genome and do not look at gene expression across the whole genome of the fungus (Carpenter et al. 2005; Eyre et al. 2010).

In my study of interactions between fungi I took a phylogenetic approach to choose fungi for experiments where mycelia was collected from *Neurospora* when contact was made with other fungi closely and distantly related to determine what genes are significantly differentially expressed, important to specific interactions, shared between interactions, and to determine if there was a correlation between genetic distance between fungi and gene expression. I wanted to characterize interactions and find highly differentially expressed genes that would be good candidates for future experiments using knockout and over expression mutants to find pathways important to fungi when coming into contact with each other for a better understanding of fungal cell biology.

I chose to use *N. crassa* as my model organism because *Neurospora* has a highly resolved phylogeny concordant with the biological species concept. When Dettman et al. first made the phylogeny three new putative species of *Neurospora* were found. The putative species were phylogenetically distinct, but not biologically (Dettman et al.

2003a; Dettman et al. 2003b). Chapter one of this dissertation involves retesting the phylogenetic species concept in *Neurospora* and determining if the phylogenetic species found in Dettman et al. 2003a are real species.

In chapter two of this thesis, using population and phylogenetic data available in the *Neurospora* community (Dettman et al. 2003a; Ellison et al. 2011) I chose to look at the interactions between *N. crassa* with an *N. crassa* of the same genotype (self-self), with a different genotype from the same population (intrapopulation), with *N. crassa* from a different population (interpopulation), and with a different species, *N. discreta* (interspecies). I used RNA sequencing (RNAseq) techniques (Mortazavi et al. 2008) to observe gene expression of *N. crassa* collected from the interactions. In chapter two I determined the genes that were significantly differentially expressed in *N. crassa* and characterized the four different interactions between *N. crassa* and the other *Neurospora* to test what differences and similarities exist between the interactions. We wanted to find pathways or cellular functions that were affected when two *Neurospora* meet and what differences are present based on genotype. An important part of chapter two was finding significantly differentially expressed genes and testing knockouts of the respective genes for phenotypic differences to relate morphological phenotypes to genes important in interactions between *Neurospora*. Knockouts (Colot et al. 2006) with an interesting phenotype could be used in for future expression analyses using my mycelial contact model.

In chapter three of this dissertation I analyzed gene expression profiles in *N. crassa* when exposed to a more distantly related, anti fungal protein (PAF) producing, growth inhibiting, 72 hour old *P. chrysogenum* and a 24 hour old *P. chrysogenum* that did not inhibit growth (Kaiserer et al. 2003; Marx et al. 2007). The expression data from the two interactions in Chapter 3 between *N. crassa* and *P. chrysogenum* were compared to the expression data from Chapter 2 to differentiate between significantly differentially expressed genes and enriched functional categories important to interactions between fungi in general and important to the inhibition of *Neurospora* by PAF producing *P. chrysogenum.* The study is a first look at global gene expression in a fungus inhibited by an anti fungal protein and one of the aims of Chapter 3 was to find pathways that are significantly affected by PAF and play an important role in growth inhibition of *N. crassa.* We related genes that were being significantly expressed to previous biochemical and cellular biology research (Binder et al. 2010; Marx 2004; Marx et al. 2007) that looked at different aspects of growth inhibition by anti fugal proteins in susceptible fungi. In Chapter 3 I also searched for genes, which were significantly differentially expressed, had relevant annotations related to PAF growth inhibition, and would be excellent candidates for further study using over expression and knock out mutants.

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CHAPTER 1

Three new phylogenetic and biological *Neurospora* species: *N. hispaniola*, *N. metzenbergii* and *N. perkinsii*

Christopher Francisco Villalta, David J. Jacobson, and John W. Taylor

Abstract

The recent recognition of provisional *Neurospora* Phylogenetic Species (PS) 1–3 provided us with the opportunity to compare genetic isolation, which underlies phylogenetic species recognition (PSR), with reproductive isolation as criteria for recognizing new species. This investigation involved first finding new individuals of PS 1–3 from a search of the Perkins culture collection, then assessing genetic isolation by PSR for old and new members of PS 1–3, and finally comparing species recognition by genetic isolation as determined by PSR to species recognition by reproductive isolation as determined by biological species recognition (BSR) and geographic distribution. To facilitate the search for additional members of the PS, we used the genetic variation originally used to discover *Neurospora* PS 1-3 to easily distinguish members of *Neurospora* PS1–3 from the closely related species *N. crassa* and *N. intermedia*. To increase our chance of success, the analysis was performed on *N. crassa* and *N. intermedia* isolates that were either not clearly assignable to species by BSR using tester strains, or that were from the same geographic locations as the known members of PS1–3. Eleven new members of *Neurospora* PS 1–3 were identified: one new PS1, nine new PS2, and one new PS3. To complement PSR, we investigated reproductive isolation using BSR in PS1–3 and the two other most closely related species, *N. intermedia* and *N. crassa* using intraspecific and interspecific crosses. PS1 and PS2 appear reproductively isolated because they successfully mated intraspecifically and not interspecifically. PS3 isolates successfully crossed with other PS3 isolates, however, they also successfully crossed with *N. crassa,* as previously reported, indicating that genetic isolation can precede reproductive isolation. We compared phylogenetic, mating, and geographical data to challenge the use of PSR as the main criterion in the formal description of species and, having failed to discredit the approach, describe the new species, *N. hispaniola* (PS1), *N. metzenbergii* (PS2), and *N. perkinsii* (PS3).

Introduction

Recently, phylogenetic species recognition (PSR) of outbreeding *Neurospora* individuals has found at least 15 genetically isolated, species-level clades where previous biological species recognition (BSR) using mating to tester strains had delimited just five, reproductively isolated species (Dettman et al. 2003a, 2006; Turner et al. 2001). These 15 phylogenetic species (PS) are found in two sister clades. The first comprises four of the five described species, *N. crassa*, *N. sitophila* (Shear and Dodge 1927), *N. intermedia* and *N. tetrasperma* (Tai 1935), and three new species of *Neurospora* tentatively labeled PS 1, 2, and 3 (Dettman et al. 2003a). The second clade comprises the fifth described species, *N. discreta* (Perkins and Raju 1986), and seven new species

of *Neurospora* tentatively labeled PS 4-10 (Dettman et al. 2006). The goal of our study was to determine if species found using PSR could meet specific confidence criteria for use in the formal description of new *Neurospora* species. The criteria that had to be met were: (1) that the new species were distinct PS according to the PSR criteria set forth in Dettman 2003a; (2) that the new species could mate successfully with other members of their species as determined through the mating tests required for biological species recognition (BSR); and (3) that barriers to interspecific mating were either intrinsic, as determined by BSR (Mayr 1996), or extrinsic as inferred from the presence of geographically distinct ranges that define allopatry. As a prelude to our testing of PSR as the basis for formally describing species, we searched existing culture collections of *Neurospora* for new PS1, PS2, and PS3 specimens, using previously determined genetic variation. With the testing of PSR completed, we then formally described and named the three phylogenetic species.

When *N. crassa, N. sitophila*, *N. intermedia,* and *N. tetrasperma* were described in 1927 and 1935, intra- and interspecific crosses showed clear differences and this information influenced the authors of the descriptions (Shear and Dodge 1927; Tai 1935), well before the publication of the Biological Species Concept in 1942 (Mayr 1942). Although morphology was the basis of the descriptions of all four species, since that time reproductive isolation measured by mating success has been shown to be a more reliable method for the identification of heterothallic *Neurospora* (Perkins and Raju 1986; Perkins and Turner 1988; Shear and Dodge 1927; Tai 1935). Morphology, however, continues to be useful for identifying *N. tetrasperma* because this pseudohomothallic species produces perithecia with asci containing four dikaryotic and binucleate spores, as opposed to the eight spored asci found in all other *Neurospora* species (Turner et al. 2001). Almost all strains in the extensive holdings of *Neurospora* collected from nature were assigned to species by matings to tester strains (Jacobson et al. 2006; Perkins and Turner 1988; Turner et al. 2001). This approach revealed the most recently described species of *Neurospora*, *N. discreta,* which was recognized and described based solely on crossing behavior that showed reproductive isolation from the other known species of *Neurospora* (Perkins and Raju 1986). Other accessions that failed to mate well with any tester were suspected of having a hybrid origin (Turner et al. 2001).

Most recently, species recognition by genetic isolation (PSR) was applied to outbreeding *Neurospora* species and compared to species recognition by reproductive isolation (BSR) using many crosses among individuals in a more thorough manner than could be achieved by crosses only to testers strains (Dettman et al. 2003b). This study found that PSR was in general agreement with a broader BSR application and that both approaches recognized more species than were recognized by crosses to mating tester strains alone (Dettman et al. 2003a; Dettman et al. 2003b). PSR found three new phylogenetic species (PS) that had been missed by crosses with tester strains. Moreover, each putative hybrid was shown to belong to a single PS, i.e., by all single locus phylogenies and there was no evidence that any were hybrids (Turner et al.

2001). These new cryptic species would never have been found without PSR because of a lack of tester strains specific to the new *Neurospora* PS and because they do not have any distinguishing morphological features (Dettman et al. 2003a; Dettman et al. 2003b).

The findings that *Neurospora* PS1 and PS2 contained a disproportionate fraction of what had been thought to be hybrid individuals and that all of the new species were narrowly endemic provided a strategy for discovering additional members of these new species within existing natural *Neurospora* collections (Turner et al. 2001). We flagged for further study nearly 200 natural isolates that were either difficult to assign to a species due to equivocal crosses to mating testers or that were collected in the geographic areas occupied by *Neurospora* PS1–3, or both. In order to evaluate this large number of candidates, we developed a PCR and restriction enzyme digest screen to rapidly exclude genuine members of *N. crassa* or *N. intermedia*. Isolates passing the screen were then sequenced for the informative loci originally used to recognize the new species (Dettman et al. 2003a) and subjected to phylogenetic analysis for assignment to species, first by the criterion of genetic isolation. To further understand the relationship of this first criterion of genetic isolation to the second criterion of intraspecific mating compatibility and intrinsic aspects of the third criterion of reproductive isolation, isolates found to belong to *Neurospora* PS 1–3 were mated among themselves and to individuals of *N. crassa* and *N. intermedia*. Where intrinsic barriers of the third criterion of interspecific reproductive isolation were not observed, biogeography was examined for potential extrinsic barriers to reproductive isolation, as inferred from allopatry. Emerging from the comparison of genetic and reproductive isolation was the finding that PSR and BSR recognize nearly the same groups and that PSR, alone, could be used as the principal criterion for the formal description of new fungal species.

Methods

Neurospora isolates dataset

We examined 188 wild *Neurospora* isolates from the Perkins collection (Appendix). The isolates used in our research were chosen for one of two reasons, ambiguity in mating tests and geographic location. The first 102 isolates were included in the study because they did not mate well with tester strains, which are commonly used to identify outbreeding species in the *Neurospora* genus (Perkins and Turner 1988). The next 86 samples were chosen for the study based on their collection at localities where PS1 (Caribbean Basin), PS2 (Caribbean Basin, Madagascar), and PS3 (Sub-Saharan Africa) have previously been found.

Conidium isolation

All the cultures used in the study are homokaryons subcultured from a single conidium isolated from cultures belonging to the David Perkins culture collection (Turner et al. 2001). Single conidia were isolated by streaking conidia from a mass culture onto 10-fold diluted Vogel's medium agar plates (Vogel 1956). Following incubation for 18–24 hours at ambient temperatures, single, germinated conidia were transferred to establish

cultures (Jacobson 1995). New isolates used in the study were deposited into the Fungal Genetics Stock Center (FGSC) and information can be found in the appendix.

DNA extraction, PCR, and Restriction enzyme digest

DNA was extracted from all strains in the study using the same methods described in Dettman *et al* 2003a. Polymerase chain reaction (PCR) was performed on genomic DNA extraction samples using the TMI loci primers (Dettman et al. 2003a). The TMI locus is one of four phylogenetically informative loci (DMG, QMA, TML, TMI) found on different linkage groups in the *Neurospora sp.* discovered, tested, and described in Dettman *et al.* 2003a. Reactions included 200 μM dNTPs, 0.4 μM of reverse and forward primers, 1X PCR buffer, and 1.0 unit of DNA polymerase in 50 μL reactions. An Eppendorf Mastercycler gradient thermocycler was used for DNA amplification with the following settings: 94°C for 2 minutes during the primary denaturation of DNA, 40 cycles of 94°C (denaturation), primer specific annealing temperature for 1 minute (Dettman et al. 2003a), 72°C for 1 minute (extension), and 72°C for 7 minutes during the final extension.

After PCR amplification of the TMI locus, the samples were all digested using 0.5μL (10 units of enzyme) NciI (New England Biolabs), 2.0 μL of NEBuffer 4 (50mM potassium acetate, 20mM Tris-acetate, 10mM magnesium acetate, 1mM Dithiothreitol, pH 7.9 at 25°C), 7.5 μL of filter-sterilized dH_2O, and 10 μL of TMI PCR product (20 μL total reaction). The reactions were left to digest overnight at 37°C and 5 μL of each reaction were electrophoresed on a 1.5% agarose gel at 150mA for 3 hours (FIGURE 1). Undigested bands (446bp long) identified isolates as *N. crassa*.

PCR products digested at TMI position 102 (344 bp long) were selected for processing with a second restriction digest enzyme, BciVI (New England Biolabs), to identify *N. intermedia* isolates. BciVI is more sensitive to contaminating molecules, so PCR products to be digested with this enzyme were cleaned of primers, single-strand PCR product, and dNTPs in ¼ ExoSAP-IT (UBS) reactions as recommended by the manufacturer. Cleaned PCR products were then digested for 4 hours at 37 °C in a thermocycler with 0.2 μL of BciVI (2 units of enzyme), 2 μL of NEBuffer 4, 7.8 μL of filter sterilized dH_2O, and 10 μL of cleaned PCR product. BciVI was inactivated for 20 minutes at 65 °C. TMI PCR product left undigested by BciVI was identified as *N. intermedia* and TMI PCR products that digested (240 bp) were identified as possibly coming from *Neurospora* PS1–3 (FIGURE 2). Positive controls for the screen were well-characterized individuals of *N. crassa* (D11, D12), *N. intermedia* (D7, D31), PS1 (D57), PS2 (D93), and PS3 (D77) (Dettman et al. 2003a) (FIGURE 1,2).

Sequencing of Informative Loci

To assign screened candidates to phylogenetic species, PCR amplifications of the TMI locus and three additional loci, DMG, QMA, and TML, were sequenced in both directions using published protocols for amplification and BigDye Terminator v3.1 cycle sequencing (ABI) (Dettman et al. 2003a). Scanning of the sequences was performed with an ABI Prism 3100 Genetic Analyzer. Sequence data were analyzed using

Sequencher 4.2.2 (Gene Codes Corp.) and consensus sequences for all four loci for each candidate isolate were assembled. New sequences from the study were deposited into Genbank and assigned accession numbers FJ35356–FJ53549.

Sequence alignment

Sequences of each of the four loci generated here were aligned, using clustalW [13] with those used by Dettman *et al.* 2003a (Dettman et al. 2003a). For each locus, the aligned, combined data file was visually inspected to optimize the alignment and remove unalignable microsatellite positions (Dettman et al. 2003a). The alignment used in the study was deposited into Treebase (www.treebase.org).

Tree building and species identification

Sequence data from the DMG, QMA, TMI, and TML loci were aligned together in one consensus file. Sequences of D104 and D138 from Dettman 2003a *et al*. were excluded because they were missing QMA sequences. Using Mr.Modeltest 3.1.7 (Nylander 2004) with Paup 4.0 (Swofford 2003) the appropriate nucleotide substitution model was chosen for the consensus sequence as a whole, which was input into Mr. Bayes (Huelsenbeck et al. 2001; Ronquist and Huelsenbeck 2003) (Bayesian Inference) and Garli (Zwickl 2006) (Maximum Likelihood) for phylogenetic analysis. The consensus alignment and the chosen nucleotide substitution model were input into Mr. Bayes for one million generations with a burnin of 250,000 generations in order to produce a consensus tree with Bayesian posterior probabilities. The same alignment and nucleotide substitution model was input into Garli and run for one million generations to determine the most likely tree. One hundred, 10000 generation bootstrap replicates were run to obtain 100 trees that were input into PAUP to determine bootstrap support numbers for the different clades. Species assignment of the putative PS isolates was determined by the placement of the unknown isolates in relation to other known isolates from the Dettman *et al*. 2003a study.

Design of crossing matrix and mating of isolates

Matings and evaluation of reproductive success were done following published protocols (Dettman et al. 2003b). The *Neurospora* strains used in the crosses were grown on synthetic crossing medium in the dark for 7 days at 24 °C (Westergaard and MItchell 1947). Reciprocal crosses were performed by collecting conidia from two cultures of opposite mating type and inoculating each culture with conidia from the mating partner. After 14 additional days at 24 °C the slants were visually inspected and graded for reproductive success according to the criteria listed in FIGURE 3.

Results

RFLP Screen for Phylogenetic Species

To design our RFLP screen, we used NEBCutter 2.0 (Vincze et al. 2003) to search among the four loci used for PSR to find restriction endonuclease recognition sites that were invariant within *N. crassa, N. intermedia* and the combined PS 1–3, but variable among these three groups. We confirmed the predictions that NciI would digest TMI

sequences not belonging to *N. crassa* at nucleotide position 102 (target sequence: 5′-CCCGG-3′) and that BciVI would digest TMI sequences not belonging to *N. intermedia* at nucleotide position 240 (target sequence: 5′-GTATCC-3′) using positive controls for *N. crassa*, *N. intermedia*, and PS 1–3 (FIGURE 1, 2). In the screen, NciI failed to digest PCR amplified TMI from 41 of the 188 PCR samples, which were considered to be *N. crassa* and were excluded from further analysis. BciVI failed to digest PCR amplified TMI from 124 of the remaining 147, which were considered to belong to *N. intermedia* and were also excluded from further analysis. The remaining 22 samples were candidates for membership in *Neurospora* PS1–3.

Phylogenetic Species Recognition

Species assignment of the 22 individuals possessing both NciI and BciIV restriction sites and possibly belonging to *Neurospora* PS 1–3, required PCR amplification and sequencing of the loci used previously for PSR (DMG, QMA, TMI, and TML (Dettman et al. 2003a). The sequences were aligned with the corresponding sequences of the 145 individuals used by Dettman *et al.* 2003a to recognize phylogenetic species. The alignments of the four loci were compiled into one consensus alignment, which was used to build a maximum likelihood (ML) tree using Garli (Zwickl 2006) and a Bayesian inference (BI) tree using Mr. Bayes (Huelsenbeck et al. 2001; Ronquist and Huelsenbeck 2003). The phylogeny found by both methods is displayed in FIGURE 3. Both the ML tree and the BI tree were in agreement with Dettman *et al.* 2003a in that each species-level clade had well supported Bayesian posterior probabilities and Maximum likelihood bootstrap proportions (FIGURE 4).

Of the 22 strains sequenced, 10 were identified as PS2 and one isolate each was identified as a member of PS1 or PS3 (FIGURE 4). The 10 remaining individuals proved to be *N. sitophila* individuals. Finding the false positive *N. sitophila* prompted us to search for false negative results among the strains excluded from further analyses by our screen. We sequenced the TMI loci of several isolates excluded by the restriction digestions as *N. crassa* (CV11, CV120, CV147) or as *N. intermedia* (CV54, CV72, CV76, CV83, CV99, CV111, CV170). None of the excluded individuals proved to be PS1, PS2, or PS3, however one was identified as belonging to *N. discreta sensu lato (*CV11).

Phylogenetic Species BSR Matrix

To determine if the new *Neurospora* PS1–3 specimens shared the same mating patterns as those reported by Dettman *et al.* 2003b, we performed crosses among six of the new PS2 individuals, the new PS1 and PS3 individuals, and two PS1, two PS2, two PS3, four *N. crassa*, and four *N. intermedia* individuals used by Dettman *et al.* 2003b. Matings were evaluated exactly as reported by Dettman *et al.* 2003b and results for 130 new crosses and 68 crosses taken from Dettman *et al.* 2003b are shown in FIGURE 3. Intraspecific matings within PS1, PS2, and PS3 were very successful and in most cases were scored as one of the two highest categories of reproductive success, i.e., 5 or 6. As in the previous study, PS3 isolates were found to mate well with each other and also with individuals from *N. crassa* subclade A (Dettman et al. 2003b).

Taxonomy

Having conducted a more extensive search of culture collections and having discovered additional members of PS1, PS2, and PS3 we feel confident in naming the three phylogenetic species. The original and newly discovered members of PS1, PS2, and PS3 all meet the criteria of genetic and reproductive isolation (intrinsic or extrinsic) necessary for a formal description. The additional specimens did not alter the *Neurospora* phylogeny of the original members and their mating success with different *Neurospora* species was similar to that of the original members (Dettman et al. 2003b).

Neurospora hispaniola Villalta, Jacobson et Taylor, sp. nov.
Fungus generatione sexuale inter individua, heterothallicus haploideus prius PS1 designatus, a speciebus Neurosporae alteris heterothallicis generatione sexuale inter individua distinguendus non per formam, sed per notionem speciei phylogenetici et quattuor locos geneticos DMG, DMA, TMI, et TML. Notae characteristicae nucleotidorum fixae in individuis notis speciei huius: positiones loci TMI 71 (T), 109 (G), 48 (T); loci TML 129 (T), 130 (G), 131 (G), 195 (T), 514 (G); loci QMA 70 (G), 290 (A), 415 (G).

Neurospora hispanola is an outbreeding heterothallic haploid fungus previously designated PS1. *N. hispaniola* is morphologically indistinguishable from the other heterothallic outbreeding species of *Neurospora*, but individuals can be assigned to *N. hispaniola* using the phylogenetic species recognition concept and these four loci: DMG, QMA, TMI, and TML (Dettman et al. 2003a). Diagnostic nucleotide characters that are fixed among the known individuals include TMI locus nucleotide positions 71 (T), 109 (G), 48 (T); TML locus nucleotide positions 129 (T), 130 (Dettman et al. 2003b) (G), 131 (G), 195 (T), 514 (G); QMA locus nucleotide positions 70 (G), 290 (A), 415 (G).

HOLOTYPE: CV55 (FGSC 10403)

Distribution: Western region of Hispaniola in the country of Haiti.

Etymology: Species is named after the island of Hispaniola where it was first collected by David Perkins.

Neurospora metzenbergii Villalta, Jacobson et Taylor, sp. nov
Fungus generatione sexuale inter individua, heterothallicus haploideus prius PS2 designatus, a speciebus Neurosporae alteris heterothallicis generatione sexuale inter individua distinguendus non per formam, sed per notionem speciei phylogenetici et quattuor locos geneticos DMG, QMA, TMI, et TML. Notae characteristicae nucleotidorum fixae in individuis notis speciei huius: positiones loci TMI 315 (G), 396 (A); loci TML 393 (G), 456 (T), 458 (G); loci QMA 124 (A), 135 (A), 146 (A), 219 (A), 318 (A), 357 (T).

Neurospora metzenbergii is an outbreeding heterothallic haploid fungus previously designated PS2. *N. metzenbergii* is morphologically indistinguishable from other heterothallic outbreeding species of *Neurospora*, but individuals can be assigned to *N. metzenbergii* using the phylogenetic species recognition concept and these four loci: DMG, QMA, TMI, and TML (Dettman et al. 2003a). Diagnostic nucleotide characters that are fixed among the known individuals include the TMI locus nucleotide positions 315 (G), 396 (A); TML locus nucleotide positions 393 (G), 456 (T), 458 (G); QMA locus nucleotide positions 124 (A), 135 (A), 146 (A), 219 (A), 318 (A), 357 (T).

Distribution: Found throughout the Yucatan peninsula in Mexico, on the western part of Hispaniola in the country of Haiti, and on the island of Madagascar.

HOLOTYPE: CV89 (FGSC 10395)

Etymology: The species is named after Robert L. Metzenberg who was a leading *Neurospora* geneticist. He collected most of the *N. metzenbergii* samples while in Mexico.

Neurospora perkinsii Villalta, Jacobson et Taylor, sp. nov.
Fungus generatione sexuale inter individua, heterothallicus haploideus prius PS3 designatus, a speciebus Neurosporae alteris heterothallicis generatione sexuale inter individua distinguendus non per formam, sed per notionem speciei phylogenetici et quattuor locos geneticos DMG, QMA, TMI, et TML. Notae characteristicae nucleotidorum fixae in populatione: positiones loci TMI 66 (T), 154 (G), 159 (A), 237 (C), 434 (G).

Neurospora perkinsii is an outbreeding heterothallic haploid fungus previously designated PS3. *N. perkinsii* is morphologically indistinguishable from other heterothallic outbreeding species of *Neurospora*, but individuals can be assigned to *N. perkinsii* using the phylogenetic species recognition concept and these four loci: DMG, QMA, TMI, and TML (Dettman et al. 2003a). Diagnostic nucleotide characters all present and fixed in the population include the TMI locus nucleotide positions 66 (T), 154 (G), 159 (A), 237 (C), 434 (G).

Distribution: All current specimens have been found in sub-Saharan Africa in the Democratic Republic of Congo and Gabon.

HAPLOTYPE: CV79 (FGSC 10406)

Etymology: N. perkinsii is named after the *Neurospora* geneticist and biologist David D. Perkins who was so important to the *Neurospora* community and who was responsible for the majority of the specimens in the Perkins collection, including the *N. perkinsii* isolates.

Discussion

We found additional members of *N. hispaniola, N. metzenbergii,* and *N. perkinsii* by searching among collections of Neurospora individuals for which mating tests were equivocal or that had been collected in endemic areas and then screening to exclude *N. crassa* and *N. intermedia*. In formally describing species preliminarily identified by PSR, we found that *N. hispaniola, N. metzenbergii*, and *N. perkinsii* were all distinct phylogenetic species according to the PSR criteria set forth in Dettman 2003a and all were able to mate successfully intraspecifically. Intrinsic interspecific mating barriers consistent with the biological species concept were found for *N. hispaniola* and *N. metzenbergii,* but not for *N. perkinsii* when mated to *N. crassa* (FIGURE 3). However, the *N. crassa* and *N. perkinsii* strains that successfully mated are genetically and geographically isolated, suggesting that the extrinsic barrier of allopatry keeps the two species reproductively isolated. In our matings, the African *N. crassa* strains that mated successfully with *N. perkinsii* were from the Ivory Coast, which is at least 2000 km from Congo. It would be interesting to see if there are any mating barriers present between *N. perkinsii* and *N. crassa* collected from Congo, because previous studies have shown that mating barriers may be stronger between species in sympatry than in allopatry (Dettman et al. 2003b).

Adding the newly discovered *Neurospora* individuals to the existing phylogeny changed neither the phylogenetic relationships nor the distinct geographic ranges of the species. The narrow endemism found in *N. hispaniola* (Haiti*), N. metzenbergii* (Yucatan and Madagascar) and *N. perkinsii* (Congo) remains, but our sampling was partially biased to those regions. As in previous studies we did not find any wild hybrid individuals.
We found cases where *Neurospora* individuals had been incorrectly assigned to species by crosses to mating testers. For individuals that belong to *N. hispaniola, N. metzenbergii,* and *N. perkinsii*, the assignment problems could be explained by a lack of tester strains for the new species, a problem that has been corrected (Dettman et al. 2003b). In the cases of the *N. sitophila* or *N. discreta* individuals that previously had been assigned to *N. crassa* or *N. intermedia*, the problem may be due to intraspecific variation in reproductive compatibility that cannot be represented by a few tester strains. In the case of *N. discreta sensu lato*, we know that there is significant genetic differentiation and isolation in this species (Dettman et al. 2003a, 2006). For *N. sitophila*, the necessary detailed studies have not been carried out. We found no misassigned *N. tetrasperma* isolates, indicating that the four-spored morphology and pseudohomothallic mating is a reliable morphological character. There are, however, genetically isolated or differentiated groups within *N. tetrasperma* (Saenz et al. 2003). Our screen could be improved to account for *N. sitophila* and *N. discreta* genotypes and, thereby, reduce the number of false positives. We were unable to detect any false negatives.

Although we found additional members of each species, the need remains to characterize more individuals of these *Neurospora* species. Obviously, *N. hispaniola and N. perkinsii* are still very under sampled, and *N. metzenbergii* individuals are split between two well separated geographic regions, Yucatan and Madagascar. Genetic

differentiation in this species correlates with geographic range (FIGURE 3), but our data show that individuals from the two geographic areas are not reproductively isolated. For example, isolate D120, collected in Madagascar, mates successfully with isolates from the Yucatan. When more Madagascar individuals are collected and characterized, as the result of new fieldwork, the species status of this group may change if the presently observed genetic differentiation is unchanged. The need for more individuals also applies to *N. sitophila*, were a sampling comparable to that provided by Dettman *et al.* for *N. crassa* or *N. intermedia* (Dettman et al. 2003a, 2006) will be needed to interpret the phylogenetic relationships of the ten "false positive" *N. sitophila* isolates discovered here.

While the species described here have narrow geographic ranges, as many as five *Neurospora* species can be found in sympatry at the same geographic location (Turner et al. 2001) and in very close proximity on the same substrate (Powell et al. 2003). However, as mentioned above no hybrids have been collected in the wild although ample opportunity for hybridization exists (Dettman et al. 2003a, 2006; Powell et al. 2003; Turner et al. 2001). *Neurospora* remains an ecological enigma because little is known about the life history or different niches occupied by any of the species, or about interactions with other organisms. Understanding the genetic distance of the different *Neurospora* species will be especially helpful in the era of high-throughput sequencing and comparative genomics where having that information can determine what organisms should have their genome sequenced.

This and previous studies have found that identification by successful mating, BSR, is similar to PSR, but that genetic isolation can precede reproductive isolation (Dettman et al. 2003a, 2006; Dettman et al. 2003b). Our results show that BSR by mating to tester strains alone can be misleading due to a lack of testers or the absence of reproductive isolation. In the last decade phylogenetic species recognition has become a popular alternative to morphological species recognition (MSR) and biological species recognition (BSR) (Taylor et al. 2000), as evidenced by studies involving *Coccidiodes* (Fisher et al. 2002), *Neurospora* (Dettman et al. 2003a, 2006; Dettman et al. 2003b), *Saccharomyces* (Ae et al. 2006), and *Schizophyllum* (James et al. 2001) (Taylor et al. 2006). The results above show that while PSR alone is powerful and accurate, it is also important, where possible, to the formal description of new species to account for reproductive isolation, biogeography, and morphology. We hope that our characterization and naming of *Neurospora* species, and the addition of additional strains to the *Neurospora* phylogenetic tree will add to the attractiveness of *Neurospora* as an interesting model organism for evolutionary biology and ecology.

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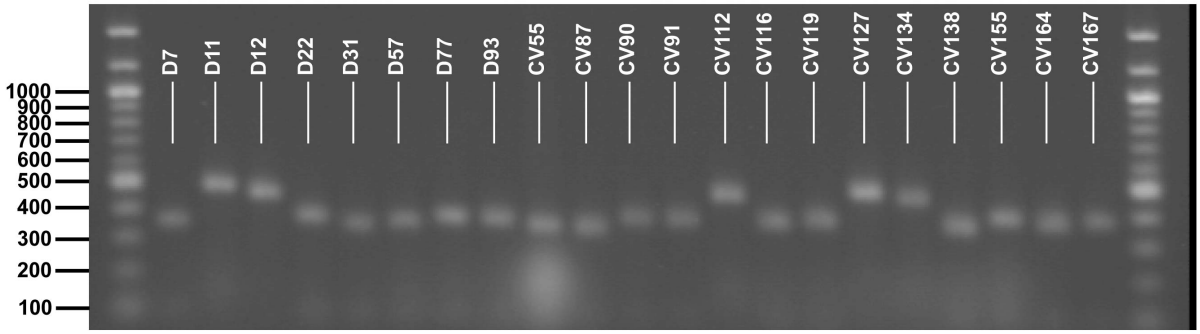

Figure 1. NciI restriction digestions of the PCR amplified *Neurospora* TMI locus electrophoresed in agarose and stained with ethidium bromide. Controls: *N. crassa* (undigested) D11, D12, *N. intermedia* (digested) D7, D22, D31, PS1 (digested) D57, PS2 (digested) D93, and PS3 (digested) D77. Unknowns: Undigested and putative *N. crassa* CV113, CV146; digested and putative *N. intermedia* or putative new PS CV114–CV119, CV142. The 466pb TMI locus is digested by Nci1 into a 344bp fragment and a 102bp fragment that is too small be seen on this gel. TMI PCR lengths may be slighty larger than 446 bp (D22) because of microsatellites (Dettman et al. 2003a). A 100 bp ladder (NEB) was used to measure PCR product lengths. The 102 bp segment is not clearly visible, nor needed for analysis.

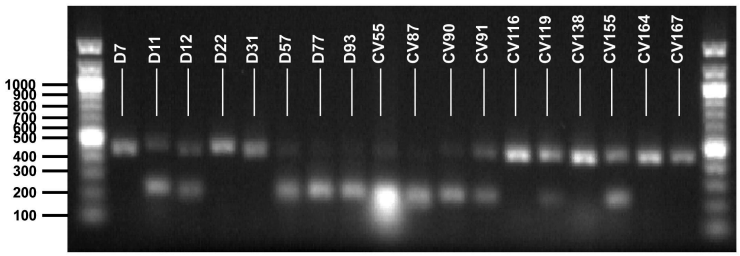

Figure 2. BciVI restriction digestions of the PCR amplified *Neurospora* TMI locus electrophoresed in agarose and stained with ethidium bromide. Controls: *N. intermedia* D7, D22, D31; digested TMI indicating *N. crassa* (D11, D12), PS1 (D57), PS2 (D93), and PS3 (D77). Unknowns: Undigested and putative *N. intermedia,* CV116, CV138, CV164, CV167; digested and putative new PS, CV55, CV87, CV90, CV91, CV119, CV155. The 446 bp was digested by BciVI into a 240 bp fragment and a 206 bp fragment. Enzyme did not always completely digest TMI PCR product, but any PCR sampled that looked digested was queued for further analysis. CV87 was eventually identified as *N. sitophila* (see Results and Discussion). A 100 bp ladder (NEB) was used to measure PCR product length.

PS1-3 Matings (BSR)

	matA-->	Nh CV55	Nh D58	Nm CV152	Nm CV155	Nm CV156	Nm D120	Np D78	Nc D107	Nc D62	Ni D122	Ni D36
	mata											
Nh	D55	**6/6**	**6/6***	1/1	6/1	1/5	0/4*	-*	0/3	-*	0/0*	-
Nm	CV89	6/0	6/1	**6/6**	**6/5**	**6/5**	**6/6**	5/5	5/6	4/5	4/1	1/5
Nm	CV119	1/1	1/2	**2/5**	**5/4**	**4/5**	**5/6**	6/6	6/5	6/6	1/1	6/5
Nm	CV148	0/5	5/1	**6/6**	**6/6**	**6/6**	**5/6**	5/5	4/4	4/4	1/5	5/6
Nm	D92	1/5	3/0*	**5/5**	**6/5**	**5/4**	**5/5***	3/3*	4/4*	3/3*	4/1	-*
Np	CV79	1/1	1/1	6/6	6/1	2/5	5/6	**6/6**	6/6	6/6	1/1	6/6*
Np	D77	1/4	3/0*	5/5	5/4	5/4	3/3*	**6/6***	5/5*	-*	3/0*	-*
Nc	D100	4/1	3/0*	5/4	5/4	2/6	3/3*	5/5*	**6/6***	**4/4***	3/2*	-*
Nc	D116	1/4	3/0*	6/6	6/5	4/1	3/3*	6/6*	**6/5***	**6/6***	0/0*	3/3*
Ni	D127	0/1	1/0*	2/2	2/1	1/2	3/3*	3/3*	3/1*	1/2*	**5/6***	**6/6***
Ni	D2	0/3	-*	-	2/1	1/1	-*	-*	3/3*	-*	**6/6***	**6/0***

6	>50% black ascospores
5	15-50% black ascospores
3&4	<1% black ascospores; & 1-15% black ascospores
2	perithecia developed ostioles, no ascospores ejected
0&1	sterile, no perithecia produced; & barren perithecia, no ostiole developed

Figure 3. BSR matrix of crosses between the new PS and previously identified *Neurospora* specimens. The grading criteria are the same as the criteria used in Dettman *et al.* 2003b. The boxes labeled in bold signify intraspecific crosses. The majority of intraspecific crosses were successful displaying cohesion between BSR and PSR. Crosses with an (*) are those taken from Dettman *et al.* 2003b in order to compare those crosses with our data. Reciprocal crosses were performed in every cell and the data is ordered as follows: *mat a* (parent*)/ mat A* (parent). Nh: *N. Hispaniola*; Nm: *N. metzenbergii*: Np: *N. perkinsii*; Nc: *N. crassa*; Ni: *N. intermedia.*

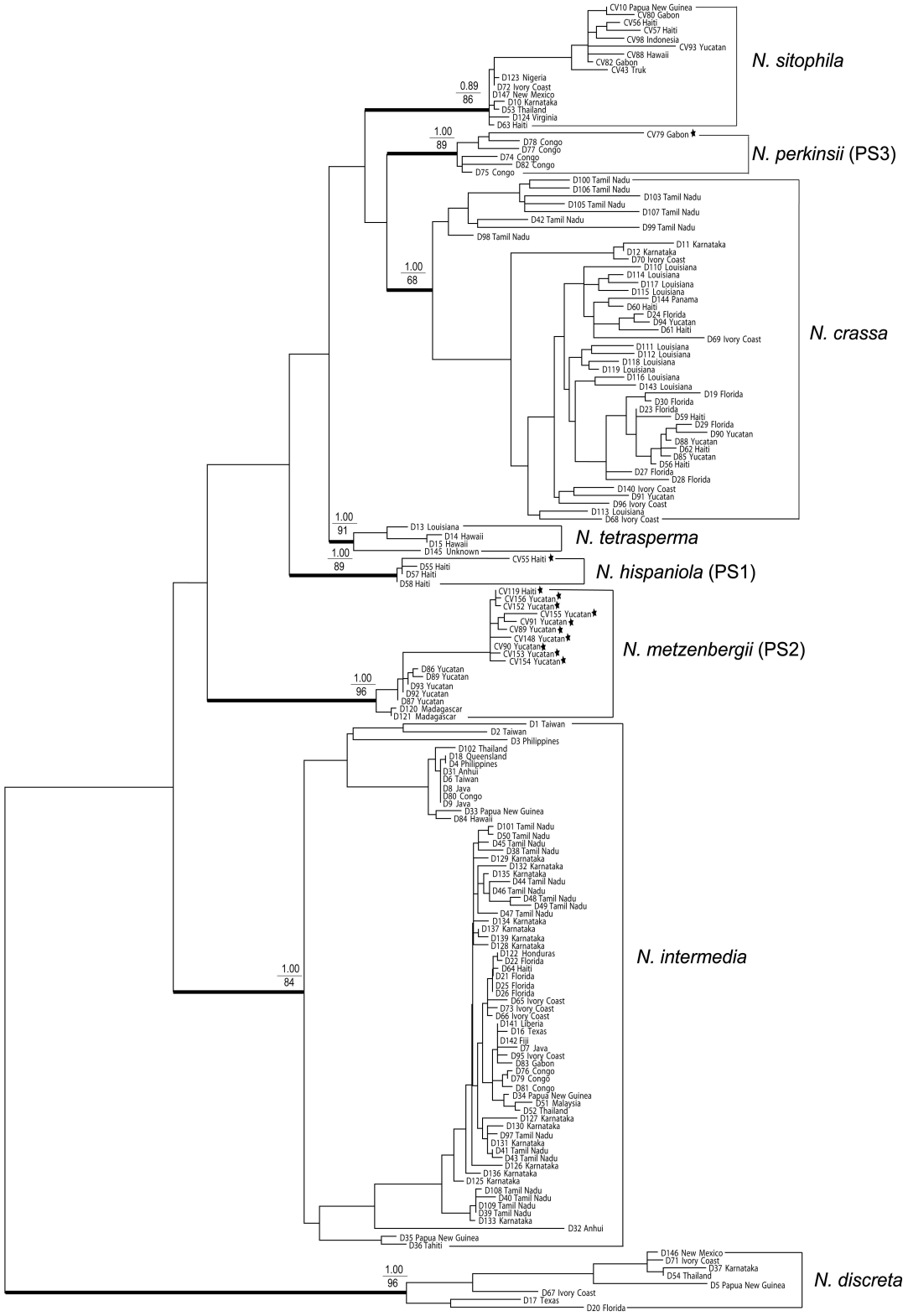
CV10 Papua New Guinea
CV80 Gabon
CV56 Haiti
CV57 Haiti
CV98 Indonesia
CV93 Yucatan
CV88 Hawaii
CV82 Gabon
CV43 Truk
N. sitophila
0.89
86
D123 Nigeria
D72 Ivory Coast
D147 New Mexico
D10 Karnataka
D53 Thailand
D124 Virginia
D63 Haiti
1.00
89
CV79 Gabon
D78 Congo
D77 Congo
D74 Congo
D82 Congo
D75 Congo
N. perkinsii (PS3)
D100 Tamil Nadu
D106 Tamil Nadu
D103 Tamil Nadu
D105 Tamil Nadu
D107 Tamil Nadu
D42 Tamil Nadu
D99 Tamil Nadu
D98 Tamil Nadu
D11 Karnataka
D12 Karnataka
D70 Ivory Coast
D110 Louisiana
1.00
68
D114 Louisiana
D117 Louisiana
D115 Louisiana
D144 Panama
D60 Haiti
D24 Florida
D94 Yucatan
D61 Haiti
D69 Ivory Coast
N. crassa
D111 Louisiana
D112 Louisiana
D118 Louisiana
D119 Louisiana
D116 Louisiana
D143 Louisiana
D19 Florida
D30 Florida
D23 Florida
D59 Haiti
D29 Florida
D90 Yucatan
D88 Yucatan
D62 Haiti
D85 Yucatan
D56 Haiti
D27 Florida
D28 Florida
D140 Ivory Coast
D91 Yucatan
D96 Ivory Coast
D113 Louisiana
D68 Ivory Coast
1.00
91
D13 Louisiana
D14 Hawaii
D15 Hawaii
D145 Unknown
N. tetrasperma
1.00
89
CV55 Haiti
D55 Haiti
D57 Haiti
D58 Haiti
N. hispaniola (PS1)
CV119 Haiti
CV156 Yucatan
CV152 Yucatan
CV155 Yucatan
CV91 Yucatan
CV89 Yucatan
CV148 Yucatan
CV90 Yucatan
CV153 Yucatan
CV154 Yucatan
N. metzenbergii (PS2)
1.00
96
D86 Yucatan
D89 Yucatan
D93 Yucatan
D92 Yucatan
D87 Yucatan
D120 Madagascar
D121 Madagascar
D1 Taiwan
D2 Taiwan
D3 Philippines
D102 Thailand
D18 Queensland
D4 Philippines
D31 Anhui
D6 Taiwan
D8 Java
D80 Congo
D9 Java
D33 Papua New Guinea
D84 Hawaii
D101 Tamil Nadu
D50 Tamil Nadu
D45 Tamil Nadu
D38 Tamil Nadu
D129 Karnataka
D132 Karnataka
D135 Karnataka
D44 Tamil Nadu
D46 Tamil Nadu
D48 Tamil Nadu
D49 Tamil Nadu
D47 Tamil Nadu
D134 Karnataka
D137 Karnataka
D139 Karnataka
D128 Karnataka
1.00
84
D122 Honduras
D22 Florida
D64 Haiti
D21 Florida
D25 Florida
D26 Florida
D65 Ivory Coast
D73 Ivory Coast
D66 Ivory Coast
D141 Liberia
D16 Texas
D142 Fiji
D7 Java
D95 Ivory Coast
D83 Gabon
D76 Congo
D79 Congo
D81 Congo
D34 Papua New Guinea
D51 Malaysia
D52 Thailand
D127 Karnataka
D130 Karnataka
D97 Tamil Nadu
D131 Karnataka
D41 Tamil Nadu
D43 Tamil Nadu
D126 Karnataka
D136 Karnataka
D125 Karnataka
D108 Tamil Nadu
D40 Tamil Nadu
D109 Tamil Nadu
D39 Tamil Nadu
D133 Karnataka
D32 Anhui
D35 Papua New Guinea
D36 Tahiti
N. intermedia
D146 New Mexico
D71 Ivory Coast
D37 Karnataka
D54 Thailand
D5 Papua New Guinea
1.00
96
D67 Ivory Coast
D17 Texas
D20 Florida
N. discreta
0.001 substitutions/site

Figure 4. The phylogenetic relationships among the out breeding species of *Neurospora*, including the 12 newly characterized isolates of *N. hispaniola*, *N. metzenbergii* and *N. perkinsii* (denoted by BLACK star) and the 145 specimens previously characterized by Dettman *et al*. 2003a. The tree was constructed using Mr. Bayes and the major branches defining each phylogenetic species (PS) are marked in bold. The numbers above each major branch indicate confidence levels; Bayesian Posterior Probability/Maximum Likelihood Bootstrap Proportions.

Appendix: Specimens used in RFLP analysis

Study ID	FGSC ID[1]	Perkins ID[2]	Original Species[3]	Phylogenetic Species	Mating Type[4]	NciI[5]	BciVI[6]	Collection Site
CV-1		P1563	N. *intermedia*?		A&a	+	–	Yalu, Papua New Guinea
CV-2		P1666	N. *intermedia*?		A&a	+	–	Kaindi Road, Papua New Guinea
CV-3		P1668	N. *intermedia*?		A&a	–		Kaindi Road, Papua New Guinea
CV-4		P1717	N. *intermedia*?		A&a	+	–	Goroka, Papua New Guinea
CV-5		P1751	N. *intermedia*?		A&a	+	–	Goroka, Papua New Guinea
CV-6		P1804	N. *intermedia*?		A&a	+	–	Baiyer River, Papua New Guinea
CV-7		P1828	N. *intermedia*?		a	+	–	Mt. Hagen, Papua New Guinea
CV-8		P1829	N. *intermedia*?		a	+	–	Mt. Hagen, Papua New Guinea
CV-9		P1982	N. *intermedia*?		A&a	+	–	Brown River Area, Papua New Guinea
CV-10	**10393**	**P2026**	***N. intermedia?***	***N. sitophila***	**A**	**+**	**+**	**Brown River Area, Papua New Guinea**
CV-11	10394	P2027	N. *intermedia*?	*N. discreta* (PS?)*	A	–		Brown River Area, Papua New

CV-11	10394	P2027	N. *intermedia*?	*N. discreta* (PS?)*	A	–		Brown River Area, Papua New Guinea
CV-12		P2745	N. *intermedia*?		A&a	+	–	Kampung Cempaka, Malaysia
CV-13		P2840	N. *intermedia*?		A&a	+	–	Mantin, Malaysia
CV-14		P2853	N. *intermedia*?		A&a	+	–	Melaka, Singapore
CV-15		P2864	N. *intermedia*?		A&a	+	–	Singapore, Singapore
CV-16		P2871	N. *intermedia*?		A&a	+	–	Singapore, Singapore
CV-17		P2891	N. *intermedia*?		A&a	+	–	Bangkok Airport, Thailand
CV-18		P2922	N. *intermedia*?		A&a	+	–	Khao Eto, Thailand
CV-19		P2933	N. *intermedia*?		A&a	+	–	Khao Yai, Thailand
CV-20		P2944	N. *intermedia*?		A&a	+	–	Khao Yai, Thailand
CV-21		P2983	N. *intermedia*?		A&a	+	–	Klong Rangsit, Thailand
CV-22		P3006	N. *intermedia*?		A&a	+	–	Pakchong, Thailand
CV-23		P3032	N. *intermedia*?		A&a	+	–	Saraburi, Thailand
CV-24		P3038	N. *intermedia*?		A&a	+	–	Wang Noi, Thailand
CV-25		P3077	N. *intermedia*?		A&a	+	–	Singapore, Singapore
CV-26		P3083	N. *intermedia*?		A&a	+	–	Kuching, Borneo
CV-27		P3085	N. *intermedia*?		A&a	+	–	Kuching, Borneo
CV-28		P3111	N. *intermedia*?		A&a	+	–	Lokawi Camp, Borneo
CV-29		P3140	N. *intermedia*?		A&a	+	–	Kota Kinabalu, Borneo
CV-30		P3154	N. *intermedia*?		A&a	+	–	Songsong, Rota
CV-31		P3164	N. *intermedia*?		A&a	+	–	Songsong, Rota
CV-32		P3169	N. *intermedia*?		A&a	+	–	Rota, Rota
CV-33		P3176	N. *intermedia*?		A&a	+	–	Rota, Rota
CV-34		P3177	N. *intermedia*?		A&a	+	–	Rota, Rota

CV-37		P3185	N. *intermedia*?		A&a	+	–	Rota, Rota
CV-38		P3215	N. *intermedia*?		A&a	+	–	Merizo, Guam
CV-39		P3225	N. *intermedia*?		A&a	+	–	Taleysay, Guam
CV-40		P3227	N. *intermedia*?		A&a	+	–	Taleysay, Guam
CV-41		P3230	N. *intermedia*?		A&a	+	–	Taleysay, Guam
CV-42		P3233	N. *intermedia*?		A&a	+	–	Moen, Truk
CV-43	**10402**	**P3252**	***N. intermedia?***	***N. sitophila***	**A**	**+**	**+**	**Moen, Truk**
CV-44		P3255	N. *intermedia*?		A&a	+	–	Moen, Truk
CV-45		P3288	N. *intermedia*?		A&a	+	–	Moen, Truk
CV-46		P3289	N. *intermedia*?		A&a	+	–	Moen, Truk
CV-47		P3300	N. *intermedia*?		A&a	+	–	Moen, Truk
CV-48		P3301	N. *intermedia*?		A&a	+	–	Moen, Truk
CV-49		P3309	N. *intermedia*?		A&a	+	–	Kolonia, Ponape
CV-50		P3321	N. *intermedia*?		A&a	+	–	Kolonia, Ponape
CV-51		P3324	N. *intermedia*?		A&a	+	–	Kolonia, Ponape
CV-52		P3338	N. *intermedia*?		A&a	+	–	Ponape, Ponape
CV-53		P3339	N. *intermedia*?		A&a	+	–	Ponape, Ponape
CV-54		P3340	N. *intermedia*?	*N. intermedia**	A&a	+	–	Ponape, Ponape
CV-55	**10403**	**P3431**	***N. intermedia?***	***N. hispaniola* (PS1)**	**A**	**+**	**+**	**Leogane, Haiti**
CV-56	**10404**	**P3472**	***N. intermedia?***	***N. sitophila***	**A**	**+**	**+**	**Bas Quarter, Haiti**
CV-57	**10405**	**P3473**	***N. intermedia?***	***N. sitophila***	**A**	**+**	**+**	**Bas Quarter, Haiti**
CV-58		P3534	N. *intermedia*?		A&a	+	–	Ran Adjame, Ivory Coast
CV-59		P3537	N. *intermedia*?		A&a	+	–	Ran Adjame, Ivory Coast
CV-60		P3546	N. *intermedia*?		a	+	–	Yopougon, Ivory Coast
CV-61		P3547	N. *intermedia*?		A&a	+	–	Universite, Ivory Coast
CV-62		P3548	N. *intermedia*?		A&a	+	–	Universite, Ivory Coast
CV-63		P3574	N. *intermedia*?		a	–		Godilisheri Ecole, Ivory

CV-63		P3574	N. *intermedia*?		a	–		Godilisheri Ecole, Ivory Coast
CV-64		P3708	N. *intermedia*?		A&a	–		Hermankono, Ivory Coast
CV-65		P3745	N. *intermedia*?		A&a	+	–	Agbanou, Ivory Coast
CV-66		P3756	N. *intermedia*?		A&a	+	–	Adiopodoume, Ivory Coast
CV-67		P3777	N. *intermedia*?		A	–		Brazzaville, Congo
CV-68		P3779	N. *intermedia*?		a	–		Brazzaville, Congo
CV-69		P3806	N. *intermedia*?		A&a	+	–	Lebanda, Congo
CV-70		P3832	N. *intermedia*?		A&a	+	–	Jacob, Congo
CV-71		P3861	N. *intermedia*?		A&a	+	–	Mantsoumba, Congo
CV-72		P3871	N. *intermedia*?	*N. intermedia**	A&a	+	–	Mantsoumba, Congo
CV-73		P3876	N. *intermedia*?		A&a	+	–	Mindouli, Congo
CV-74		P3883	N. *intermedia*?		A&a	+	–	Missafou, Congo
CV-75		P3892	N. *intermedia*?		A&a	+	–	Kinkala,Congo
CV-76		P3896	N. *intermedia*?	*N. intermedia**	A&a	+	–	Missafou, Congo
CV-77		P3904	N. *intermedia*?		a	+	–	Kinkala,Congo
CV-78		P3945	N. *intermedia*?		A&a	+	–	Liberville, Gabon
CV-79	**10406**	**P3947**	***N. intermedia?***	***N. perkinsii* (PS3)**	**a**	**+**	**+**	**Liberville, Gabon**
CV-80	**10407**	**P3948**	***N. intermedia?***	***N. sitophila***	**a**	**+**	**+**	**Liberville, Gabon**
CV-81		P3949	N. *intermedia*?		a	+	–	Liberville, Gabon
CV-82	**10408**	**P3952**	***N. intermedia?***	***N. sitophila***	**a**	**+**	**+**	**Liberville, Gabon**
CV-83		P3953	N. *intermedia*?	*N. intermedia**	A	+	–	Libreville, Gabon
CV-84		P3954	N. *intermedia*?		A&a	+	–	Libreville, Gabon
CV-85		P3961	N. *intermedia*?		a	+	–	MadingoMarket, Dominican Republic
CV-86		P4077	N. *intermedia*?		A	–		Torani

CV-87		P4098	N. *intermedia*?	*N. sitophila*	a	+	+	Laie, Hawaii
CV-88	**10394**	**P4099**	***N. intermedia?***	***N. sitophila***	**a**	**+**	**+**	**Laie, Hawaii**
CV-89	**10395**	**P4166**	***N. intermedia?***	***N. metzenbergii* (PS2)**	**a**	**+**	**+**	**Macantoc, Mexico**
CV-90	**10396**	**P4171**	***N. intermedia?***	***N. metzenbergii* (PS2)**	**a**	**+**	**+**	**Coba, Mexico**
CV-91	**10397**	**P4172**	***N. intermedia?***	***N. metzenbergii* (PS2)**	**a**	**+**	**+**	**Coba, Mexico**
CV-92		P4173	N. *intermedia*?		a	–		Coba, Mexico
CV-93	**10409**	**P4181**	***N. intermedia?***	***N. sitophila***	**A**	**+**	**+**	**Chemax, Mexico**
CV-94		P4388	N. *intermedia*?		A&a	+	–	Adiopodoume, Ivory Coast
CV-95		P4390	N. *intermedia*?		a	+	–	Adiopodoume, Ivory Coast
CV-96		P4411	N. *intermedia*?		a	+	–	Adiopodoume, Ivory Coast
CV-97		P4535	N. *intermedia*?		a	+	–	Bani, Dominican Republic
CV-98	**10398**	**P4594**	***N. intermedia?***	***N. sitophila***	**a**	**+**	**+**	**Sulawesi Indonesia**
CV-99		P4665	N. *intermedia*?	*N. intermedia**	a	+	–	Kampang Pengakalan Kuin, Malaysia
CV-100		P4666	N. *intermedia*?		A&a	+	–	Kampang Pengakalan Kuin, Malaysia
CV-101		P4771	N. *intermedia*?		A	+	–	Madurai, India
CV-102		P4772	N. *intermedia*?		a	–		Madurai, India
CV-103		P1143	N. *intermedia*		A	+	–	Garrochales, Puerto Rico
CV-104		P1149	N. *intermedia*		A	+	–	La Prada, Puerto Rico
CV-105		P1157	N. *intermedia*		a	+	–	La Prada, Puerto Rico
CV-106		P1174	N. *intermedia*		a	+	–	Aguadilla, Puerto Rico
CV-		P1175	N. *intermedia*		A	+	–	Aguadilla,

107								Puerto Rico
CV-108		P1200	N. *intermedia*		a	+	–	San German, Puerto Rico
CV-109		P1215	N. *intermedia*		a	+	–	Betances, Puerto Rico
CV-110		P1216	N. *intermedia*		A	+	–	Betances, Puerto Rico
CV-111		P1250	N. *intermedia*	*N. intermedia**	A	+	–	Playa Cortada, Puerto Rico
CV-112		P1291	N. *crassa*		a	–		Colonia Paraiso, Puerto Rico
CV-113		P1295	N. *crassa*		A	–		Colonia Paraiso, Puerto Rico
CV-114		P3430	N. *intermedia*		A	+	–	Leogane, Haiti
CV-115		P3439	N. *intermedia*		a	+	–	Merger, Haiti
CV-116		P3442	N. *intermedia*		A	+	–	Puilboreau Mt., Haiti
CV-117		P3443	N. *intermedia*		a	+	–	Puilboreau Mt., Haiti
CV-118		P3450	N. *intermedia*		a	+	–	Pescail,Haiti
CV-119	**10399**	**P3453**	***N. crassa***	***N. metzenbergii* (PS2)**	**a**	**+**	**+**	**Pescail, Haiti**
CV-120		P3454	N. *crassa*	*N. crassa**	A	–		Pescail, Haiti
CV-121		P3455	N. *intermedia*		A	+	–	Pescail, Haiti
CV-122		P3471	N. *crassa*		a	–		Bas Quartier, Haiti
CV-123		P3479	N. *crassa*		A	–		Berard, Haiti
CV-124		P3488	N. *intermedia*		A	+	–	Haut Du Cap, Haiti
CV-125		P3509	N. *intermedia*		a	+	–	Gran Sous, Haiti
CV-126		P3962	N. *crassa*		a	–		Jaco, Costa Rica
CV-127		P3963	N. *crassa*		A	–		Jaco-1, Costa Rica
CV-128		P3979	N. *crassa*		a	–		Agudas, Costa Rica
CV-129		P3981	N. *crassa*		A	–		Agudas, Costa Rica
CV-		P3984	N. *crassa*		A	–		Esterillo

CV-130		P3984	N. *crassa*		A	–		Esterillo Este, Costa Rica
CV-131		P3992	N. *crassa*		a	–		Esterillo Este, Costa Rica
CV-132		P3993	N. *crassa*		a	–		Esterillo Este, Costa Rica
CV-133		P3994	N. *crassa*		A	–		Esterillo Este, Costa Rica
CV-134		P4003	N. *crassa*		a	–		Esterillo Este, Costa Rica
CV-135		P4021	N. *crassa*		A	–		Covolar, Costa Rica
CV-136		P4035	N. *crassa*		a	+	–	Puerto Ayachucho, Venezuela
CV-137		P4052	N. *intermedia*		a	+	–	Mt. Ayanganna, Guyana
CV-138		P4055	N. *intermedia*		a	+	–	Big Emma, Guyana
CV-139		P4056	N. *intermedia*		a	+	–	Imbaima Dai, Guyana
CV-140		P4063	N. *intermedia*		A	+	–	Canje River, Guyana
CV-141		P4065	N. *crassa*		A	–		Digitima Creek, Guyana
CV-142		P4068	N. *intermedia*		a	+	–	Digitima Creek, Guyana
CV-143		P4070	N. *intermedia*		A	+	–	Ekwarun, Guyana
CV-144		P4082	N. *intermedia*		A	+	–	Lookout Village, Guyana
CV-145		P4087	N. *crassa*		A	–		Maripasoula, French Guiana
CV-146		P4088	N. *crassa*		a	–		Maripasoula, French Guiana
CV-147		P4108	N. *crassa*	*N. crassa**	A	–		Chemax, Mexico
CV-148	**10410**	**P4112**	***N. intermedia***	***N. metzenbergii***	**a**	**+**	**+**	**Chemax, Mexico**

CV-149		P4125	N. *crassa*		a	–		Kabah, Mexico
CV-150		P4127	N. *crassa*		A	–		Kabah, Mexico
CV-151		P4149	N. *intermedia*		A	–		Sayil, Mexico
CV-152	**10411**	**P4153**	***N. intermedia***	***N. metzenbergii* (PS2)**	**A**	**+**	**+**	**Uxmal, Mexico**
CV-153	**10412**	**P4156**	***N. intermedia***	***N. metzenbergii* (PS2)**	**a**	**+**	**+**	**Uman, Mexico**
CV-154	**10400**	**P4167**	***N. intermedia***	***N. metzenbergii* (PS2)**	**a**	**+**	**+**	**Macantoc, Mexico**
CV-155	**10401**	**P4168**	***N. intermedia***	***N. metzenbergii* (PS2)**	**A**	**+**	**+**	**Macantoc, Mexico**
CV-156	**10413**	**P4176**	***N. intermedia***	***N. metzenbergii* (PS2)**	**A**	**+**	**+**	**Coba, Mexico**
CV-157		P4180	N. *crassa*		a	–		Chemax, Mexico
CV-158		P4503	N. *intermedia*		a	+	–	Lookout Village, Guyana
CV-159		P4529	N. *intermedia*		A	+	–	Bani, Dominican Republic
CV-160		P4531	N. *intermedia*		a.	+	–	Bani, Dominican Republic
CV-161		P4538	N. *intermedia*		A	+	–	Bani, Dominican Republic
CV-162		P4539	N. *intermedia*		a	+	–	Bani, Dominican Republic
CV-163		P4540	N. *intermedia*		a	+	–	Bani, Dominican Republic
CV-164		P4541	N. *intermedia*		A	+	–	Bani, Dominican Republic
CV-165		P4542	N. *intermedia*		a	+	–	Bani, Dominican Republic
CV-166		P4543	N. *intermedia*		a	+	–	Bani, Dominican Republic
CV-167		P4544	N. *intermedia*		A	+	–	Bani, Dominican Republic

CV-168		P4547	N. *intermedia*		A	+	–	Bani, Dominican Republic
CV-169		P4552	N. *intermedia*		a	+	–	Bani, Dominican Republic
CV-170		P4553	N. *intermedia*	*N. intermedia**	A	–		Bani, Dominican Republic
CV-171		P4581	N. *crassa*		A	–		Arena Reser, Trinidad
CV-172		P4582	N. *intermedia*		a	+	–	Walter Air Force Base, Trinidad
CV-173		P4583	N. *intermedia*		a	+	–	Caroni Swamp, Trinidad
CV-174		P4584	N. *intermedia*		A	+	–	Caroni Swamp, Trinidad
CV-175		P4585	N. *crassa*		A	–		Caroni Swamp, Trinidad
CV-176		P4586	N. *intermedia*		A	+	–	Caroni Swamp, Trinidad
CV-177		P4588	N. *crassa*		A	–		Orinoco Delta, Venezuela
CV-178		P4590	N. *intermedia*		a	+	–	Ile St. Joseph, French Guiana
CV-179		P4591	N. *intermedia*		A	+	–	Ile St. Joseph, French Guiana
CV-180		P4595	N. *intermedia*		a	–		Caroni Swamp, Trinidad
CV-181		P4694	N. *crassa*		a	–		Old Man Bay, Grand Cayman BWI
CV-182		P4723	N. *crassa*		A	–		Old Man Bay, Grand Cayman BWI
CV-183		P4765	N. *intermedia*		a	+	–	Colonia Paraiso, Puerto Rico

CV-184		P4773	N. *intermedia*		a	+	–	Friendship Village, Guyana
CV-185		P4776	N. *intermedia*		A	+	–	Friendship Village, Guyana
CV-186		P4782	N. *crassa*		a	–		Old Man Bay, Grand Cayman BWI
CV-187		P4783	N. *crassa*		a	–		Old Man Bay, Grand Cayman BWI
CV-188		P4784	N. *crassa*		A	+	–	Old Man Bay, Grand Cayman BWI

1. FGSC IDs are only present for samples that were identified using PSR with the 4 polymorphic loci.
2. Perkins IDs correspond to the numbers assigned to specimens belonging to the Perkins culture collection. The full culture collection catalog can be obtained from the FGSC via email (questions@fgsc.net).
3. Originally all the isolates used in the study were identified by BSR with tester strains of varying *Neurospora* species. Some specimens have a "?" in their identification which means that the specimen could not be clearly identified, but the name given appears to be the best assumption
4. Specimens that had A&a for mating type means that when the specimen was places in the Perkins collection it was saved as a mixture of both A and a mating types. All Specimens used in our study came from a single conidial isolate.
5. In the NciI column a "+" means the TMI PCR product was digested by NciI and a "–" means it was not digested.
6. In the BciVI column a "+" means the TMI PCR product was digested by BciVI and a "–" means it was not digested. If the cell is shaded in grey in the BciVI column it means that the digest was not performed for the particular TMI PCR sample.

* The specimen was identified by PSR only with the TMI loci as a control to ensure that RE digests were working correctly. A question mark is present for CV11 because while the specimen is likely *N. discreta* we did not analyze it further to determine what species clade it belongs to.

Specimens in bold were those that had all 4 loci sequenced and were placed into the FGSC collection.

CHAPTER TWO

Mycelial interactions among *Neurospora* individuals of increasing genetic distance lead to transcriptional changes that reveal shared responses, highlight specific pathways, and lead to discovery of gene functions

Christopher Francisco Villalta

Abstract

Observations of fungi in nature indicate that individual fungi constantly interact with other individuals of their own species (wood decay studies) and other species (endophyte and mycorrhizal studies). Laboratory studies of interactions between different fungal individuals have focused on heterokaryon incompatibility interactions, and mating. To study interactions among fungal individuals over a range of genetic relatedness, from self-self interactions to those among genetically different individuals in the same population, different populations, and different species, we have used *Neurospora*. To assess the activity associated with these interactions, we focused on gene expression as measured by transcription profiling using RNAseq. A large amount of gene downregulation occurs between NcA1 growing alone and NcA1 growing with another fungal colony, but with no mycelia contact. Overall there was a trend of gene downregulation in our experiment, but a subset of the downregulated genes were upregulated when the mycelia came into contact. The least change in gene expression occurred in the self-self interaction and the greatest change in gene expression occurred in the interpopulation interaction. In nonself interactions between *Neurospora* we found an upregulation of genes related to melanin synthesis and reactive oxygen metabolism while there was a downregulation of genes related to polar growth and cellular signaling. We observed melanin synthesis occurring in NcA1 interacting with *N. crassa* from a different population. We also found a glutaredoxin and putative bzip transcription factor upregulated during mycelial contact in nonself interactions that in gene deletion mutants led to a phenotype of reduced aerial mycelia in *N. crassa* during contact with the other *Neurospora*. In wild type *N. crassa* these genes had similar expression patterns to the genes related to melanin synthesis and reactive oxygen species metabolism. When we compared expression patterns between each interaction we found that the expression profiles did not correlate with genetic distance between the four *Neurospora* interactions. Our study has provided a model for observing and characterizing interactions between fungi at the gene expression level and we were able to successfully relate gene expression patterns to a phenotype. The gene expression data helped us choose knockout mutants and find two genes of interest that appear important to *Neurospora* during interactions with other fungi.

Introduction

The level of fungi present over small spatial scales and the diversity of fungi present leave little doubt that various fungi come into contact with each other and interact. Indirect evidence of the amount of potential contact comes from studies that have looked at saprobe diversity in nature and found a diverse number of fungi from different

species growing alongside each other (Hyde et al. 2006). When dead plant material from *Proteaceae* was collected in South Africa, 535 fungal isolates displaying a great deal of diversity were found on the material collected from one type of plant (Lee et al. 2004). While collecting endophytic fungi from *Pinus taeda* Arnold et al. isolated a mean total of 50 endophytes belonging to 12 morphotaxa per tree (Arnold et al. 2007).

We are interested in understanding how different fungi interact when they encounter each other. Fungi coming into contact with each other is an important event for an individual colony (Rayner 1991) because it could be coming into contact with a competitor, a potential mate, a fungus with fungal degrading enzymes (Inbar and Chet 1995) or mycotoxins (Marx et al. 2007), a relative with a similar genotype, a distantly related fungus, a fungus with an aggressive genome (Debets and Griffiths 1998; James et al. 2008; Rayner 1991), or a fungus infected with a transmissible mycovirus (Ghabrial 1994; Suzaki et al. 2005). Contact with another fungus could be beneficial or detrimental and based on previous research we suspect a fungal colony will not treat every fungus it comes into contact with in the same exact manner.

Previous researchers have been interested in the role relationships between fungi play in the outcome of interactions, such as whether a self-self, intraspecific, and interspecific interaction will lead to an antagonistic response, e.g., a dark staining zone where mycelial contact occurs or a compatible response, e.g., the fusion of hyphae and proliferation of aerial mycelia (Rayner 1991; Rayner et al. 1994; Rayner and Turton 1982). Researchers have also investigated interactions between wood decay fungi that result in one fungus being replaced or a deadlock between species (Boddy 2000), interspecific interactions between grape vine disease causing fungi that leads to reactive oxygen species production (ROS) (Freitas et al. 2009), and interactions of *Podospora arsenia* and *Coprinopsis cinerea* with other fungi resulting in melanin synthesis and peroxide accumulation (Silar 2005).

Studies of interactions between fungi have found several physiological responses, most notably the presence of dark lines at the zone of contact in studies involving *Podospora* (Silar 2005), wood decaying Basidiomycetes (Boddy 2000; Rayner et al. 1994), and *Neurospora* (Micali and Smith 2003) that could be a result of oxidative stress induced melanin synthesis (Boddy 2000; Score et al. 1997). Melanin synthesis during times of oxidative stress has been studied in the pathogenic fungi *Cryptococcus* and *Histoplasma*, which both produce melanin for protection during times of oxidative stress in the host (Langfelder et al. 2003). In several of the interaction studies fungi of different genotypes or belonging to different species had antagonist responses to the other fungi where reactive oxygen species (ROS) appear to play a role, although whether the accumulation of ROS is involved directly with cell death or as part of a signaling response mechanism (Hansberg et al. 1993; Takemoto et al. 2007) has not been resolved and is probably dependent on the type of fungus and interaction (Freitas et al. 2009; Hutchinson et al. 2009; Silar 2005).

Some studies involving interactions between fungi do take species and phylum into account such as knowing whether fungi belong to the Basidiomycota (Boddy 2000) or that two fungi in an interaction, such as *P. anserina* and *P. chrysogenum*, are different species (Silar 2005), but none have compared interactions between fungi using a detailed phylogenetic framework. Other studies take intraspecific relationships between fungi into account, but are only interested in certain genes, such as those involved in a heterokaryon incompatibility (HI) response, an antagonistic response, where cell lysis is induced because of a difference in *het* (heterokaryon incompatible) loci between two fungi that come into mycelial contact (Glass et al. 2000; Micali and Smith 2003). We are interested in using a phylogenetic framework in choosing fungi over a range of genetic relatedness to see how interactions between fungi that are increasingly genetically distant affect interactions at the gene expression level.

Only a few researchers have looked at gene expression during interactions between filamentous fungi. Two studies where gene expression was observed were microarray analyses involving intraspecific interactions between *Trametes* and other Basidiomycota (Eyre et al. 2010) and a second study that looked at the interspecific interactions between *Heterobasidion* and *Phlebiopsis* (Adomas et al. 2006). Both studies were limited because the microarrays were based on cDNA sequences from expressed mRNA and not a full genome (Kasuga et al. 2005). We are interested in studying how fungi of close genetic relatedness interact in comparison to phylogenetically distant fungi at the gene expression level using RNAseq (Mortazavi et al. 2008; Wang et al. 2009).

In our study we used *Neurospora crassa* because it has a well annotated genome (Galagan et al. 2003) and is an important model organism with a rich history of knowledge (Davis 2000). We are interested in studying global gene expression changes from a genomic perspective and observing the changes in gene expression that occur when different fungi interact. We are also interested in finding specific genes that play an important role in the interactions for more detailed studies.

Neurospora is an ideal model system for the exploration of the genetic responses to fungal-fungal interactions because *Neurospora* is already a good laboratory, evolutionary, and ecological model organism. *Neurospora* is one of the first fungal colonizers after a fire and can be found five to ten days after a fire growing on semi burnt vegetation (Jacobson et al. 2004). *Neurospora* comes into contact with other fungi while growing after a fire including other Neurospora individuals from the same species (Powell et al. 2003), individuals from other *Neurospora* species (Turner et al. 2001), and more distantly related fungi (*Aspergillus niger*, Angora 2007 Fire, Lake Tahoe, personal observation). The phylogenetic structure of the heterothallic *Neurospora* genus is well documented (Dettman et al. 2003a, 2006; Ellison et al. 2011; Menkis et al. 2009; Villalta et al. 2009) allowing us to pick *Neurospora* specimens from a range of genetic and phylogenetic distance, such as, intrapopulation, interpopulation, and interspecific. *N. crassa* has a fully sequenced genome (Galagan et al. 2003) and a large amount of

transcription data from *N. crassa* is available because of full genome microarray studies (Hutchinson et al. 2009; Kasuga et al. 2005). Intraspecific interactions have been studied in terms of HI, but in genetically manipulated strains of *Neurospora* (Glass et al. 2000; Hutchinson et al. 2009; Micali and Smith 2003). HI will play an important factor in any interaction that takes place between fungi. In addition to being useful in framing hypotheses, *N, crassa* can be used to test hypotheses about the role of specific genes because there are gene deletion strains for nearly all nonessential genes in N. crassa (Colot et al. 2006) (McCluskey et al. 2010). Overall *Neurospora* is a good laboratory, evolutionary, and ecological model organism.

Our experimental protocol involved growing a *Neurospora crassa* 2489 (NcA1) colony in four separate taxonomically defined interactions: 1) a self-self interaction with another genetically identical colony, 2) an interspecific interaction with a *Neurospora* colony of a different genotype from the same population, 3) an interpopulation interaction with a *Neurospora* colony from a different population within the *N. crassa* species, and 4) an interspecific interaction with a *Neurospora* from a different species. We chose all pairs of isolates based on their position in the *Neurospora* phylogeny. In addition we sampled timing effects of interactions by comparing gene expression profiles of NcA1 growing alone, with NcA1 growing on the same media as another *Neurospora* before coming into mycelia contact, and with NcA1 within an hour of coming into contact with another *Neurospora*.

The main goals of our study are to identify genes that are similarly expressed between all four interactions, versus genes that are differentially expressed only in the context of genetically different fungi, and then to determine if genes are related to important cellular processes. Finally, we aimed to test hypotheses about the roles of select significantly upregulated genes in nonself interactions found by our transcriptomic approach using strains in which these genes had been knocked out, both by examining colony phenotypes and their transcriptomes. We hypothesized that self-self interactions would have fewer changes in expression compared to intrapopulation, interpopulation and interspecific interactions. We also hypothesized that intraspecific encounters would elicit a stronger response than interspecific encounters.

Methods

Strains utilized and culture conditions

Wild type *Neurospora* strains, all mat A, were obtained from the FGSC: *N. crassa* FGSC 2489 (NcA1) and FGSC 8875 (NcA2) from the Caribbean population of the NcA clade, *N. crassa* FGSC 8867 (NcC) from the Indian NcC clade, and *N. discreta* FGSC 8579 (Nd) (McCluskey et al. 2010). To study fungal-fungal interactions, we followed the *Neurospora* large plate protocol (http://www.yale.edu/townsend/Links/ffdatabase/downloads.html) (Kasuga and Glass 2008). *Neurospora* strains from stock cultures were inoculated into slants of Vogel's medium (Vogel 1956) with 1% sucrose and incubated at 30 °C for three days followed by five days at 25 °C in constant light to suppress synchronous gene expression

associated with circadian rhythms (Kasuga et al. 2005). Conidia were collected from the slant by vortexing the culture with 1 mL of ddH_2O to give a concentration of approximately 5 x 10^6 conidia/mL. To prepare mycelium for experiments, 100 mL of the suspended conidia were evenly distributed across the large, 23 cm x 23 cm, plastic culture plates of Bird's medium (Metzenberg 2004) that had been overlain with cellophane. Conidia were spread by shaking 5 mm glass beads across the plate for 10 seconds, retrieving the beads, and incubating the plates at 25 °C in constant light for 24 hours. To conduct experiments, strips of mycelium and cellophane (0.5 cm x 22 cm) were cut from this plate and placed on one side of a fresh, large plate of Bird's media overlaid with cellophane. In experiments involving interactions, a similar strip of the second strain (Table 1) was placed on the opposite side of the plate at a distance determined from preliminary experiments to allow the colonies to meet at approximately 27 hours. In each experiment involving two fungi, mycelium was collected from the NcA1 partner at two different times from replicate plates, once before mycelial contact (24 hours) and again after mycelial contact (~27 hours) (Figure 1). Mycelium from plates where NcA1 was grown alone was collected at 24 hours (Figure 1). Collection at 24 hours involved removing a strip of mycelium (1 cm x 22 cm) that was 0 – 3 hours old from the colony edge. In replicate plates, approximately three hours later, within an hour of contact between the two colonies, a 1 cm x 22 cm strip of mycelia from NcA1 was collected 0.5 cm away from the zone of contact to avoid collecting mycelium from the second colony. The strips of mycelia were split into 7 cm x 1 cm segments, placed in 1.5 mL tubes, frozen in liquid nitrogen, and stored at -80 °C. Three bioreplicates were conducted for each experiment.

To test for melanin, L-Dopa plates (Chun and Madhani 2010) were used, in which melanin producing colonies exhibit a brown pigmentation that signals the occurrence of melanin synthesis. Experiments involving L-DOPA plates used the protocol described above with the following exceptions: conidia were prepared from cultures grown for only three days at 25 °C; plates were smaller (10 cm x 10 cm) and a Bird's medium plate was inoculated with 50 μl of approximately 5 x 10^6 condia/mL; cellophane strips from Bird's media were smaller (0.5 cm x 10 cm) and placed on L-DOPA plates overlain with cellophane; mycelia were observed for phenotype after seven days.

Single gene knockout mutants made in NcA1 (*N. crassa* FGSC 2489) were ordered from the FGSC *Neurospora* knockout collection (Colot et al. 2006; McCluskey et al. 2010) (Table 2). As in the case of wild type experiments, all knockout strains were mat A. Experiments involving these strains used the same experimental conditions as with wild strains and we used the same protocols as the L-DOPA plate experiments except that Bird's medium was used and mycelia were observed for phenotype at 24 and 48 hours.

RNA extraction and RNAseq library construction

To extract RNA, mycelium in each 1 cm x 7 cm strip was broken in 1 mL of TRIzol (Invitrogen Life Technologies) using a MiniBeadBeater and Zirconia/Silica beads (0.2 g,

0.5 mm diameter, Biospec products), twice for 30 seconds at maximum speed (Kasuga et al. 2005). The total RNA was extracted following a protocol adapted from the TRIzol manufacturer's protocol, in which, following the gentle shaking of incubating samples, the samples were further disrupted in chloroform using a MiniBeadBeater (Kasuga and Glass 2008). A 1 μL sample of the total RNA was electrophoresed on a 1.5% agarose gel at 150 mA and quantified using a Nanodrop ND-1000 Spectrophotometer (ThermoScientific). We used the RNeasy Mini Kit (Qiagen) to clean total RNA of cell debris and fragments. Messenger RNA (mRNA) was purified from the total RNA, fragmented, synthesized into cDNA, and processed into RNAseq libraries following the Illumina mRNA Sequencing sample preparation guide (September 2009 version). We quantified concentration of cDNA libraries with the Qubit Fluorometer (Invitrogen Life Technologies). Libraries were then sent to the University of California, Berkeley QB3 Functional Genomics Laboratory where insert size was determined (~200bp), and where DNA concentration was again measured using the Bioanalyzer 2000 (Agilent). Libraries were sequenced on individual lanes from single ends to 76 base pairs (bp) at the University of California, Berkeley QB3 Vincent J. Coates Genomics Sequencing Laboratory using the Illumina Genome Analyzer platforms and sequencing technology.

Mapping of libraries and processing of samples

Libraries were mapped to the NcA1 (*N. crassa* FGSC 2489) genome (Galagan et al. 2003) with TopHat v1.3.1 (Trapnell et al. 2009) parameters set to two splice mismatches, minimum intron length of 40, a maximum intron length of 200, and three threads. We compared mapping between the Nc genome and to the Nd genome (*N. discreta* FGSC 8579 sequenced by JGI) using TopHat with zero initial read mismatches, zero segment mismatches, and one allowed multiple hit.

The total amount of raw non-normalized read counts mapping to each gene in NcA1 was calculated using a Perl script and coverage information (.wig) from Tophat and gene coordinates from the NcA1 version 10 genome annotation (.gff3). For each comparison of transcription for a pair of conditions, raw read counts for the three bioreplicates from each of the two conditions were compiled into a dataset. Genes with no read counts in any of the six libraries were removed. Individual genes were normalized by the upper-quartile (75^{th} percentile) specific to their library (Bullard et al. 2010). To determine if transcription profile variation was lower within conditions than between conditions, as expected. We first employed MA plots of the pairwise difference in gene expression against the level of gene expression for libraries within conditions and across conditions (Figure 2) (Cleveland and Delvin 1988). We also fit LOESS lines was to the coordinates in each plot and we used a Pearson's chi square test (chitest in R) to determine if our y-coordinates from the LOESS line fit the zero y-axis with a sum of the critical values having a p-value greater than 0.05. Our expectation was that there would not be significant expression differences between the majority of genes and therefore the LOESS line should fit the zero y-axis. Library sequencing error or mapping errors would cause a significant deviation from the zero y-axis (Figure 2).

In the second, we used box plots to evaluate bioreplicate variation within condition compared to variation among all conditions. To make the box plots, for each gene we log transformed the normalized read counts and calculated the median of the log transformed read counts for the three libraries in each condition (condition median) and for all six libraries (all median), together. To compare differences in interquartile ranges (IQRs), for each gene we plotted the differences between, the normalized and log transformed read counts, and the median for that condition (condition median difference), and the difference between the counts for both conditions and the median for the counts for both conditions (all median difference) using R v2.12.1 (RDevelopmentCoreTeam 2011) (Figure 3).

Differential expression analysis

Methods of assessing differential gene expression assume a negative binomial distribution of gene expression for genes with at least five counts (Bullard et al. 2009). To determine if our data for each experimental condition followed a negative binomial distribution, we compared observed data to a negative binomial distribution simulated using rnbinom in R given the observed number of genes, mean read counts and dispersion as calculated using edgeR in R Bioconductor (Robinson et al. 2009). The experimental read counts and simulated read counts were fit separately to a negative binomial distribution using the glm.nb package in R and p-values were collected for how well each gene fit a negative binomial distribution. To attempt to reject the null hypothesis of no difference, which would require that the sum of the critical values had a p-value less than 0.05, we used a Pearson's chi square test with the p-values from the fit of the observed and simulated data.

To analyze differences in expression we used two negative binomial models in R Bioconductor, DESeq (Anders and Huber 2010) and edgeR. We excluded genes where counts were 0 in all libraries. For genes where counts for just some libraries were 0, the 0 values were increased to 1 (Anders and Huber 2010; Bullard et al. 2009; Robinson et al. 2009). Instead of using the default settings in DESeq and edgeR counts were normalized using the upper quartile and we used tag wise dispersion in edgeR. To avoid raising the false discovery rate, p-values were adjusted for multiple hypothesis testing using the Benjamini and Hochberg method in R (Benjamini and Hochberg 1995).

To find genes that were significantly (adjusted p -value< 0.05) differentially expressed, we used DESeq and edgeR, separately or in combination and further filtered for genes significantly differentially expressed more than 1.5 fold between conditions to produce six gene lists, DESeq alone, edgeR alone, DESeq or edgeR (gene found in at least one analysis), DESeq and 1.5 fold, edgeR and 1.5 fold, and DESeq or edgeR both 1.5 fold.

Coding expression differences between experimental conditions

Gene expression levels for NcA1 alone and for NcA1 before and after encountering a second mycelium were compared in three ways, alone versus before mycelial contact, before mycelial contact versus after-contact and alone versus after mycelial contact.

Where expression levels showed a significant difference, they were coded as either D (down) or U (up). Where there was no significant difference, they were coded N (none). Thus, each gene was assigned a three letter code, for example, UDN representing differential expression in the order alone versus before-contact, before-contact versus after-contact, and alone versus after-contact. Where expression differences were considered in both DESeq and edgeR, a fourth possible code was used for conflicting results, X.

The patterns of expression represented by the three letter codes were used to compare transcription across the four comparisons by bar charts of pattern frequency (Figure 4), by similarity in gene patterns (Figure 5), and by distance among conditions based on shared gene patterns (Figure 6). Bar charts were based on the proportion of three-letter codes for genes that were present in DESeq alone, edgeR alone, DESeq or edgeR, DESeq and 1.5 fold, edgeR and 1.5 fold, and DESeq or edgeR both 1.5 fold.

To compare expression differences for each gene across the four interactions, we collected all the genes that were significantly differentially expressed in either DESeq or edgeR with a greater than 1.5 fold change in each interaction to determine their three letter expression code. The list of three letter significant expression codes for each interaction were assigned a single character, akin to abbreviations for each codon of an amino acid, saved as an alignment in fasta format, and visualized using Jalview 2.6.1 (Figure 6) (Clamp et al. 2004).

To estimate the distances among the gene expression patterns for the four encounters we converted the alignment from fasta format to Phylip format using trimAL version 1.2 revision 59 (Capella-Gutierrez et al. 2009), made a distance matrix using Distmat from the EMBOSS 3.6.0 package (Rice et al. 2000), made an unrooted neighbor-joining tree with Neighbor from the Phylip 3.68 package (Felsenstein 1989), and visualized the tree with Mesquite version 2.74 (Figure 7) (Maddison and Maddison 2010). Bootstrap support for the internal branches was based on 100 data sets resampled with replacement using Phylip 3.68.

Comparison to known transcription factors

Putative transcription factor domains were analyzed with hmmscan (Finn et al. 2006) and aligned to homologs from other fungi found during a phammer search (Finn et al. 2006). These new sequences were aligned to bzip domains from *N. crassa* (Nc), *Aspergillus nidulans* (An), *Saccharomyces cerevisiae* (Sc), *Schizosaccharomyces pombe* (Sp), *Candida albicans* (Ca), *Magnaporthe grisea* (Mg), and *Yarrowia lipolytica* (Yl) (Tian et al. 2011) using Muscle (Edgar 2004). The alignment was visualized in Jalview, converted to nexus format with trimAL, analyzed phylogenetically with MrBayes (default settings, 10 million generations, burnin of one hundred thousand generations) and visualized in Mesquite.

Putative coiled coil regions were evaluated using COILS (Lupas et al. 1991) and the MTIDK database with a 2.5 fold weighting of positions a and d in the putative coiled coil regions. The higher the p-value the greater the potential a sequence has to form a coiled-coil region.

Comparison to other Neurospora expression studies using the hypergeometric distribution

To determine if there was a significant overlap between significantly differentially expressed genes found in our data and those found in other expression studies we used a hypergeometric model (phyper in R) with a threshold of p-value less than 0.05 adjusted for multiple hypothesis testing using the Benjamini and Hotchberg method (Fury et al. 2006; Johnson et al. 1992). When comparing our data to microarray expression data we restricted our analyses to genes that were found expressed in both datasets for an equal comparison.

Functional Enrichment

To determine if the collection of genes showing the same expression patterns during the fungal interactions was enriched for specific functions, we compared our data to functional categories determined for NcA1 in FunCat version 2.1 (Ruepp et al. 2004) from MIPS (Mewes et al. 2004) using phyper and a p-value less than 0.05 as the threshold for significance after Benjamini and Hochberg correction for multiple hypothesis testing. The more overlap there was between our set of genes of interest and a known set of genes with a shared known functional relationship the more significant the p-value and the smaller the likelihood that the overlap was a result of chance.

Gene expression box plots

For selected genes we used the three bioreplicates from each condition to calculate the median expression level and the IQR for *NcA1* growing alone, before-contact with another fungus, and after-contact with another fungus and graphed the results as box plots (Figure 11).

Results

Growth and interaction phenotypes

The average growth rates of the *Neurospora* strains in our experiments were 2.2 +/- 0.2 mm/hr for NcA1, 1.8 +/- 0.1 mm/hr for NcA2, 2.1 +/- 0.4 mm/hr for NcC and 1.5 +/- 0.1 mm/hr for Nd. The growth rate of NcA1 was unaffected by the presence of other fungi and was 2.2 mm/hr on average irrespective of the interaction occurring and whether growth rates were measured before mycelial contact at 24 hours growth or after mycelial contact at ~27 hours. NcA1, NcA2, and NcC had identical macroscopic phenotypes with colonies that had an abundance of aerial hyphae at the growth front and where the mycelium contacted the sides of the culture dish, which produced bright orange conidia. Nd differed by having a shorter, more uniform layer of aerial hyphae that produced larger amounts of salmon colored conidia at 24 and 27 hours than the Nc

strains. There were no obvious phenotypic differences at the macroscopic level among the interactions between NcA1 and other *Neurospora*, except for the aforementioned differences in color and structure of Nd mycelia.

RNAseq library sequencing and read mapping
A total of 27 RNAseq libraries were sequenced, which included three biological replicates each of NcA1 growing alone, before (24 hour) contact, or after-contact (27hour) with itself (NcA1), another member of the Louisiana population of the *N. crassa* A clade (NcA2), a member of the basal *N. crassa* C clade (NcC), or *N. discreta* (Nd) (Table 3). Illumina next generation sequencing of the 27 libraries produced an average of 21,096,570 reads, of which an average of 17,792,966 (80%) reads mapped to the NcA1 (*N. crassa* 2489) genome. We found expression in the combined libraries for 97% (9,562 out of 9,907) of annotated *N. crassa* genes (Galagan et al. 2003).

To determine if NcA1 mRNA sequences were contaminated by those of the fungus encountered by NcA1 we compared libraries of NcA1 alone, NcA1 interacting with Nd before mycelial contact, and NcA1 interacting with Nd after-contact by mapping reads to Sanger sequenced genomes of both species. We gathered the total number of uniquely mapping 76 bp sequences that mapped to unique regions of Nd, NcA1, and sequences that mapped to unique regions shared between Nd and NcA1. Even in samples where NcA1 was grown alone there were sequences present that mapped to Nd, e.g., in library CV265, 62,486 sequences mapped to Nd, in library CV310 64,152 sequences mapped to Nd, and in library CV314 6,043 mapped to Nd. Half of the sequences in Nd were shared and likely conserved between NcA1 and Nd with the remaining sequences specific to Nd making up less than 1% of total unique sequences and most likely the result of sequencing error (Table 4). Results were similar in the other samples where NcA1 was grown in the presence of Nd. All but two libraries had reads mapping to 1% or less unique regions in the Nd genome half the sequences mapping to Nd mapped to Nc as well.. Libraries CV26 and CV38 where NcA1 was growing with Nd before-contact were exceptional (Table 4). Both libraries had a higher percentage of unique reads (7% and 8%, respectively) mapping to Nd and of those only 20% were shared with NcA1. When both samples were mapped to NcA1 for expression analysis both CV26 (68%) and CV38 (65%) had a lower percentage of genes mapping to NcA1 in comparison to the 80% average, meaning the libraries contain more sequencing errors than the other libraries (Table 3). The low percentage of unique reads mapping to Nd gave us confidence that cutting mycelia 0.5cm away from the interaction zone in the after-contact conditions prevented mRNA contamination from the other *Neurospora*.

Comparison of mycelia contact data to hyphal tip microarray expression data
Transcription profiling of NcA1 growing alone and of NcA1 before-contact with another fungus included the colony margin but transcription profiling of NcA1 after-contact with another fungus omitted 0.5 cm of the colony margin to avoid collecting mRNAs from the other fungus in the interaction. To determine if expression changes that we detected included those due to omitting the colony margin, we compared our results to those

from a previous microarray study of NcA1 growing alone over a period of 27 hours (Table 5) (Kasuga and Glass 2008). Using a hypergeometric approach, we found no significant overlap between genes we found to have significant changes in expression after-contact with a different colony as compared to either growth alone or before-contact and those genes that Kasuga and Glass (Kasuga and Glass 2008) had shown to have significant, differential expression between colony margins collected at 0-3 hr versus 0-1 hr of growth, Neither was there any overlap between the pool of genes from the microarray study (Kasuga and Glass 2008) and NcA1 before-contact versus NcA1 alone. From this comparison we conclude that the differential transcription that we measured is due to interactions between fungi and not to inclusion or exclusion of the colony margin in comparisons.

Testing consistency among bioreplicates

Gene expression variation among bioreplicates within a condition was lower than that between conditions as judged from MA plots (Figure 2). Our expectation was while we would find differential expression between conditions in *Neurospora*, the majority of genes would be similarly expressed and LOESS lines fit to comparisons of libraries among and between conditions would not significantly deviate from the zero y-axis. We fit LOESS lines to all the points in the MA plot and determined that our data did not deviate significantly from the zero y-axis (Pearson's Chi square test, p-value>0.05), which could be evidence of a sequencing or mapping error in one of the libraries (Figure 2).

Similarly, comparison of IQR calculated for variation among bioreplicates within a condition showed variation to be less than those calculated for comparisons between conditions (Figure 3, Table 5).

Genes found differently expressed using DESeq and edgeR

Our use of DESeq and edgeR to estimate expression differences was justified in that data for all nine experimental conditions fit a negative binomial distribution as well as data simulated to fit that distribution (p-value>0.05, Pearson's Chi square test).

We found that DESeq recognized more genes as significantly (adjusted p-value < 0.05) differentially expressed than edgeR. This is true in spite of the fact that DESeq missed some genes that were recognized by edgeR. However, almost all of the genes recognized by both methods had at least a 1.5 fold change in expression (Figure 4, Table 6). Significantly differentially expressed genes were evenly distributed amongst genes found at all expression levels as displayed in smear and MA plots (Figure 4). For our comparative analyses, we limited our comparisons to genes found to be significantly differentially expressed by either DESeq or edgeR and with a change in expression of 1.5 fold or greater, as practiced by Tian et al (2011).

The most striking difference seen when comparing NcA1 alone versus before-contact with another *Neurospora* (NcA1, NcA2, NcC, and Nd) was the widespread

downregulation of transcription (Figure 8a-b). Although genes that were downregulated in just one of the four interactions were most common, there were 372 downregulated genes shared by all interactions. These 372 genes were significantly enriched for 38 FunCat terms that included 20 terms related to “Metabolism,” five terms related to “Energy,” five terms related to “Cellular transport, transport facilitation and transport routes,” six terms related to “Cell rescue, virulence, and defense,” and two related to “Protein with binding function or cofactor requirement” (Supplemental table 1).

In the comparisons between NcA1 before-contact and after-contact with another *Neurospora* we found that the amount of upregulation and downregulation occurring was more balanced than NcA1 growing alone compared to before-contact. The most divergent interaction occurred between NcA1 versus NcC and had the most upregulated genes when we compared before to after-contact (Fig 8c). There were 33 upregulated genes shared between all four interactions and they were significantly enriched for “Extracellular metabolism,” “Extracellular protein degradation,” “Amino acid and amino acid derivatives transport,” “Peptide transport” and “Virulence disease factors.”

When NcA1 alone was compared to NcA1 after-contact with any of the four partner *Neurospora* strains we again found that there were more genes downregulated than upregulated, similar to NcA1 alone versus before-contact, but with far fewer genes. The difference between the two comparisons is largely the result of upregulation of gene expression occurring as mycelia come into contact. In some cases, genes not significantly differentially expressed less than 1.5 fold in alone versus before or before versus after were significantly differentially expressed in the cumulative comparison of alone versus after. There were 69 genes whose downregulation was shared by all interactions and they were now significantly enriched for the FunCat term “Chemical agent resistance.”

From the results above it is clear that gene expression is changing throughout the interactions. To compare patterns of changes in gene expression, we coded significant changes as down (D) or up (U) and used N for no significant change for the three comparisons of gene expression: alone versus before-contact, before-contact versus after-contact and alone versus after-contact. Of the 27 combinations of D, U and N, seven would be impossible to observe (NUD, NDU, DNU, DDU, UND, UUN, and UUD), no gene was seen with a UDU pattern and we did not consider unchanged genes (NNN). To see the proportion of genes within each of the 18 remaining trends, bar graphs were made of the trends (Figure 5).

These graphs can be used to compare different methods of assessing significance of gene expression change. The graphs of patterns for genes found using DESeq and at least 1.5 fold change, or using DESeq or edgeR and at least 1.5 fold change are nearly identical, but the bar graph using edgeR and at least 1.5 fold change differed visibly, because edgeR had fewer genes (Figure 5). Excluding the 1.5 fold change filter had no visible effect on any analysis (data not shown). The bar graphs further justified our

decision to use genes with a significant adjusted p-value less than 0.05 from edgeR or DESeq with at least a 1.5 fold change in expression, in our analyses.

These graphs can also be used to judge the similarity of patterns, irrespective of the total number of genes, in the four different interactions. The interactions where NcA1 encountered NcA2 and where NcA1 encountered Nd appeared most similar to each other and are more similar to NcA1 versus NcA1 than to the interaction between NcA1 and NcC, which was most divergent. NcA1 versus NcC has a higher percentage of genes with a DNN expression pattern in comparison to the other trends (Figure 5c). NcA1 versus NcC also had a higher percentage of genes with DUN and a lower percentage of genes with a NUN trend, but was lacking UNU and DDD trends. A larger percentage of NcA1 versus NcC genes are being highly upregulated between before versus after-contact compared to the other interactions.

Similarities in patterns of gene expression, now including NNN, could be observed when genes were ordered and then grouped by their expression patterns (Figure 6). The self-self interaction, NcA1 versus NcA1, had the most genes with no significant changes in expression, i.e., with the NNN pattern, far more than any of the interactions between different individuals. The interspecific interaction, NcA1 versus Nd had the second most genes without significant changes in expression, and the intraspecific interactions, NcA1 versus NcA2 and NcA1 versus NcC had the fewest genes lacking significant changes in expression. Clearly, the fewest changes in gene expression occur in self-self interactions and the most occur in intraspecific interactions. Visually, the intraspecific interaction NcA1 versus NcC appeared to be the most distinct in terms of patterns of gene expression, and this difference was also seen when the data were used to make a distance tree (Figure 7).

When a distance tree was made from the ordered expression patterns profiles, NcA1 versus NcA1 (self-self) and NcA1 versus Nd (different species) were separated by the shortest distance (Figure 7) because the majority of genes shared between the two interactions had an NNN expression pattern (Figure 6). NcA1 versus NcC was still the most distant of the interactions in the distance matrix (Figure 7a).

Analysis of Expression pattern alignments with FunCat

Significant enrichment for FunCat terms was assessed for each group of genes sharing the same pattern of expression for any of the four interactions, e.g., genes in NcA1 versus NcA1 with a DUN expression trend (402 genes) were significantly enriched for the FunCat term “Metabolism of tyrosine” (p-value = 0.009) due to the presence of six genes related to tyrosine metabolism. To see how significantly enriched FunCat terms for each pattern were shared across the four interactions we again used Venn diagrams (Figure 9) (Supplemental table 2).

From the Venn diagram (Figure 9), it is clear that most of the significantly enriched FunCat terms from each expression pattern were unique to each of the four interactions.

There was only one FunCat term, “C compound and carbohydrate metabolism,” with the NNN pattern shared among all four interactions. More interestingly among the intra (NcA2, NcC) and interspecific interactions (Nd) were 17 shared, FunCat terms. Of these 17, 12 showed upregulation during contact with the pattern DUN. Four of these terms were related to melanin synthesis, “Metabolism,” “Secondary Metabolism,” “Metabolism of phenylalanine,” and “Metabolism of tyrosine” and three these terms were related to ROS metabolism “Detoxification,” “Oxidative stress response,” and “Oxygen and radical detoxification.” In contrast, in the self interaction (NcA1 versus NcA1) many of the same functional categories showed no significant increased expression. For example, FunCat terms related to melanin synthesis, including “Secondary metabolism,” “Metabolism of polyketides,” “Metabolism of phenylalanine,” and “Metabolism of tyrosine,” were significantly enriched among genes that lacked upregulation (patterns NNN, NND or DND, Table 7) in the self interaction.

In addition to the FunCat terms “Oxidative stress response” and “Oxygen and radical detoxification” other terms related to ROS were seen shared between intraspecific interactions. We found that genes in NcA1 versus NcA2 and NcC with the DUN pattern shared the FunCat term “Glutathione conjugation reaction,” which could help protect NcA1 from free radicals (Herrero et al. 2006) and “Peroxidase reaction,” which points to an the increase in peroxidase production to mediate the breakdown of increased H_2O_2 (Hiscox et al. 2010) (Table 7). Genes unique to the intraspecific interaction NcA1 versus NcC with a DUN expression pattern were enriched for “Superoxide metabolism” which was more evidence that ROS production is occurring in NcA1 when interacting with *Neurospora* of a different genotype (Silar 2005) (Table 7). In the interaction between same genotype, NcA1 versus NcA1, we found no FunCat enrichment, shared or unique to the interaction, related to ROS metabolism.

Within the 17 significant FunCat terms that also shared the same expression patterns in interactions between NcA1 and other *Neurospora* there were two FunCat terms with a NDN expression pattern “Budding cell polarity and filament formation” related to polar cell growth and “Small GTPase mediated signal transduction” related to cellular signaling (Table 7). Downregulation of polar growth is consistent with our observation that *Neurospora* colonies ceased forward growth when they came into contact with each other, whether the partners were the same genetic individual or not. When we looked through the FunCat terms for all the interactions we found more evidence that genes related to mycelial growth were being downregulated in nonself interactions, but there was no enrichment for any FunCat terms related to upregulation or downregulation of mycelial growth in NcA1 versus NcA1 (Table 7). The observation that “Small GTPase mediated signal transduction” was downregulated for all nonself interactions contradicted our hypothesis that cellular signaling would be increased in interactions between genetically different individuals and caused us to search for other FunCat terms related to cell signaling significantly enriched for different expression patterns in each interaction (Table 7). In all of the nonself interactions we found a significant enrichment for FunCat terms related to cell signaling “MAPKKK cascade,” “Second

messenger mediated signal transduction," "Polyphosphoinositol mediated signal transduction," "Cellular signaling," "Kinase activator," "GTPase inhibitor GIP," "Small GTPase mediated signal transduction" in patterns with downregulation of genes (NND and NDN). However, among the intraspecific interaction between NcA1 and NcC we found that genes showing no change in expression (NNN) were enriched for "Cellular communication signal transduction mechanism" and "Cellular signaling" signifying that in a group of genes related to cell signaling no change in expression was occurring. There were no significantly enriched FunCat terms related to cellular signaling for any expression pattern groups for self-self interactions, meaning there were not enough significant changes occurring with the same expression pattern for any FunCat enrichment in the interaction where NcA1 encounter itself.

Testing interactions for melanin production

All four interactions were replicated on L-DOPA plates to test the hypothesis that melanin synthesis was upregulated in interactions involving genetically different fungi. After seven days of incubation we found that the only interaction that produced the brown pigment consistent with melanin synthesis was between NcA1 and NcC (Figure 10). Melanin was not produced when NcA1 was grown alone.

Knockout screen

We chose 12 genes for gene deletion studies that displayed significant upregulation of at least 1.5 fold when NcA1 encountered NcA2, NcC or Nd. Several of the genes shared a DUN expression pattern, such as the FunCat terms that displayed enrichment for melanin synthesis and ROS (Table 2), but the 12 genes were initially chosen irrespective of their three letter expression patterns. The 12 genes were judged to be important to transcription regulation, mating, secondary metabolism, membrane proteins, signal transduction, and protein secretion after determining their functions using FunCat, the annotation information on the Broad *Neurospora* website (http://www.broadinstitute.org/annotation/genome/neurospora/MultiHome.html), and matching genes to annotated homologs using NCBI BLAST (Altschul et al. 1997). We obtained knockouts made in NcA1 for each of the 12 genes from the FGSC (McCluskey et al. 2010) to look for changes in the interaction phenotypes when the knockout strains interacted with intra- and interspecific partners (Table 2). Two of the single knockout strains, ΔNCU01074 and ΔNCU01219, showed a wild type phenotype when grown alone, but a changed phenotype of reduced aerial mycelium compared to wild type NcA1 in interactions with NcA2, NcC or Nd at 24 hours and more so at 48 hours. The phenotype is stronger in ΔNCU01074 than in ΔNCU01219.

Considering that the two genes showed a morphological phenotype in interactions, no function had been ascribed to NCU01074 in the Broad *Neurospora* database or MIPS *Neurospora* genome database (http://mips.helmholtz-muenchen.de/genre/proj/ncrassa/), but we found that it has a significant BLAST match to bzip TFs (NCBI nr database, e-value < 0.05). NCU01219 is a glutaredoxin (Broad *Neurospora* website) that showed significant differential expression in two previous

studies that looked at ROS and programmed cell death (PCD) in *Neurospora* (Hutchinson et al. 2009; Videira et al. 2009).

Phylogenetic analysis of NCU01074 transcription factor

We used hmmscan and phylogenetics to investigate our hypothesis that NCU01074 is a bzip TF because previous studies of TFs in *Neurospora* had not found this gene (Colot et al. 2006; Tian et al. 2011). Use of hmmscan detected a significant similarity (threshold e-value of 8.6E-0.5) between the NCU01074 inferred amino acid sequence and that of the bzip TF Pfam domain, PF00170.10, For phylogenetic analysis, we compared NCU01074 in *N. crassa* and its homologs in *N. discreta* (NCU01074 Nd) and *N. tetrasperma* (NCU01074 Nt) with sequences from the Taphrinomycotina *S. pombe*, the Saccharomycotina, *S. cerevisiae*, *Y. lipolytica* and *C. albicans*, and the Pezizomycotina, *A. niger* and *M. grisea*, that either had been found in our phmmer search or were bzip TF domains sequences used in Tian et al. 2011. We rooted our tree to ensure that *S. cerevisiae* bzip TFs from the YAP (named after the eight *S. cerevisiae* YAP proteins) family of bzip TF that originated from six duplication events from GCN4 remained monophyletic and separate from the non-YAP clade (Tan et al. 2008; Tian et al. 2011). NCU01074 and its homologs in *N.discreta* and *N. tetrasperma* fell into the YAP clade that includes YAP5 and YAP7 from *S. cerevisiae* and the closest relative to NCU01074 and its homologs, MG00587 from *M. grisea*, which we had found in our phmmer homolog search. The similarity between bzip TF domains can be seen in the sequence alignment (Figure 14) especially between amino acids 7-39 in the alignment. Finally, known bzip TFs have a leucine zipper, coiled coil region. Using COILS we found that amino acid positions 55-71 and 26-50 in NCU01074 had high probabilities of being coiled coil regions (Figure 15)

Comparison of mycelia contact data to heterokaryon incompatibility data

Intraspecific pairings of NcA1 with NcA2 or NcC should elicit HI reactions. Transcription during HI has been studied not in interactions between haploid strains, as we have done, but in genetically constructed heterokaryons whose nuclei with different *het* alleles are compatible at 34˚C but incompatible at 20˚C (Hutchison et al. 2009). To determine if there was significant overlap (adjusted p-value <0.05) in the pool of genes that we have found to be differentially transcribed and those showing differential transcription when comparing a compatible heterokaryon with an incompatible heterokaryon grown at the restrictive temperature (Hutchison et al. 2009) by determining how well the overlap between both datasets fit a hypergeometric distribution. We compared genes significantly differentially expressed more than 1.5 fold in each of our interactions to genes that were significantly differentially expressed in an incompatible heterokaryon following 20 minutes, 30 minutes, or 1 hour of growth at the restrictive temperature (20°C) as compared to a compatible heterokaryon (Hutchinson et al. 2009)(Table 8). We found significant overlap between genes downregulated at all HI time points and genes downregulated in NcA1 growing alone versus before-contact for all four interactions, in NcA1 growing alone versus after-contact for NcA1 versus NcA1 and Nd, and in before versus after-contact for NcA1 versus NcA1. No significant overlap

occurred between genes significantly upregulated in the three time points of the HI study and genes significantly upregulated in interactions between NcA1 and *Neurospora* with a different genotype. This result was surprising in that the NcA1 versus NcA1 interaction, which is a self-self interaction, should not elicit an HI response and that genes upregulated in an HI response would be expected to be upregulated in our intraspecific interactions.

Discussion

Fungi downregulate transcription when they perceive another fungus

The most striking trend in significant changes in gene expression when fungi approach each other was the downregulation of many genes. Depending on the partner, between 546 and 1,310 genes are downregulated when *N. crassa* (NcA1), growing alone, meets another fungus, whether itself, another member of its population, a member of a different population, or a member of a different species (Figure 8a-b). The second obvious result was that many of these downregulated genes (372) are shared by all four interactions. The shift in transcription occurred before hyphal contact, so *Neurospora* must be reacting to changes in the environment caused by the other fungi. Many of the downregulated genes are related to the FunCat terms “Metabolism,” “Energy,” “Cellular transport, transport facilitation, and transport routes,” “Cell rescue, virulence, and defense,” and “Protein with binding function or cofactor requirement,” so it is possible that *Neurospora* is sensing fewer nutrients, the presence of altered nutrients, or the presence of other metabolic products released by the other fungus. Distinguishing among these possibilities could be very interesting and might involve characterizing the effect on NcA1 of fractions of diffusible metabolites secreted by the other fungi.

Another striking result was that one of the four interactions, NcA1 versus NcC, had far more uniquely upregulated (160 genes) and downregulated genes (526) from the comparison of NcA1 alone to before-contact than any other interaction, a total that was also larger than the number of genes shared by all four interactions. As will be discussed later, this intraspecific, but interpopulation comparison, NcA1 versus NcC, appears to be the most divergent, a claim also supported by the large number of both upregulated (189) and downregulated (1,310) genes in the alone versus before-contact comparison (Figures 8a-b).

Majority of gene upregulation occurred between before compared to after mycelial contact

The next large change in expression occurs when the fungi come into actual contact and here the theme is shared upregulation of genes. Whereas shared downregulation out weighed upregulation by 372 to two when NcA1 growing alone was compared to before-contact, upregulation outweighed downregulation by 33 to three when after-contact was compared to before-contact (Figure 8). This set of 33 upregulated genes was enriched in FunCat for “Extracellular protein degradation,” “Amino acid derivatives transport,” “Peptide transport,” and “Virulence factors.” Since the genes are shared by all four interactions they must be important to NcA1 during initial mycelial contact.

Neurospora is known to secrete many enzymes (Borkovich et al. 2004) that are involved in sensing and deconstructing biopolymers. It is possible that NcA1 views the other fungus as a source of food, or that it begins to secrete extracellular proteins involved in the break down of the cell wall of the other fungus in preparation for hyphal fusion events (Glass et al. 2000). The most interesting interactions involve genetically different partners (NcA2, NcC, and Nd) and in these interactions 67 genes were upregulated in common. These genes were enriched for 23 FunCat terms (Supplementary figure 1) of which 14 were related to "Metabolism," five were "Cell rescue, defense, and virulence," and three were "Interaction with the environment." Of the FunCat terms related to "Metabolism," seven are relevant to melanin synthesis, with higher level FunCat terms, such as, "Metabolism of phenylalanine," "Metabolism of tyrosine," and "Metabolism of secondary metabolism products derived from primary amino acids" (Supplemental table 1). The five FunCat terms within the Level 1 hierarchical category "Cell rescue, defense, and virulence" were important because all five of them, "Oxidative stress response," "Disease and virulence defense," "Resistance proteins," "Detoxification," and "Oxygen and radical detoxification" were related to ROS metabolism. The shared group of 67 genes was also enriched for "Cellular sensing and response to external stimulus" and "Chemoreception and response," which would be expected to be involved when *Neurospora* contacts another fungus. During contact, as it did before-contact, the interaction NcA1 versus NcC again had the most upregulated genes (459) and the most uniquely upregulated genes (209).

Upregulation of genes involved in melanin synthesis and ROS metabolism as fungi come into contact is a response we found in *Neurospora*. It seems to be a general response that ROS are produced when fungi interact and melanin production has been shown to be a protective mechanism that fungi use against high levels of ROS (Casadevall et al. 2000; Hiscox et al. 2010; Hutchinson et al. 2009; Langfelder et al. 2003; Micali and Smith 2003; Silar 2005).

After-contact, the proportion of genes with shared gene expression changes swung back to downregulation (no upregulated genes to 69 downregulated) and very few genes were upregulated (83 genes) compared to downregulated (1,582 genes). Among the 69 downregulated genes the only significantly enriched FunCat term was "Chemical agent resistance," evidence that no toxins are being produced when *Neurospora* interact with each other.

NcA1 versus NcC had the most unique gene response

NcA1 interacting with NcA1 has the fewest genes significantly differentially expressed when all three comparisons, between NcA1 growing alone compared to before-contact, NcA1 before-contact compared to after-contact, and NcA1 growing alone compared to after-contact are taken into account because it was a self-self interaction between same genotypes. The interaction between NcA1 and Nd had the next fewest changed genes; yet, it was the most distant relative to NcA1.

The intraspecific interactions between NcA1 with NcA2 and NcC had the most genes significantly differentially expressed, one plausible explanation for this is they could both be potential partners or competitors to NcA1, yet both interactions did not share the most genes in common. More RNAseq data on NcA1 during other intraspecific interactions would be needed to test this hypothesis. NcA1 versus NcC shared the least amount of genes with the other interactions and when we looked at expression patterns we saw the same trend because NcA1 versus NcC shared the least genes (533 or 33%) and their expression patterns in common with the other three interactions. The gene expression response in NcA1 versus NcC was also unique because the interaction did not align well with any of the other interactions (Figure 6). We speculate that the interaction is a result of allopatry between NcA1 and NcC, which are more phylogenetically and geographically distant (NcC from Tamil Nadu, India) than NcA1 and NcA2.

FunCat enrichment related to melanin synthesis, ROS metabolism, mycelial growth, and cell signaling

Characterizing gene expression patterns in interactions using FunCat allowed us to focus on small groups of genes with shared patterns of expression. By grouping genes by their expression patterns (Figure 6) and then finding which FunCat terms were enriched in each expression pattern for each interaction, we could determine which expression patterns and corresponding FunCat terms were shared or unique to each interaction (Figure 10, Supplemental table 2). All four interactions shared only one expression pattern, NNN, with the same FunCat term, for “C compound and carbohydrate metabolism,” meaning that in all interactions, there is no change in the breakdown and synthesis of carbohydrates between NcA1 growing alone and with another fungus. In contrast, 17 FunCat terms were shared when NcA1 encountered genetically different fungi, and 12 of them included upregulation of genes during contact (DUN). Of the 12 FunCat terms, 4 are related to melanin synthesis, “Metabolism,” “Secondary Metabolism,” “Metabolism of phenylalanine,” and “Metabolism of tyrosine” and 3 are related to ROS metabolism, “Detoxification,” “Oxidative stress response,” and “Oxygen and radical detoxification. In interactions between NcA1 and non-NcA1 *Neurospora,* genes related to melanin synthesis and ROS metabolism were initially downregulated when NcA1 growing alone was compared to before-contact, but were then upregulated between before-contact compared to after mycelial contact, and showed no change between alone compared to after mycelial contact.

Having found that genes related to melanin synthesis were upregulated in interactions between genetically different fungi, we investigated genes with relevant FunCat terms in the self-self interaction and found that none were upregulated, for example, FunCat terms “Secondary metabolism” (NNN), “Metabolism of polyketides” (NND), “Metabolism of phenylalanine” (DND) and “Metabolism of tyrosine” (DND) (Table 7). We also found additional FunCat terms with a DUN expression trend related to melanin synthesis when we limited the search to interpopulation or interspecific interactions (Table 7).

As noted above, interactions between NcA1 and genetically different *Neurospora* displayed three significantly enriched FunCat terms related to ROS metabolism in genes with the DUN expression pattern. In the self-self interaction, we found no enrichment of any FunCat terms related to ROS production (Table 7). When we restricted our search to intraspecific (nonself) encounters we found genes with a DUN expression pattern that were enriched for “glutathione conjugation reaction,” “peroxidase reaction” and “superoxide metabolism” (Table 7).

Shifting to the gene expression patterns that lacked any upregulation among the 17 significant FunCat terms shared by interactions between NcA1 and genetically different *Neurospora* we found three significantly enriched FunCat terms with a NDN expression pattern “Budding cell polarity and filament formation related to polar cell growth” related to mycelial growth and “small GTPase mediated signal transduction” related to cellular signaling (Table 7). Downregulation of genes with FunCat terms related to polar cell growth is relevant because upon contact between mycelia *Neurospora* appears to stop growing. Downregulation of genes with the FunCat term, “Cell signaling,” seems counter intuitive given that signaling ought to be involved in fungal interactions and given that the intraspecific interaction NcA1 versus NcC revealed no change (NNN) in genes with FunCat terms related to “Cell signaling” or “Cellular communication signal transduction.” There was no significant upregulation for signaling in any of the four interactions and its possible that the downregulation in intraspecific interactions and lack of change (no enrichment) in expression in self-self interactions could reflect a lack of need for signaling once mycelia have come into contact.

The expression patterns and their corresponding FunCat terms for each interaction provide strong evidence that genes related to melanin synthesis and ROS metabolism are being upregulated in interactions between NcA1 and *Neurospora* of a different genotype, while they are being downregulated or showing no significant change in NcA1 versus NcA1 when mycelial contact is made. We found the opposite was true for genes related to hyphal growth and cellular signaling, which were downregulated in NcA1 when interacting with different genotype *Neurospora* and stayed the same or showed no enrichment in NcA1 versus NcA1.

We think when *Neurospora* encounters another *Neurospora* of a different genotype that the hyphal growth front senses the other fungus, which leads to a slow down in cell signaling related to mycelial growth while an upregulation of genes related to ROS metabolism occurs leading to the production and the induction of melanin synthesis to protect *Neurospora* from the increase in ROS. The over production and breakdown of ROS could be occurring as a result of PCD caused by a HI response as was seen in heterokaryons undergoing HI (Glass et al. 2000; Hutchinson et al. 2009). Previous research has also found that ROS produced by NADPH oxidases plays a role in several cell response like hyphal growth, hyphal differentiation, and more importantly hyphal defense, which could be occurring in our interactions (Takemoto et al. 2007). Our interactions could be similar to interactions between *Podospora* and other fungi where

an increase in ROS and cell death was detected after-contact between fungi (Silar 2005).

FunCat analysis leads to interactions that induce melanin production
There was significant enrichment for FunCat terms related to melanin synthesis in NcA1 when interacting with *Neurospora* of a different genotype. Previous studies have shown that melanin can serve a protective function against ROS in *Cyrptococcus neoformans* (Casadevall et al. 2000), *Coprinopsis cinerea (Silar 2005)*, and *Aspergillus fumigatus* (Schmaler-Ripke et al. 2009). In the interactions between NcA1 and nonself there was an increase in the metabolism of phenylalanine and tyrosine when mycelia before-contact were compared to mycelia after-contact, while in self-self interactions we found there was an overall significant decrease. Phenylalanine and tyrosine are important in the production of melanin (Butler and Day 1998) and finding upregulation with the same DUN expression pattern in the metabolism of both amino acids in the same interactions where we found an increase in ROS production in the DUN expression pattern was significant. We think NcA1 is protecting itself from free radicals being produced by NcA1 or the *other Neurospora* by increasing the production of melanin (Butler and Day 1998; Langfelder et al. 2003). In addition to finding gene expression evidence for melanin synthesis we found visual evidence that NcA1 when interacting with NcC produces melanin, but we did not find evidence for melanin production in NcA1 interacting with NcA2 and Nd. There was a subset of 254 genes with the DUN expression pattern specific to NcC that were significantly upregulated between before compared to after mycelial contact that could allow the final steps of melanin synthesis to occur in NcC. Within those genes we found 4 genes that were related to “Metabolism of secondary products derived from L-phenylalanine and L-tyrosine” with an adjusted p-value of 0.59. While the FunCat enrichment was not significant the 4 genes a 4-coumarate-CoA ligase (NCU03295), ketoreductase (NCU03358), nitrilase (NCU03358), and a type III polyketide synthase (NCU04801) could play a significant role in allowing full melanin synthesis to occur in NcA1 when encountering NcC (Funa et al. 2007; Galagan et al. 2003). The production of melanin in NcA1 versus NcC and not in the other two interactions could be a result of NcC being allopatric to NcA1 and causing a stronger response in gene expression.

Alone versus before-contact significantly overlapped Heterokaryon incompatibility data
We compared gene expression changes in fungal encounters to those previously reported for interactions among nuclei with incompatible HI because our interspecific encounters involved incompatible heterokaryons and the production of ROS is related to PCD, which occurs in *Neurospora* after fusion as a result of a HI (Hutchinson et al. 2009). We did see a significant overlap in downregulated genes from the previous HI study and from our data on NcA1 growing alone compared to NcA1 in any encounter and from our data on NcA1 self encounter before-contact compared to after-contact. It may be that what has been assumed to be a HI effect is a combination of a general fungal encounter reaction of downregulation plus a specific HI. Hutchison et al 2009 did not pair different individuals, but used strains that had been genetically manipulated to

contain two, heterokaryon incompatible nuclei, so they would not necessarily observe the encounter reaction in addition to the HI. It may also be that other significant differences between our study and that of Hutchison et al. (2009) confound comparisons. For example, we used two strains that must have incompatible alleles at many *het* loci, whereas Hutchison et al. used nuclei with just one incompatible *het* locus. We also know from the work of Hall et al. 2010 that our strains had different alleles even at the *het* locus studied by Hutchison et al. In addition, we measured gene expression changes by RNAseq whereas Hutchison and colleagues used microarrays.

Knockouts of bzip transcription factor and glutaredoxin confer mutant phenotype
From the 12 knockouts (Table 2) of genes that are significantly upregulated in nonself interactions in *Neurospora* (Figure 8 a, c, e) we found two knockouts with a phenotype different from wild type NcA1: NCU0174, a previously undescribed TF and NCU01219, a glutaredoxin. NCU0174 had a DUN expression pattern in NcA1 versus NcC and Nd and NCU01219 shared the same pattern for NcA1 versus NcA2 and NcC. Both genes shared the same expression pattern with genes related to melanin synthesis and ROS metabolism and could be related to those processes. The two genes play an important role in mycelial contact because during interactions the knockouts produced significantly less aerial mycelia than wild *type Neurospora*. The mutant phenotype was not as pronounced in ΔNCU01219 as in the ΔNCU01074. Even though both genes only showed significant expression in nonself interactions we saw the same mutant phenotype in the self interaction. Both genes are likely more important to interactions between *Neurospora* of a different genotype, but still play an important role in interactions between the same genotype. The mutant phenotype appeared specific to fungi interacting with each other because the two knockouts retained a wild type phenotype when grown alone.

In our study we performed a phylogenetic analysis and found that NCU01074 is a newly described putative bzip TF and is closely related to of the YAP bzip TF family in *S. cerevisiae*. NCU01074 appears to have a truncated coiled coil region in comparison to the other YAP family bzip TFs (Figure 12) and needs further characterization. NCU01219 was already annotated as a glutaredoxin, a group of proteins that can act as antioxidants and defend against ROS through the oxidation of glutathione (Herrero et al. 2006). NCU01219 was found significantly downregulated at one hour post induction of the HI response and significantly upregulated at 8 hours post induction of the HI response (Hutchinson et al. 2009). We also found NCU01219 was significantly upregulated when *Neurospora* was exposed to phytosphingosine, an inducer of programmed cell death, which can lead to an increase in ROS (Videira et al. 2009). The lack of aerial hyphae in the mutants could be a result of an increase in ROS interfering with cellular signaling and gene regulation in the knockouts.

Changes in transcription and relation to genetic distance.
N. crassa represented by NcA1 behaves differently when it comes into contact with itself (NcA1), NcA2 another specimen with a different genotype from the same population,

NcC from another population of *N. crassa*, and Nd a different species from the same genus. Our initial expectation was that intraspecific interactions between NcA1 and nonself (NcA2 and NcC) would have the most similar gene expression patterns because both strains interacting with NcA1 were from the same species and had a different genotype from NcA1. We expected the intraspecific interaction between NcA1 and Nd have the most divergent expression patterns from the other interactions because NcA1 and Nd are distant relatives (Dettman et al. 2003a, 2006; Villalta et al. 2009). The self-self interaction was expected to have the least changes in gene expression because the interaction occurred between two compatible fungi of the same genotype (Glass et al. 2000). Contrary to our expectations there was no correlation between genetic distance and differences in gene expression between interactions,

Instead, we found that NcA1 interacting with NcC is the most divergent of the interactions at the gene expression level (Figure 8). NcA1 interacting with NcA2 and Nd shared the most significantly expressed genes and expression patterns in common (Figure 5,9), but only when the NNN expression pattern was included. NcA1 interacting with NcA1 and Nd shared the most in common because they shared many genes with no significant differential expression (NNN) and had the fewest significantly differentially expressed genes (Figure 6,7). That NcA1 interacting with NcA2 and Nd has the most significantly differentially expressed genes in common was surprising because NcA2 and Nd are distantly related species. There could be a biogeographic reason why NcA1 interacting with NcA2 and Nd have similar expression patterns because both NcA2 and Nd are from North America where as NcC is from Tamil Nadu, India. Previous research has found that genetic distance between species and allopatry and sympatry within species can affect mating success in *Neurospora* (Dettman et al. 2003b; Turner et al. 2011). The big difference in gene expression between NcA1 versus NcA2 and NcA1 versus NcC could be because NcA1 is reacting strongly to a member of its species it did not evolve with (NcC) and has never encountered before. In this case, the same individual (NcA1) or different individual (NcA2) from the same population elicits a milder response than the allopatric member of the species (NcC), and the same mild response is seen when the species are different (Nd), but from the same location.

Future research directions from our interaction studies
Our characterization of the four interactions between *Neurospora* is a start in using *Neurospora* to better understand how fungi interact with each other in the wild. The next step will be observing gene expression in more interactions between NcA1 and *Neurospora* from the *N. crassa* C clade in India, *N. crassa* A from the Louisiana population, more isolates from *N. discreta* found in the south eastern United States, and *N. crassa* along with *N. discreta* from Europe to study if biogeography is playing a role in the gene expression differences and what role being a different species, allopatry, and sympatry play during interactions between *Neurospora.*

A significant result in our study was finding FunCat enrichment for genes related to melanin synthesis in the three nonself interactions and finding that NcA1 in one of three

the nonself interactions produced melanin when grown on L-DOPA plates with NcC, but not when grown alone. We found four genes significantly upregulated in NcA1 versus NcC, but not in the other interactions, that could play a role in allowing full melanin production to occur in NcA1 when encountering NcC. It would be interesting to test NcA1 knockouts of these four genes interacting with NcC to determine if they have an affect on melanin production, which could help understand melanin synthesis in fungi better.

Besides looking at melanin production another important future study would be to measure the amount of ROS production occurring in all four interactions and determine if it corresponds to the upregulation of ROS metabolism we saw in interactions between nonself *Neurospora* when comparing before versus after-contact. The ROS, superoxide and peroxide, can be detected using histochemical dyes (http://www.fgsc.net/fgn37/munkres1.html) (Silar 2005).

While we found a mutant phenotype of reduced aerial mycelia in knockouts of the putative bzip TF NCU01074 and the glutaredoxin NCU01219, it needs to be tested to see if the phenotype is linked to the gene deletions and not a result of mutations that occurred during the creation of the two knockouts (Colot et al. 2006). Any future study of the knockouts should involve crossing each knockout with FGSC 4200 a strain of opposite mating type (mat a) isogenic to FGSC 2489. Five random progeny with hygromycin resistance and five susceptible progeny, all mat A, would be chosen to test if the phenotype observed in the original knockouts is still present.

In wild type NcA1, the gene, NCU01074 in interactions with NcC and Nd and, the gene, NCU01219 in interactions with NcA2 and NcC followed a DUN expression pattern (Figure 11 c, d, f, g). In nonself interactions between *Neurospora* there are many genes with a DUN expression trend (Figure 5, 6) and they were significantly enriched for functions related to melanin synthesis and ROS metabolism. It would be interesting to test if knockout mutants and their progeny exhibit a change in melanin synthesis, superoxide production, and peroxide production in comparison to wild type. Another experiment would be the chromatin immunoprecipitation and sequencing (ChipSeq) of DNA bound to the NCU01074 TF, to determine if sequences bound to the TF and found in ChipSeq corresponds to the gene expression differences between future RNAseq data of interactions with NCU01074 knockout and our current wild type NcA1 interaction data.

Summary of discussion

Observing how *Neurospora* interacts with other fungi is important because in nature a fungus will come into contact with several different fungi including close and distant relatives. Even without noticeable morphological changes this study has shown that significant gene downregulation occurs when two fungi come into close proximity and a subset of these genes are upregulated once mycelial contact is made between the two fungi. There was not a significant correlation between gene expression and genetic

distance between NcA1 and the other fungi it encountered. We found that when interacting with *Neurospora* of a different genotype, genes in NcA1 involved in ROS metabolism and melanin synthesis showed increased expression while genes related to growth and cell signaling showed decreased expression. In the interpopulation interaction we found evidence of melanin production that corresponded with the upregulation in genes related to melanin synthesis. While we did not find a significant signal for a HI response we believe HI is occurring in our nonself *Neurospora* interactions. Testing knockouts of significantly upregulated genes in nonself interactions between *Neurospora* we found a putative bzip TF NCU01074 and a previously identified glutaredoxin NCU01219 that were both upregulated during contact between mycelia and when knocked out led to a reduction in aerial mycelia.

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Fungus 1	Fungus 2*	Relation to NcA1
NcA1 (Neurospora crassa subclade A 2489)	**No Fungus**	-
NcA1	**NcA1**	Same genotype
NcA1	**NcA2** (Neurospora crassa subclade A D115)	Different genotype, same population
NcA1	**NcC** (Neurospora crassa subclade C D107)	Different population, same species
NcA1	**Nd** (Neurospora discreta 8579)	Different species, same genus

Table 1. Fungal interactions observed and relationships of fungi involved.
*All fungi are matA.

NCUid[1,2]	NcA1 vs. NcA1[3]	NcA1 vs. NcA2	NcA1 vs. Nd	NcA1 vs. NcC	FGSC[4]	Information[5]
NCU00732	DND	DUD	DUD	DUN	18706	Trichothecene C-15 hydroxylase, secondary metabolite production
NCU02903	DUN	DUN	DUN	DUN	11643	Integral membrane protein, involved in cellular signaling/signal transduction, recognition of host in *Magnaporthe*
NCU05830	NNU	UNN	NNN	NNN	13712	Cellular export and secretion, general amino acid permease GAP1, non vesicular cellular import
NCU05919	DND	DUN	DUN	DUN	13504	HET domain, involved in cellular signaling
NCU00805	NNU	UNN	NNN	NNN	16635	Transcription factor
NCU05964	DNN	DUN	DUN	DUN	13536	Developmental regulator VosA, pheromone response, mating type determination
NCU07511	DUN	DUN	DUN	NNN	11981	Related to tol protein, has a het domain, pheromone response, mating type determination, sex specific proteins
NCU04034	DND	DND	DUN	DUD	17146	Membrane-associating domain; MARVEL domain - such as Occludin and MAL family proteins. May be part of the machinery of membrane apposition events, such as transport

						vesicle biogenesis.
NCU08739	DUD	DUD	DUD	DUD	11951	Endothiapepsin, secreted protein, breaks up peptides at aspartate, knockout does not make sexual structures and has sick growth.
NCU02338	DUD	DUD	DUD	DUD	13784	Shares domain with necrosis inducing protein, secreted protein
NCU01219	NNN	DUN	DUN	NNN	17728	Glutaredoxin involved in regulation of signal transduction.
NCU01074	DNN	DNN	DUN	DUN	17482	bzip transcription factor

Table 2. Knockouts derived from NcA1 (Colot et al. 2006) used in our study.
[1] All knockouts are matA.
[2] Gene of interest.
[3] Expression patterns, e.g., DUN where "D" refers to downregulation between before versus alone, "U" refers to upregulation between before versus after, and "N" refers to no change in gene expression between alone versus after.
[4] Fungal Genetics Stock Center strain number.
[5] Gene annotations from *Neurospora crassa* Broad website, FunCat, MIPS, or NCBI BLAST.

Interaction[1]	Library ID[2,3]	Raw Reads[4]	Accepted Reads[5]	Percent Mapped[6]
NcA1 vs. NcA1-B	CV46	10,482,493	9,547,931	91%
NcA1 vs. NcA1-B	CV49	16,850,106	11,574,652	69%
NcA1 vs. NcA1-B	CV56	13,438,338	9,838,178	73%
NcA1 vs. NcA1-A	CV168	17,958,142	7,802,061	43%
NcA1 vs. NcA1-A	CV195	16,991,211	9,092,923	54%
NcA1 vs. NcA1-A	CV201	15,964,296	12,504,677	78%
NcA1 vs. NcA2-B	CV67	11,596,951	8,270,520	71%
NcA1 vs. NcA2-B	CV69	6,612,691	3,624,155	55%
NcA1 vs. NcA2-B	CV80	12,869,406	10,123,111	79%
NcA1 vs. NcA2-A	CV95	11,867,206	10,117,124	85%
NcA1 vs. NcA2-A	CV97	27,322,528	23,297,274	85%
NcA1 vs. NcA2-A	CV111	12,895,312	8,452,305	66%
NcA1 vs. NcC-B	CV229	33,204,027	28,512,322	86%
NcA1 vs. NcC-B	CV235	32,980,614	30,449,139	92%
NcA1 vs. NcC-B	CV246	33,592,628	31,567,815	94%
NcA1 vs. NcC-A	CV278	30,823,070	29,069,554	94%
NcA1 vs. NcC-A	CV299	33,867,857	30,379,546	90%
NcA1 vs. NcC-A	CV331	27,544,271	24,983,201	91%
NcA1 vs. Nd-B	CV26	8,697,019	5,914,771	68%
NcA1 vs. Nd-B	CV31	8,196,140	6,295,339	77%

NcA1 vs. Nd-B	CV38	16,321,593	10,679,700	65%
NcA1 vs. Nd-A	CV100	22,384,151	20,359,303	91%
NcA1 vs. Nd-A	CV130	26,820,819	24,686,467	92%
NcA1 vs. Nd-A	CV319	27,203,861	25,248,965	93%
NcA1 alone	CV265	33,988,284	31,554,971	93%
NcA1 alone	CV310	28,972,495	27,626,064	95%
NcA1 alone	CV314	30,161,889	28,838,004	96%

Table 3. RNAseq libraries sequenced and analyzed.
[1] Condition we sampled in each library, where "B" denotes before contact between two fungi, "A" denotes after contact between fungi, and the label "alone" refers to NcA1 growing alone.
[2] Sample identification.
[3] All RNAseq libraries are from NcA1.
[4] The number of 76 bp reads collected for each sample from one sequencing lane in the genome analyzer.
[5] The number of reads that mapped to the NcA1 genome using Tophat.
[6] The percentage of reads that mapped back to the NcA1 genome.

Condition [1]	**ID[2,3]**	**Map to Nd[4]**	**Percent Mapped to Nd[5]**	**Map to Nd and Nc[6]**	**Percent Mapped to Nd and Nc[7]**	**Map to NcA1[8]**	**Percent Mapped to NcA1[9]**	**Total Unique Reads[10]**
NcA1 alone	CV265	114,741	1%	52,255	0%	5,713,181	49%	11,768,494
NcA1 alone	CV310	117,168	1%	53,016	0%	4,579,641	40%	11,309,789
NcA1 alone	CV314	12,035	0%	5,992	0%	4,717,078	43%	11,081,757
NcA1 vs. Nd-B	CV26	548,154	8%	124,828	2%	1,406,042	20%	7,098,823
NcA1 vs. Nd-B	CV31	57,841	1%	26,925	0%	1,677,394	31%	5,401,776
NcA1 vs. Nd-B	CV38	818,290	7%	168,202	1%	2,018,109	17%	11,864,983
NcA1 vs. Nd-A	CV100	107,279	1%	49,474	1%	3,814,354	53%	7,170,809
NcA1 vs. Nd-A	CV130	121,464	1%	55,182	1%	4,682,824	47%	9,876,331
NcA1 vs. Nd-A	CV319	123,787	1%	56,518	1%	4,557,179	44%	10,322,346

Table 4. Unique reads that map to shared and unique regions in NcA1 (*N. crassa*) and Nd (*N. discreta*).
[1] Condition we sampled in each library, where "B" denotes before contact between two fungi, "A" denotes after contact between fungi, and the label "alone" refers to NcA1 growing alone.
[2] Sample identification.
[3] All libraries are from NcA1.
[4] Unique read sequences that map to unique regions in Nd.
[5] Percent of unique reads that map to Nd out of total unique reads sequenced.
[6] Unique reads that map to shared regions between NcA1 and Nd.
[7] Percent of unique reads that map to shared regions of Nc and Nd, out of total unique reads sequenced.
[8] Unique read sequences that map to unique regions in NcA1.
[9] Percent of unique reads that map to NcA1 out of total unique reads sequenced.

Interaction Study[1]	Genes Up/Down Regulated[2]	Number of Genes[3]	Colony Dissection Study[4]	Genes Up/Down Regulated[5]	Number of Genes[6]	Total Genes in Colony Study[7]	Overlap[8]	Adjusted P-value[9]
NcA1 vs. NcA1-A & NcA1 vs. NcA1-B	UP	32	3hr mycelia & 1hr mycelia	UP	699	4,674	4	1.00E+00
NcA1 vs. NcA1-A & NcA1 vs. NcA1-B	DOWN	62	3hr mycelia & 1hr mycelia	DOWN	462	4,674	4	1.00E+00
NcA1 vs. NcA1-B & NcA1 alone	UP	295	3hr mycelia & 1hr mycelia	UP	699	4,674	43	1.00E+00
NcA1 vs. NcA1-B & NcA1 alone	DOWN	33	3hr mycelia & 1hr mycelia	DOWN	462	4,674	0	1.00E+00
NcA1 vs. NcA1-A & NcA1 alone	UP	208	3hr mycelia & 1hr mycelia	UP	699	4,674	30	1.00E+00
NcA1 vs. NcA1-A & NcA1 alone	DOWN	13	3hr mycelia & 1hr mycelia	DOWN	462	4,674	0	1.00E+00
NcA1 vs.	UP	299	3hr	UP	699	4,674	6	1.00E+00

NcA2-A & NcA1 vs. NcA2-B			mycelia & 1hr mycelia					
NcA1 vs. NcA2-A & NcA1 vs. NcA2-B	DOWN	170	3hr mycelia & 1hr mycelia	DOWN	462	4,674	6	1.00E+00
NcA1 vs. NcA2-B & NcA1 alone	UP	430	3hr mycelia & 1hr mycelia	UP	699	4,674	65	1.00E+00
NcA1 vs. NcA2-B & NcA1 alone	DOWN	54	3hr mycelia & 1hr mycelia	DOWN	462	4,674	4	1.00E+00
NcA1 vs. NcA2-A & NcA1 alone	UP	363	3hr mycelia & 1hr mycelia	UP	699	4,674	25	1.00E+00
NcA1 vs. NcA2-A & NcA1 alone	DOWN	20	3hr mycelia & 1hr mycelia	DOWN	462	4,674	0	1.00E+00
NcA1 vs. NcC-A & NcA1 vs. NcC-B	UP	93	3hr mycelia & 1hr mycelia	UP	699	4,674	5	1.00E+00
NcA1 vs. NcC-A & NcA1 vs. NcC-B	DOWN	262	3hr mycelia & 1hr mycelia	DOWN	462	4,674	11	1.00E+00
NcA1 vs. NcC-B & NcA1 alone	UP	647	3hr mycelia & 1hr mycelia	UP	699	4,674	90	1.00E+00
NcA1 vs. NcC-B & NcA1 alone	**DOWN**	**102**	**3hr mycelia & 1hr mycelia**	**DOWN**	**462**	**4,674**	**20**	**2.59E-02**
NcA1 vs. NcC-A & NcA1 alone	UP	48	3hr mycelia & 1hr mycelia	UP	699	4,674	6	1.00E+00
NcA1 vs. NcC-A & NcA1 alone	DOWN	0	3hr mycelia & 1hr mycelia	DOWN	462	4,674	0	1.00E+00
NcA1 vs. Nd-A & NcA1 vs. Nd-B	UP	221	3hr mycelia & 1hr mycelia	UP	699	4,674	7	1.00E+00
NcA1 vs. Nd-A &	DOWN	124	3hr mycelia &	DOWN	462	4,674	4	1.00E+00

NcA1 vs. Nd-B			1hr mycelia					
NcA1 vs. Nd-B & NcA1 alone	UP	252	3hr mycelia & 1hr mycelia	UP	699	4,674	41	1.00E+00
NcA1 vs. Nd-B & NcA1 alone	DOWN	31	3hr mycelia & 1hr mycelia	DOWN	462	4,674	1	1.00E+00
NcA1 vs. Nd-A & NcA1 alone	UP	244	3hr mycelia & 1hr mycelia	UP	699	4,674	22	1.00E+00
NcA1 vs. Nd-A & NcA1 alone	DOWN	9	3hr mycelia & 1hr mycelia	DOWN	462	4,674	0	1.00E+00

Table 5. Comparison of genes expressed in "Interaction Study" and "Colony Dissection Study" using a hypergeometric distribution.
[1] Condition observed in "Interaction Study."
[2] Genes up or down regulated in "Interaction Study."
[3] Total number of genes significantly differentially (>1.5 fold) expressed in NcA1 during our "Interaction Study."
[4] Condition observed in "Colony Dissection Study" (Kasuga and Glass 2008).
[5] Genes up or down regulated in "Colony Dissection Study."
[6] Total number of genes significantly differentially (>1.5 fold) expressed more than 1.5 fold in NcA1 during our "Dissection Study."
[7] Total number of genes with expression detected in "Colony Dissection Study."
[8] Overlap between significantly expressed genes from "Interaction Study" and "Colony Dissectio Study."
[9] Adjusted p-values of how well the overlap (significant if adjusted p-value<0.05) between the two studies fit a hypergeometric model.

		Condition 1 Range of Median Difference		**Condition 2 Range of Median Difference**	
Condition 1[1]	**Condition 2[2]**	**Condition 1[3]**	**All 1[4]**	**Condition 2**	**All 2**
NcA1 alone	**NcA1 vs. NcA1-B**	0.20	0.43	0.10	0.22
NcA1 vs. NcA1-B	**NcA1 vs. NcA1-A**	0.10	0.20	0.13	0.22
NcA1 alone	**NcA1 vs. NcA1-A**	0.20	0.42	0.13	0.25
NcA1 alone	**NcA1 vs. NcA2-B**	0.20	0.42	0.10	0.21
NcA1 vs. NcA2-B	**NcA1 vs. NcA2-A**	0.10	0.21	0.14	0.24
NcA1-alone	**NcA1 vs. NcA2-A**	0.20	0.39	0.14	0.25
NcA1-alone	**NcA1 vs. NcC-B**	0.20	0.39	0.08	0.18
NcA1 vs. NcC-B	**NcA1 vs. NcC-A**	0.08	0.16	0.17	0.37

NcA1-alone	**NcA1 vs. NcC-A**	0.20	0.34	0.17	0.33
NcA1-alone	**NcA1 vs. Nd-B**	0.20	0.41	0.16	0.27
NcA1 vs. Nd-B	**NcA1 vs. Nd-A**	0.15	0.29	0.13	0.28
NcA1	**NcA1 vs. Nd-A**	0.20	0.37	0.12	0.24

Table 6. Interquartile Range (IQR) of median differences calculated from median within conditions and among all conditions.
[1] The first condition in the comparison (three bioreplicates).
[2] Second condition in the comparison (three bioreplicates).
[3] The IQR calculated from the difference between log transformed normalized read counts (three each) and median of three log transformed normalized read counts from the same condition for each gene.
[4] The IQR calculated from the difference between log transformed normalized read counts (three each) and median of six log transformed normalized read counts from the same condition 1 and condition 2 for each gene.

Comparisons[1]		**Significant (<0.05 adjusted p-value)[2]**		**Significant (<0.05 adjusted p-value and >1.5 fold)[3]**		
Condition 1	**Condition 2**	**Upregulated**	**Downregulated**	**Upregulated**	**Downregulated**	**Total Genes[4]**
NcA1 vs. NcA1-B	**NcA1 vs. NcA1-A**	111	41	111	41	9235
NcA1	**NcA1 vs. NcA1-B**	55	587	55	587	9377
NcA1	**NcA1 vs. NcA1-A**	33	396	33	396	9375
NcA1 vs. NcA2-B	**NcA1 vs. NcA2-A**	378	493	350	452	9233
NcA1	**NcA1 vs. NcA2-B**	90	935	90	917	9377
NcA1	**NcA1 vs. NcA2-A**	31	643	31	643	9375
NcA1 vs. NcC-B	**NcA1 vs. NcC-A**	473	160	459	160	9440
NcA1	**NcA1 vs. NcC-B**	239	1409	189	1310	9436
NcA1	**NcA1 vs. NcC-A**	0	97	0	97	9437
NcA1 vs. Nd-B	**NcA1 vs. Nd-A**	260	387	260	387	9273
NcA1	**NcA1 vs. Nd-B**	63	546	63	546	9376
NcA1	**NcA1 vs. Nd-A**	19	446	19	446	9395

Table 7. Genes found significantly differentially expressed and greater than 1.5 fold.
[1] Two conditions compared to determine differential expression using DESeq and edgeR.
[2] Genes found significantly upregulated or downregulated (adjusted p-value <0.05) in DESeq or edgeR.
[3] Genes found significantly upregulated or downregulated (adjusted p-value <0.05) in DESeq or edgeR.
[4] Total amount of genes found expressed in among the six libraries in each comparison.

Interaction[1]	Expression Trend[2]	FUNCAT[3]
Melanin synthesis[4]		
NcA1 vs. NcA1	DND	**01.01.09.04** - Metabolism of phenylalanine **01.01.09.05** - Metabolism of tyrosine
NcA1 vs. NcA1	NNN	**01.20 -** Secondary metabolism
NcA1 vs. NcA1	NND	**01.20.05.11** - Metabolism of polyketides
NcA1 vs. NcC	DUN	**01.20.35** - Metabolism of secondary products derived from L-phenylalanine and L-tyrosine **01.20.35.01** - Metabolism of phenylpropanoids **01.01.09.04.02** - Degradation of phenylalanine
NcA1 vs. Nd	DNN	**01.01.09.04** - Metabolism of phenylalanine
NcA1 vs. NcA1 NcA1 vs. Nd	DNN	**01.01.09.04.02** - Degradation of phenylalanine
NcA1 vs. NcC NcA1 vs. Nd	DUN	**01.01.09.04.01** - Biosynthesis of phenylalanine
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	DUN	**01.20.17** - Metabolism of secondary products derived from primary amino acids **01.01.09.04** - Metabolism of phenylalanine **01.01.09.05** - Metabolism of tyrosine
Reactive Oxygen Species synthesis and breakdown		
NcA1 vs. NcC	DUN	**01.20.37.01** - Metabolism of thioredoxin, glutaredoxin, and glutathione **32.07.07.07** - Superoxide metabolism
NcA1 vs. Nd	DNN	**01.20.37.01** - Metabolism of thioredoxin, glutaredoxin, and glutathione
NcA1 vs. NcA2 NcA1 vs. NcC	DUN	**32.07.07.03** - Glutathione conjugation reaction **32.07.07.05** - Peroxidase reaction
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	DUN	**32.01.01** - Oxidative stress response **32.07.07** - Oxygen and radical detoxification
Polar Cell Growth		
NcA1 vs. NcC	NDN	**42.01** - Cell Wall

		43.01.03 - Fungal and other eukaryotic cell type differentiation **40.01.03.01** - Regulation of directional cell growth
NcA1 vs. Nd	NND	**42.01** - Cell Wall
NcA1 vs. Nd	NDN	**42.29** - Bud growth tip **42.04** - Cytoskeleton structural proteins **40.01.03.03** - Guidance of longitudinal cell extension, e.g. pollen tube guidance, axonal pathfinding
NcA1 vs. NcA2 NcA1 vs. NcC	NDN	**40.01** - Cell growth, morphogenesis
NcA1 vs. NcC NcA1 vs. Nd	NDN	**43.01** - Fungal microorganismic cell type differentiation **10.03.03** - Cytokinesis, cell division, septum formation, and hydrolysis.
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	NDN	**43.01.03.05** - Budding cell polarity and filament formation
Cell Signaling		
NcA1 vs. NcA2	NND	**30.01.05.01.03** - MAPKKK cascade
NcA1 vs. NcC	NNN	**30** - Cellular communication signal transduction mechanism **30.01** - Cellular signaling
NcA1 vs. NcC	NDN	**30.01.09** - Second messenger mediated signal transduction **30.01.09.11** - Polyphosphoinositol mediated signal transduction
NcA1 vs. Nd	NDN	**30.01** - Cellular signaling **18.02.01.01.05** - Kinase activator
NcA1 vs. NcA2 NcA1 vs. Nd	NDN	**18.02.01.02** - Enzyme inhibitor **18.02.01.02.01** - GTPase inhibitor GIP
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	NDN	**30.01.05.05.01** - Small GTPase mediated signal transduction

Table 8. Enriched FunCat terms related to important cellular processes.
[1] Interaction or interactions of interest.
[2] The expression pattern present in genes significantly differentially expressed in a specific interaction/s (Column 1).
[3] FunCat terms enriched (adjusted p-value of <0.05) for genes with specific expression pattern (Column 2) from a specific interaction/s (Column 1).
[4] Cellular processes of interest.

Interaction Study[1]	Genes Up/Down Regulated[2]	Number of Genes[3]	HET Study[4]	Genes Up/Down Regulated[5]	Number of Genes[6]	Total Genes in HI Study[7]	Overlap[8]	Adjusted P-value[9]
NcA1 vs. NcA1-B & NcA1 vs. NcA1-A	UP	54	HET 30 mins	UP	375	3515	4	1.00E+00
NcA1 vs. NcA1-B &	UP	43	HET 45	UP	475	3112	4	1.00E+00

NcA1 vs. NcA1-A			mins					
NcA1 vs. NcA1-B & NcA1 vs. NcA1-A	UP	43	HET 1 hr	UP	463	3112	4	1.00E+00
NcA1 vs. NcA1-B & NcA1 vs. NcA1-A	**DOWN**	**25**	**HET 30 mins**	**DOWN**	**453**	**3515**	**8**	**1.16E-02**
NcA1 vs. NcA1-B & NcA1 vs. NcA1-A	**DOWN**	**25**	**HET 45 mins**	**DOWN**	**513**	**3112**	**9**	**1.60E-02**
NcA1 vs. NcA1-B & NcA1 vs. NcA1-A	**DOWN**	**25**	**HET 1 hr**	**DOWN**	**522**	**3112**	**11**	**1.53E-03**
NcA1 vs. NcA2-B & NcA1 vs. NcA2-A	UP	173	HET 30 mins	UP	375	3515	21	5.81E-01
NcA1 vs. NcA2-B & NcA1 vs. NcA2-A	UP	157	HET 45 mins	UP	475	3112	20	1.00E+00
NcA1 vs. NcA2-B & NcA1 vs. NcA2-A	UP	157	HET 1 hr	UP	463	3112	19	1.00E+00
NcA1 vs. NcA2-B & NcA1 vs. NcA2-A	DOWN	177	HET 30 mins	DOWN	453	3515	18	1.00E+00
NcA1 vs. NcA2-B & NcA1 vs. NcA2-A	DOWN	166	HET 45 mins	DOWN	513	3112	16	1.00E+00
NcA1 vs. NcA2-B & NcA1 vs. NcA2-A	DOWN	166	HET 1 hr	DOWN	522	3112	19	1.00E+00
NcA1 vs. NcC-B & NcA1 vs. NcC-A	UP	252	HET 30 mins	UP	375	3515	30	5.81E-01
NcA1 vs. NcC-B & NcA1 vs. NcC-A	UP	237	HET 45 mins	UP	475	3112	30	1.00E+00
NcA1 vs. NcC-B & NcA1 vs.	UP	237	HET 1 hr	UP	463	3112	27	1.00E+00

NcC-A								
NcA1 vs. NcC-B & NcA1 vs. NcC-A	DOWN	59	HET 30 mins	DOWN	453	3515	5	1.00E+00
NcA1 vs. NcC-B & NcA1 vs. NcC-A	DOWN	54	HET 45 mins	DOWN	513	3112	7	1.00E+00
NcA1 vs. NcC-B & NcA1 vs. NcC-A	DOWN	54	HET 1 hr	DOWN	522	3112	4	1.00E+00
NcA1 vs. Nd-B & NcA1 vs. Nd-A	UP	123	HET 30 mins	UP	375	3515	15	5.81E-01
NcA1 vs. Nd-B & NcA1 vs. Nd-A	UP	105	HET 45 mins	UP	475	3112	13	1.00E+00
NcA1 vs. Nd-B & NcA1 vs. Nd-A	UP	105	HET 1 hr	UP	463	3112	16	9.41E-01
NcA1 vs. Nd-B & NcA1 vs. Nd-A	DOWN	139	HET 30 mins	DOWN	**453**	3515	21	5.06E-01
NcA1 vs. Nd-B & NcA1 vs. Nd-A	DOWN	134	HET 45 mins	DOWN	**513**	3112	21	1.00E+00
NcA1 vs. Nd-B & NcA1 vs. Nd-A	DOWN	134	HET 1 hr	DOWN	**522**	3112	25	5.81E-01
NcA1 alone & NcA1 vs. NcA1-B	UP	26	HET 30 mins	UP	375	3515	0	1.00E+00
NcA1 alone & NcA1 vs. NcA1-B	UP	25	HET 45 mins	UP	475	3112	1	1.00E+00
NcA1 alone & NcA1 vs. NcA1-B	UP	25	HET 1 hr	UP	463	3112	0	1.00E+00
NcA1 alone & NcA1 vs. NcA1-B	**DOWN**	**256**	**HET 30 mins**	**DOWN**	**453**	**3515**	**62**	**9.75E-07**
NcA1 alone & NcA1 vs.	**DOWN**	**239**	**HET 45 mins**	**DOWN**	**513**	**3112**	**69**	**1.80E-06**

NcA1-B								
NcA1 alone & NcA1 vs. NcA1-B	**DOWN**	**239**	**HET 1 hr**	**DOWN**	**522**	**3112**	**67**	**1.40E-05**
NcA1 alone & NcA1 vs. NcA1-A	UP	14	HET 30 mins	UP	375	3515	0	1.00E+00
NcA1 alone & NcA1 vs. NcA1-A	UP	11	HET 45 mins	UP	475	3112	0	1.00E+00
NcA1 alone & NcA1 vs. NcA1-A	UP	11	HET 1 hr	UP	463	3112	0	1.00E+00
NcA1 alone & NcA1 vs. NcA1-A	**DOWN**	**190**	**HET 30 mins**	**DOWN**	**453**	**3515**	**43**	**2.63E-04**
NcA1 alone & NcA1 vs. NcA1-A	**DOWN**	**179**	**HET 45 mins**	**DOWN**	**513**	**3112**	**51**	**7.37E-05**
NcA1 alone & NcA1 vs. NcA1-A	**DOWN**	**179**	**HET 1 hr**	**DOWN**	**522**	**3112**	**56**	**2.09E-06**
NcA1 alone & NcA1 vs. NcA2-B	UP	49	HET 30 mins	UP	375	3515	0	1.00E+00
NcA1 alone & NcA1 vs. NcA2-B	UP	46	HET 45 mins	UP	475	3112	3	1.00E+00
NcA1 alone & NcA1 vs. NcA2-B	UP	46	HET 1 hr	UP	463	3112	0	1.00E+00
NcA1 alone & NcA1 vs. NcA2-B	**DOWN**	398	**HET 30 mins**	**DOWN**	**453**	**3515**	**70**	**7.78E-03**
NcA1 alone & NcA1 vs. NcA2-B	**DOWN**	366	**HET 45 mins**	**DOWN**	**513**	**3112**	**88**	**1.69E-04**
NcA1 alone & NcA1 vs. NcA2-B	**DOWN**	366	**HET 1 hr**	**DOWN**	**522**	**3112**	**84**	**2.27E-03**
NcA1 alone & NcA1 vs. NcA2-A	UP	19	HET 30 mins	UP	375	3515	0	1.00E+00
NcA1 alone & NcA1 vs. NcA2-A	UP	18	HET 45 mins	UP	475	3112	2	1.00E+00

NcA1 alone & NcA1 vs. NcA2-A	UP	18	HET 1 hr	UP	463	3112	0	1.00E+00
NcA1 alone & NcA1 vs. NcA2-A	DOWN	268	HET 30 mins	DOWN	**453**	3515	33	1.00E+00
NcA1 alone & NcA1 vs. NcA2-A	DOWN	246	HET 45 mins	DOWN	**513**	3112	27	1.00E+00
NcA1 alone & NcA1 vs. NcA2-A	DOWN	246	HET 1 hr	DOWN	**522**	3112	32	1.00E+00
NcA1 alone & NcA1 vs. NcC-B	UP	78	HET 30 mins	UP	375	3515	1	1.00E+00
NcA1 alone & NcA1 vs. NcC-B	UP	65	HET 45 mins	UP	475	3112	3	1.00E+00
NcA1 alone & NcA1 vs. NcC-B	UP	65	HET 1 hr	UP	463	3112	6	1.00E+00
NcA1 alone & NcA1 vs. NcC-B	**DOWN**	**581**	**HET 30 mins**	**DOWN**	**453**	**3515**	**95**	**1.28E-02**
NcA1 alone & NcA1 vs. NcC-B	**DOWN**	**518**	**HET 45 mins**	**DOWN**	**513**	**3112**	**127**	**9.75E-07**
NcA1 alone & NcA1 vs. NcC-B	**DOWN**	**518**	**HET 1 hr**	**DOWN**	**522**	**3112**	**133**	**1.13E-07**
NcA1 alone & NcA1 vs. NcC-A	UP	0	HET 30 mins	UP	375	3515	0	1.00E+00
NcA1 alone & NcA1 vs. NcC-A	UP	0	HET 45 mins	UP	475	3112	0	1.00E+00
NcA1 alone & NcA1 vs. NcC-A	UP	0	HET 1 hr	UP	463	3112	0	1.00E+00
NcA1 alone & NcA1 vs. NcC-A	DOWN	41	HET 30 mins	DOWN	453	3515	2	1.00E+00
NcA1 alone & NcA1 vs. NcC-A	DOWN	39	HET 45 mins	DOWN	513	3112	3	1.00E+00
NcA1 alone & NcA1 vs. NcC-A	DOWN	39	HET 1 hr	DOWN	522	3112	3	1.00E+00
NcA1 alone & NcA1 vs.	UP	24	HET 30	UP	375	3515	0	1.00E+00

Nd-B			mins					
NcA1 alone & NcA1 vs. Nd-B	UP	22	HET 45 mins	UP	475	3112	1	1.00E+00
NcA1 alone & NcA1 vs. Nd-B	UP	22	HET 1 hr	UP	463	3112	0	1.00E+00
NcA1 alone & NcA1 vs. Nd-B	**DOWN**	**232**	**HET 30 mins**	**DOWN**	**453**	**3515**	**59**	**4.15E-07**
NcA1 alone & NcA1 vs. Nd-B	**DOWN**	**215**	**HET 45 mins**	**DOWN**	**513**	**3112**	**72**	**4.03E-09**
NcA1 alone & NcA1 vs. Nd-B	**DOWN**	**215**	**HET 1 hr**	**DOWN**	**522**	**3112**	**69**	**1.00E-07**
NcA1 alone & NcA1 vs. Nd-A	UP	11	HET 30 mins	UP	375	3515	2	3.40E-01
NcA1 alone & NcA1 vs. Nd-A	UP	9	HET 45 mins	UP	475	3112	2	4.39E-01
NcA1 alone & NcA1 vs. Nd-A	UP	9	HET 1 hr	UP	463	3112	2	4.32E-01
NcA1 alone & NcA1 vs. Nd-A	**DOWN**	**184**	**HET 30 mins**	**DOWN**	**453**	**3515**	**33**	**5.67E-02**
NcA1 alone & NcA1 vs. Nd-A	**DOWN**	**173**	**HET 45 mins**	**DOWN**	**513**	**3112**	**39**	**4.53E-02**
NcA1 alone & NcA1 vs. Nd-A	**DOWN**	**173**	**HET 1 hr**	**DOWN**	**522**	**3112**	**43**	**8.51E-03**

Table 9. Comparison of genes expressed in “Interaction Study” and “Heterokaryon Incompatibility (HI) study” using a hypergeometric distribution.
[1] Condition observed in “Interaction Study.”
[2] Genes up or down regulated in “Interaction Study.”
[3] Total number of genes significantly differentially (>1.5 fold) expressed in NcA1 during our “Interaction Study.”
[4] Condition observed in “HI study” at 30 minute, 45 minute, and 1 hour timepoints (Hutchinson et al. 2009).
[5] Genes up or down regulated in “Colony Dissection Study.”

[6] Total number of genes significantly differentially (>1.5 fold) expressed more than 1.5 fold in NcA1 during our “HI study.”
[7] Total number of genes with expression detected in “HI study.”
[8] Overlap between significantly expressed genes from “Interaction Study” and “HI study.”
[9] Adjusted p-values of how well the overlap (significant if adjusted p-value<0.05, **bold**) between the two studies fit a hypergeometric model.

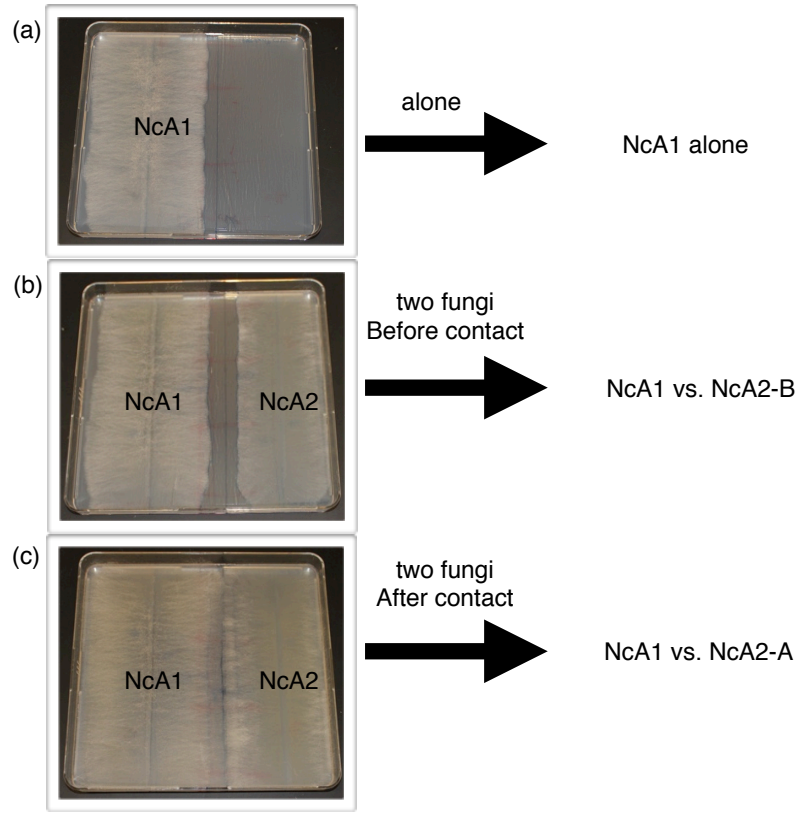


Figure 1. The three points where mycelia and gene expression data were collected for NcA1 during interactions with the four different fungi.
Mycelia was collected when (a) NcA1 was growing alone, (b) while NcA1 was growing with another *Neurospora*, but before contact, (c) and right after mycelia contact (NcA1 versus NcA2 used as an example in figure).

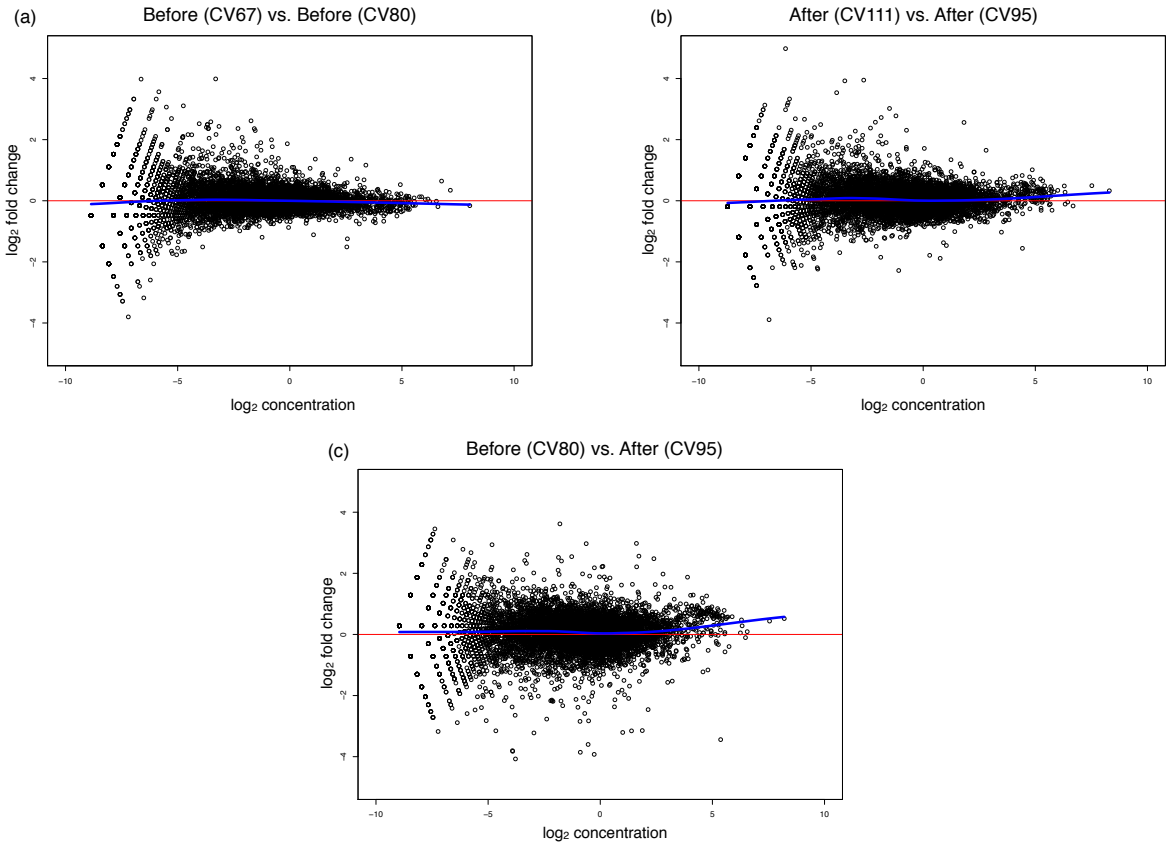
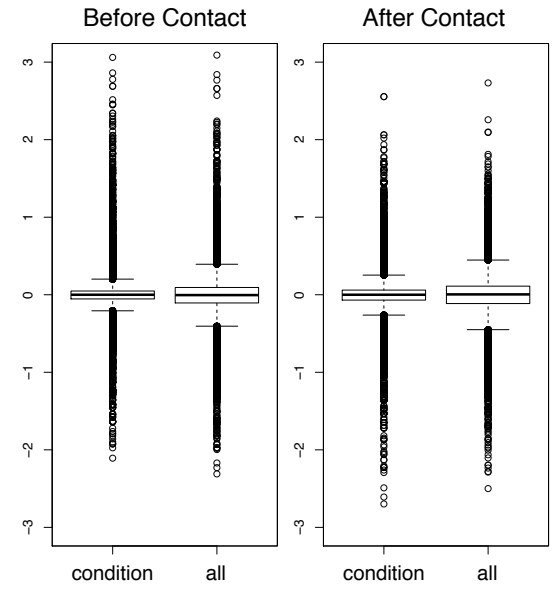
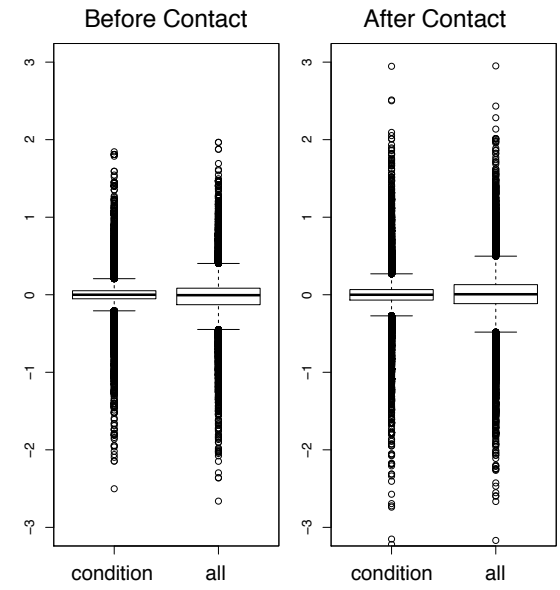


Figure 2. Pairwise comparison between bioreplicates within condition and between conditions with MA plots.
(a) MA plot of two before-contact bioreplicates, RNAseq libraries CV67 and CV80. (b) MA plot of two after-contact bioreplicates, RNAseq libraries CV111 and CV95. (c) A comparison between one before-contact (CV80) and one after-contact (CV95) RNAseq library, The red line demarcates the zero y-axis and the blue line is a LOESS line fit to the data and does not significantly deviate from the zero y-axis. Libraries were from NcA1 versus NcA2.

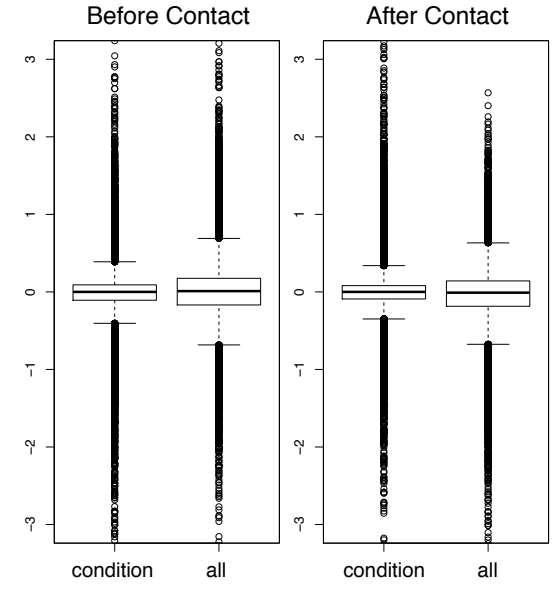
(a) NcA1 vs. NcA1



(b) NcA1 vs. NcA2



(c) NcA1 vs. NcC



(d) NcA1 vs. Nd

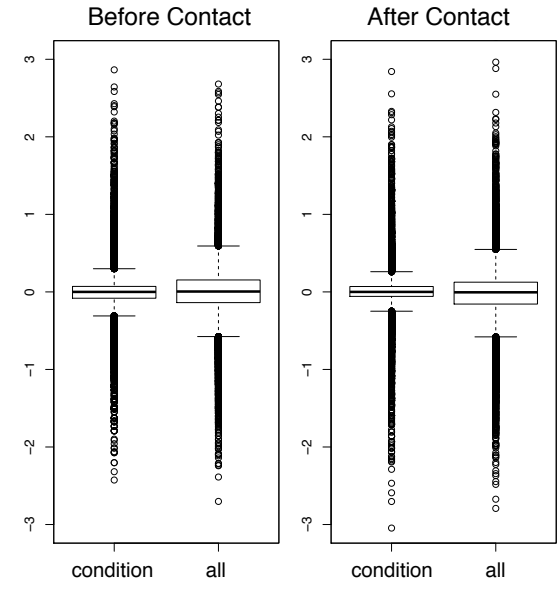


Figure 3. Box plots of log transformed median differences.
Box plots displaying the log transformed median differences for each gene calculated from the difference between the three log transformed read counts (before-contact and after-contact) with the log transformed median of the three “condition” bioreplicates (before-contact and after-contact) and “all” six libraries for the interactions of (a) NcA1 versus NcA1, (b) NcA1 versus NcA2, (c) NcA1 versus NcC and (d) NcA1 versus Nd. The box plots are composed of the median (center thick black line), the first and third quartile (bottom and top of the box), and the upper and lower whiskers (the paddles above and below the hinges separated by a dashed vertical line). The open circles above and below the whiskers are outliers that do not fall within the 95% confidence intervals of data.

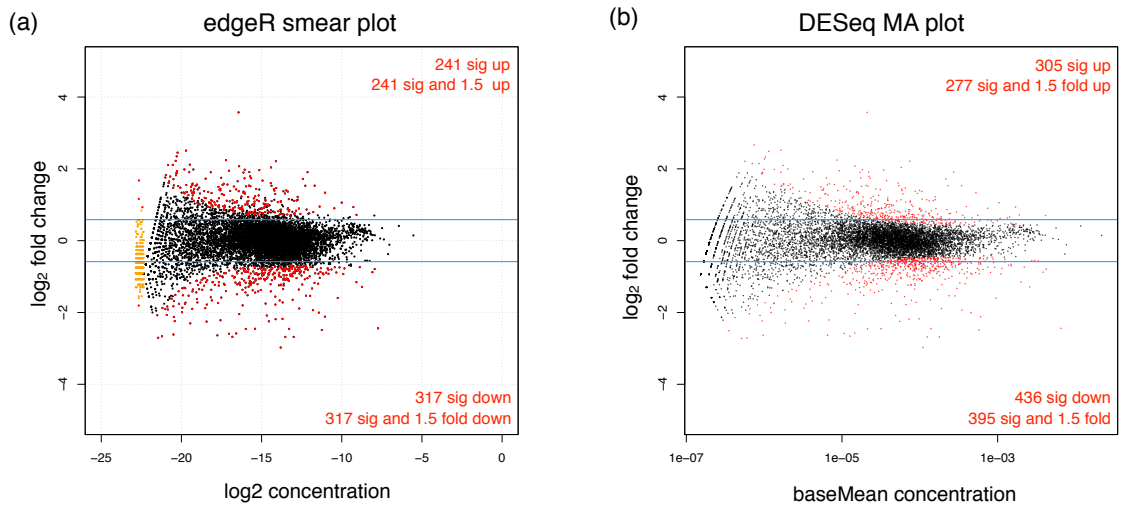


Figure 4. MA and Smear plots from DESeq and edgeR.
Plots graphing the mean expression for each gene, from before and after mycelia contact for NcA1 versus NcA2 on the x-axis and the $\log_2$ fold change in expression between before versus after mycelia contact for each gene. The light blue lines in both plots demarcate the boundary for 1.5 fold differential expression. Points colored in red were genes found significantly (adjusted p-value <0.05) differently expressed. Numbers at the top and bottom right of the plots are the total number of genes found significantly differentially expressed and greater than 1.5 fold. (a) The left plot from edgeR is called a

“smear plot” and is analogous to MA plots. In the smear plots genes that have zero counts for each bioreplicate in at least one condition. (b) The MA plot to the right was made using DESeq and the x-axis displays the same information as (a) except instead of being displayed as $\log_2$ concentration DESeq displays it as the “baseMean”.

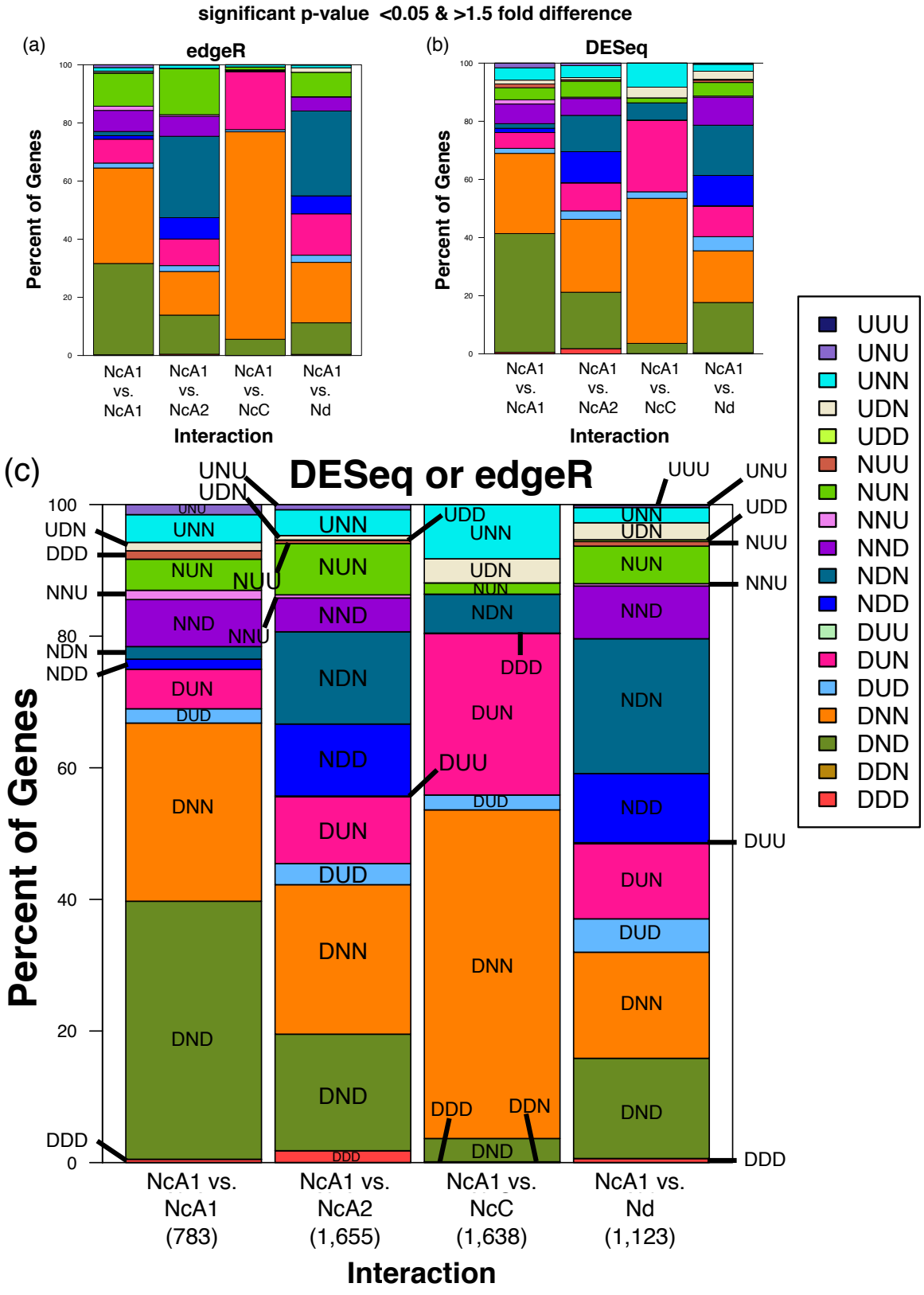


Figure 5. Bar charts of expression pattern proportions.

Expression patterns of genes that were found to have significantly different expression in one of the three comparisons (i.e., alone vs before, before vs after, alone vs after) based on (a) edgeR, (b) DESeq, and (c) DESeq or edgeR (significant in at least one of the programs). Each bar chart has four vertical bars with interactions they represent labeled on the x-axis and the total number of genes differentially expressed in parentheses. All four bars are split into subsections with different colors representing one of nineteen expression patterns. The size of the subsection on the y-axis represents the percentage of genes that fall into each expression pattern category for each interaction out of all the significant genes found in each interaction. A key is present on the left that matches the color of each subsection to a specific expression trend where “U” signifies a gene is significantly up regulated, “D” signifies a gene is significantly down regulated, and “N” signifies a gene was not significantly differentially expressed. In (c) where we look at data from edgeR and DESeq the bar chart subsections are labeled with their corresponding expression pattern.

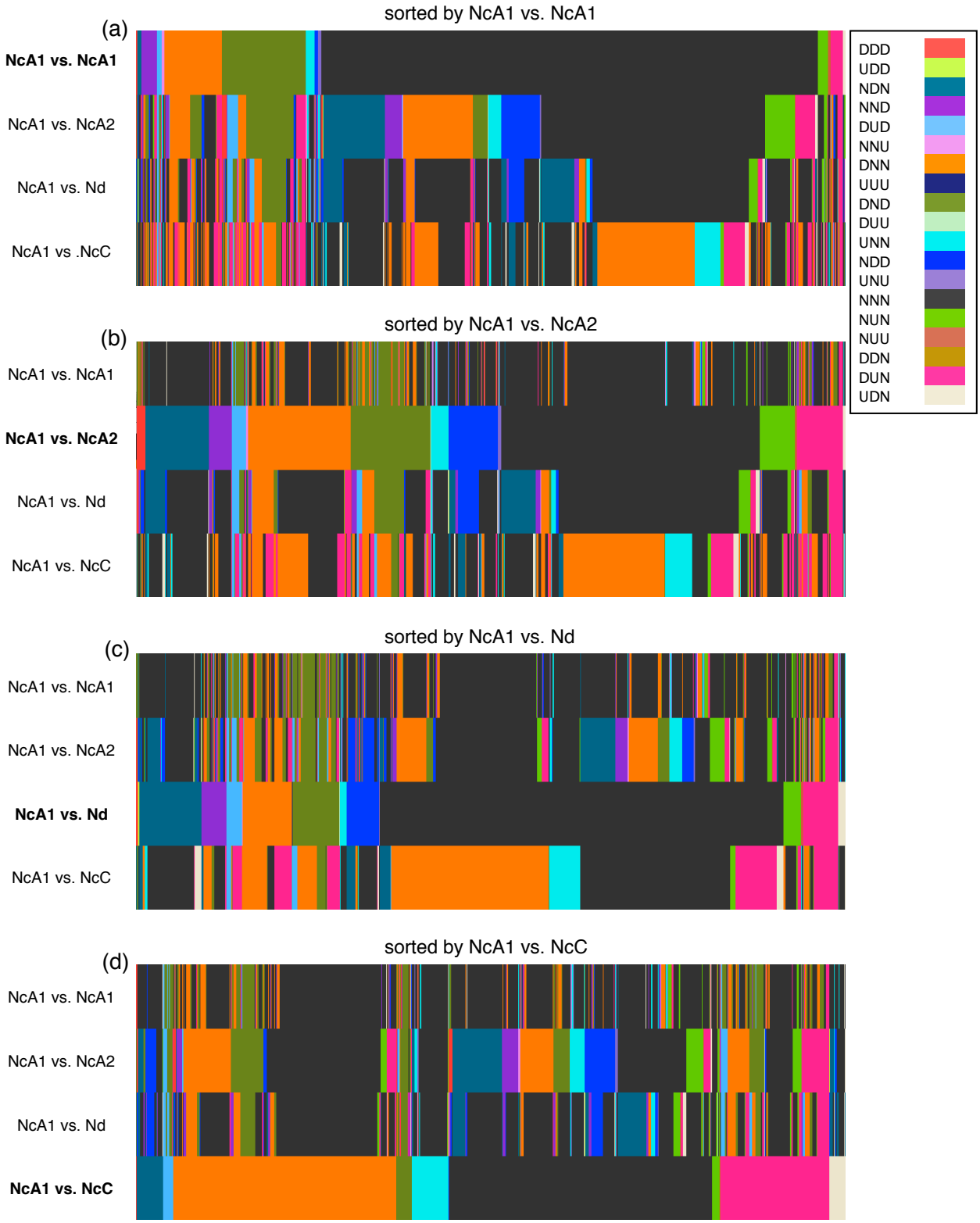

(a)
sorted by NcA1 vs. NcA1
NcA1 vs. NcA1
NcA1 vs. NcA2
NcA1 vs. Nd
NcA1 vs .NcC
(b)
sorted by NcA1 vs. NcA2
NcA1 vs. NcA1
NcA1 vs. NcA2
NcA1 vs. Nd
NcA1 vs. NcC
(c)
sorted by NcA1 vs. Nd
NcA1 vs. NcA1
NcA1 vs. NcA2
NcA1 vs. Nd
NcA1 vs. NcC
(d)
sorted by NcA1 vs. NcC
NcA1 vs. NcA1
NcA1 vs. NcA2
NcA1 vs. Nd
NcA1 vs. NcC
DDD
UDD
NDN
NND
DUD
NNU
DNN
UUU
DND
DUU
UNN
NDD
UNU
NNN
NUN
NUU
DDN
DUN
UDN

Figure 6. Four ordered of expression profile made from the 2,604 significant differentially expressed genes.
The ordered expression profiles go from 1-2,604, left to right, and the colors in each profile represent different expression patterns for each gene and the key at the upper right hand side of the figure displays what color represents each particular expression trend. The letter “U” in the key represents genes that are significantly up regulated, the letter “D” in the key represents genes that are significantly down regulated, and the letter “N” represents genes that were found not be significantly differentially expressed. Each of the four alignments in the figure was sorted in a particular order starting with (a) NcA1 interacting with NcA1, (b) NcA2, (c) NcC, and (d) Nd, which are highlighted in bold for the respective figure. The expression profiles were made in Jalview 2.6.1.

(a)

Distance Matrix

	NcA1 vs. NcA1	NcA1 vs. NcA2	NcA1 vs. NcC	NcA1 vs. Nd
NcA1 vs. NcA1	0.00	56.45	64.36	41.21
NcA1 vs. NcA2	56.45	0.00	76.46	57.10
NcA1 vs. NcC	64.36	76.46	0.00	69.28
NcA1 vs. Nd	41.21	57.10	69.28	0.00

(b)

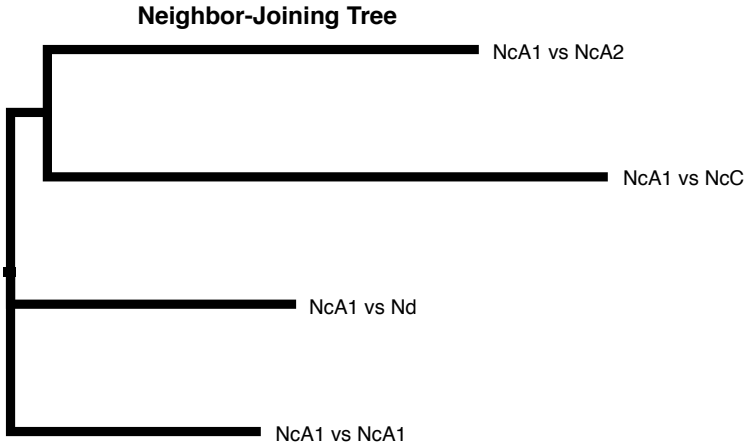


Figure 7. Distance matrix and neighbor joining tree of relationship between four expression pattern profiles.
(a) An uncorrected distance matrix compiled in Distmat from the expression pattern profiles of our four interactions (Figure 8). (b) Neighbor-Joining tree produced in Phylip using the distance matrix data from (a). The unrooted tree has 100% bootstrap support.

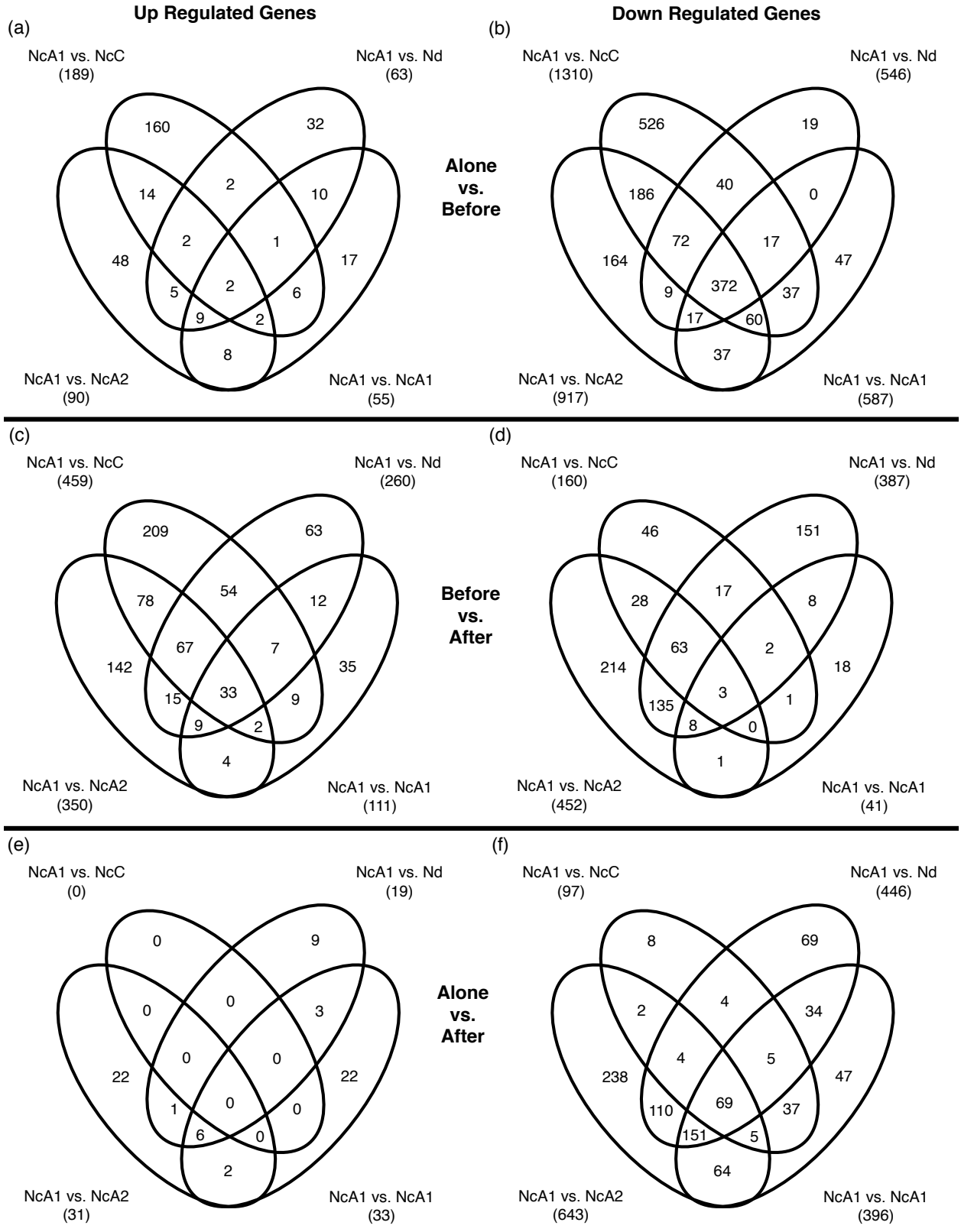

Up Regulated Genes
Down Regulated Genes
(a)
(b)
NcA1 vs. NcC
(189)
NcA1 vs. Nd
(63)
NcA1 vs. NcA2
(90)
NcA1 vs. NcA1
(55)
160
32
14
2
10
2
1
48
17
5
2
6
9
2
8
NcA1 vs. NcC
(1310)
NcA1 vs. Nd
(546)
NcA1 vs. NcA2
(917)
NcA1 vs. NcA1
(587)
526
19
186
40
0
72
17
164
47
9
372
37
17
60
37
Alone
vs.
Before
(c)
(d)
NcA1 vs. NcC
(459)
NcA1 vs. Nd
(260)
NcA1 vs. NcA2
(350)
NcA1 vs. NcA1
(111)
209
63
78
54
12
67
7
142
35
15
33
9
9
2
4
NcA1 vs. NcC
(160)
NcA1 vs. Nd
(387)
NcA1 vs. NcA2
(452)
NcA1 vs. NcA1
(41)
46
151
28
17
8
63
2
214
18
3
135
1
8
0
1
Before
vs.
After
(e)
(f)
NcA1 vs. NcC
(0)
NcA1 vs. Nd
(19)
NcA1 vs. NcA2
(31)
NcA1 vs. NcA1
(33)
0
9
0
0
3
0
0
22
22
1
0
0
6
0
2
NcA1 vs. NcC
(97)
NcA1 vs. Nd
(446)
NcA1 vs. NcA2
(643)
NcA1 vs. NcA1
(396)
8
69
2
4
34
4
5
238
47
110
69
37
151
5
64
Alone
vs.
After

Figure 8. Comparisons of shared and unique upregulated and downregulated genes between interactions.
Venn diagrams that compare the genes significantly differentially (>1.5 fold) upregulated and downregulated in the four interactions: NcA1 interacting with NcA1 (lower-right ellipse), NcA2 (lower left ellipse), NcC (upper left ellipse), and Nd (upper right ellipse). Each ellipse represents a different interaction and the number within each section represents the number of genes shared by each interaction (ellipse) that is overlapping or is unique to a particular interaction. We made Venn diagrams for up regulated genes (left) and down regulated genes (right) for the three comparisons between experimental conditions: NcA1 growing alone (a-b), NcA1 growing with another fungus, but before contact (c-d), and NcA1 growing with another fungus after mycelia contact (e-f).

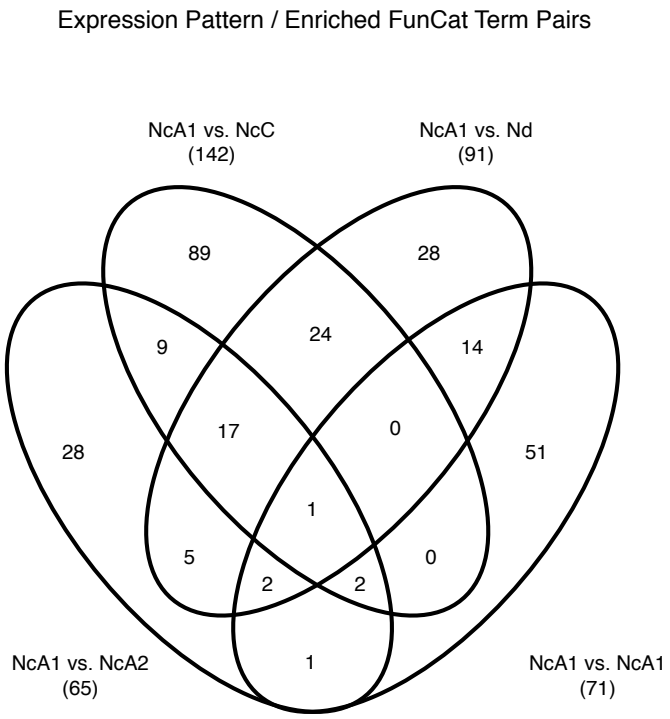


Figure 9. Comparisons of shared and unique FunCat terms enriched for expression patterns in each interaction.
FunCat analysis on each group of genes with a specific expression pattern for each interaction and collected significantly (adjusted p-value<0.05) enriched FunCat terms for each expression pattern in each interaction (Supplemental Table 2). We produced a Venn diagram from the four lists of enriched FunCat terms with specific expression patterns to compare, which FunCat terms and expression pattern were distinct to each interaction or shared between them.

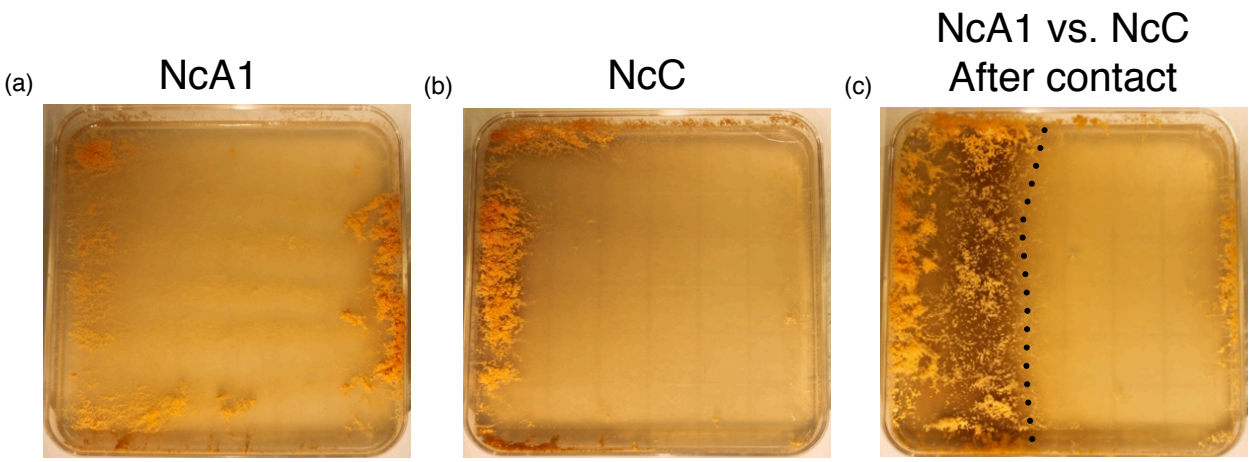


Figure 10. L-Dopa production in NcA1 when interacting with NcC.
Seven day old cultures on L-Dopa plates of (a) NcA1 and (b) NcC grown alone and after (c) mycelial contact between the two fungi. The dotted line denotes where the two *Neurospora* (NcA1 on the left, NcC on the right) came into contact. NcA1 and NcC did not have any brown pigmentation while in the interaction between NcA1 and NcC there was a significant amount of brown pigment present in NcA1, which is evidence of melanin synthesis.

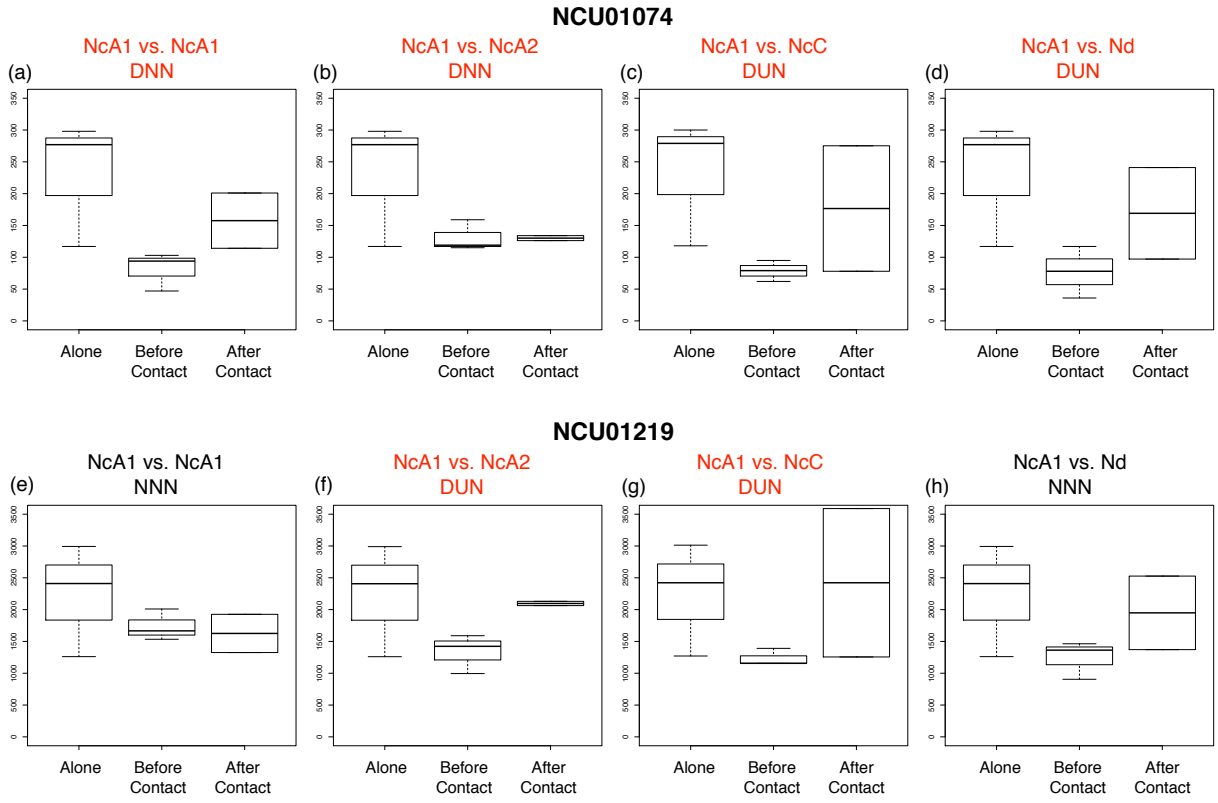


Figure 11. Gene expression box plots for NCU01074 (bzip transcription factor) and NCU01219 (glutaredoxin) in wild type NcA1 interacting with NcA1 (a,e), NcA2 (b,f), NcC (c,g), and Nd (d,h). Each box plot is a representation of three bioreplicates of RNAseq data for each interaction when NcA1 is growing alone, before mycelia contact with another *Neurospora*, and after mycelia contact with another *Neurospora*. In the heading below the type of interaction the box plot represents the expression pattern for the particular gene in that specific interaction is listed (e.g. In NcA1 versus NcA1, the gene NCU01074 was found to have the expression pattern DNN). why not give the annotation for these two genes in the legend.

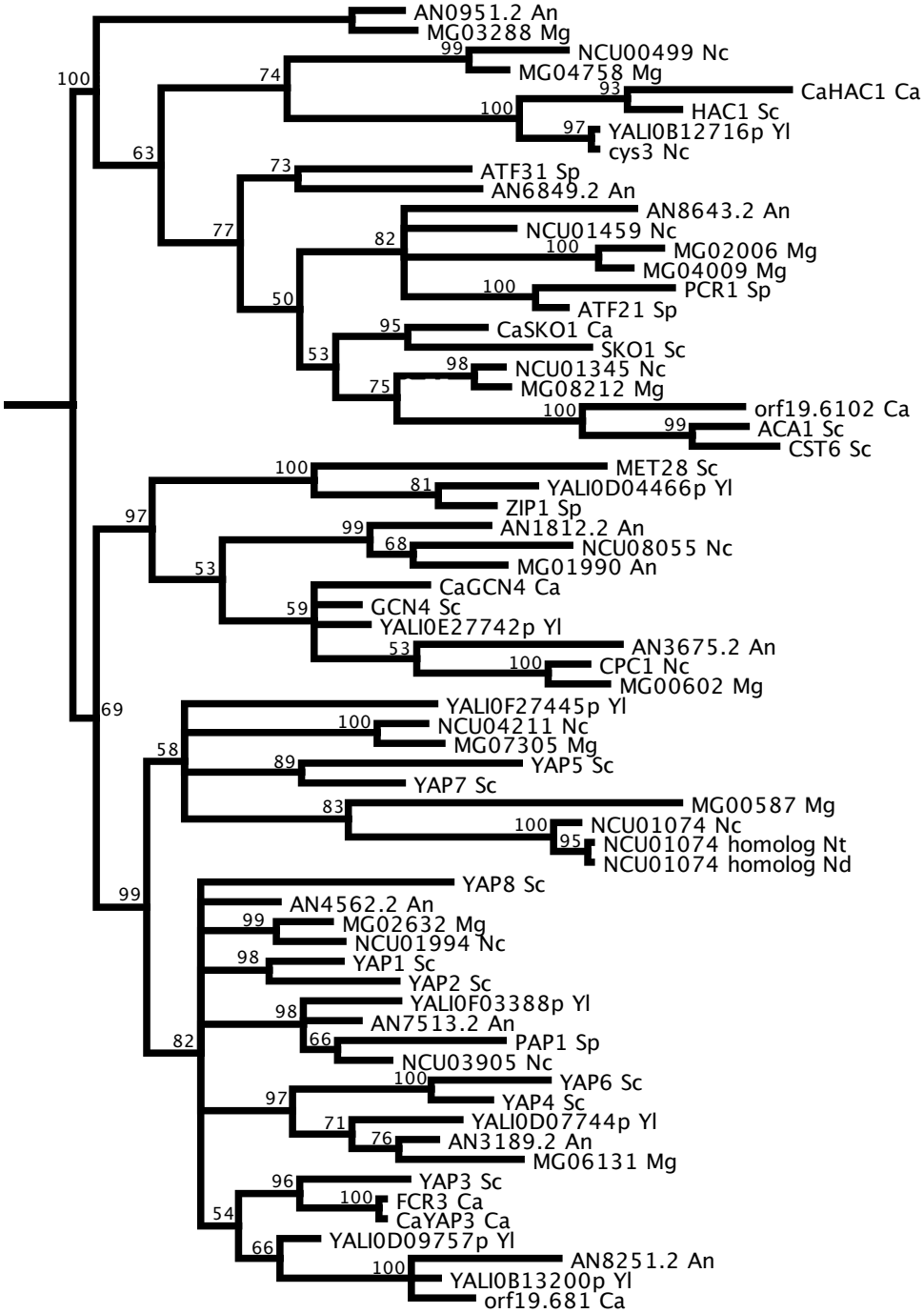


Figure 12. Phylogenetic tree of YAP and non-YAP bzip transcription factor domains.

The phylogenetic relationships among bzip transcription factor domains found in the Ascomycetes *N. crassa* (Nc), *Aspergillus nidulans* (An), *Saccharomyces cerevisiae* (Sc), *Schizosaccharomyces pombe* (Sp), *Candida albicans* (Ca), *Magnaporthe grisea* (Mg), and *Yarrowia lipolytica* (Yl). The tree includes sequences from NCU01074, its homologs in *N. discreta* and *N. tetrasperma*, and sequences from Tian *et al.* 2010. Mrbayes was used to construct the tree topology and determine confidence levels and branches with posterior probability of 50% or greater are listed next to each branch. The tree was rooted in Mesquite with the knowledge that all *S. cerevisiae* YAP proteins belong in the same monophyletic clade (Tan et al. 2008; Tian et al. 2011).

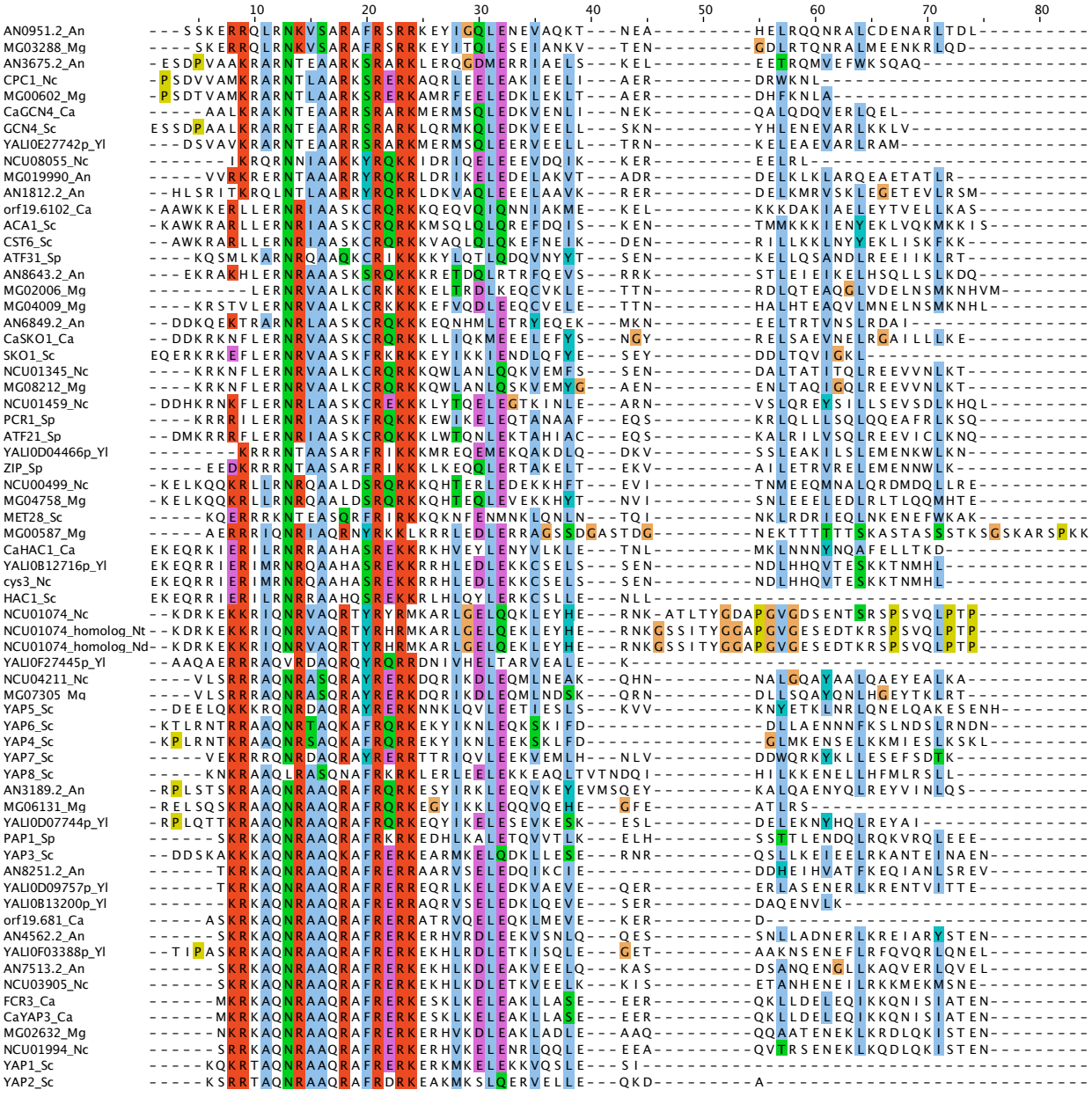


Figure 13. Alignment of YAP and non-YAP bzip transcription factor domains.
Alignment of bzip transcription factor domains from the Ascomycetes *N. crassa* (Nc), *Aspergillus nidulans* (An), *Saccharomyces ceriviseae* (Sc), *Schizosaccharomyces pombe* (Sp), *Candida albicans* (Ca), *Magnaporthe grisea* (Mg), and *Yarrowia lipolytica* (Yl). The alignment includes sequences from NCU01074, its homologs in *N. discreta* and *N. tetrasperma*, and sequences from Tian et al. 2010. The alignment was used in the construction of the MrBayes tree (Figure 14). The sequences were aligned using Muscle and displayed using Jalview.

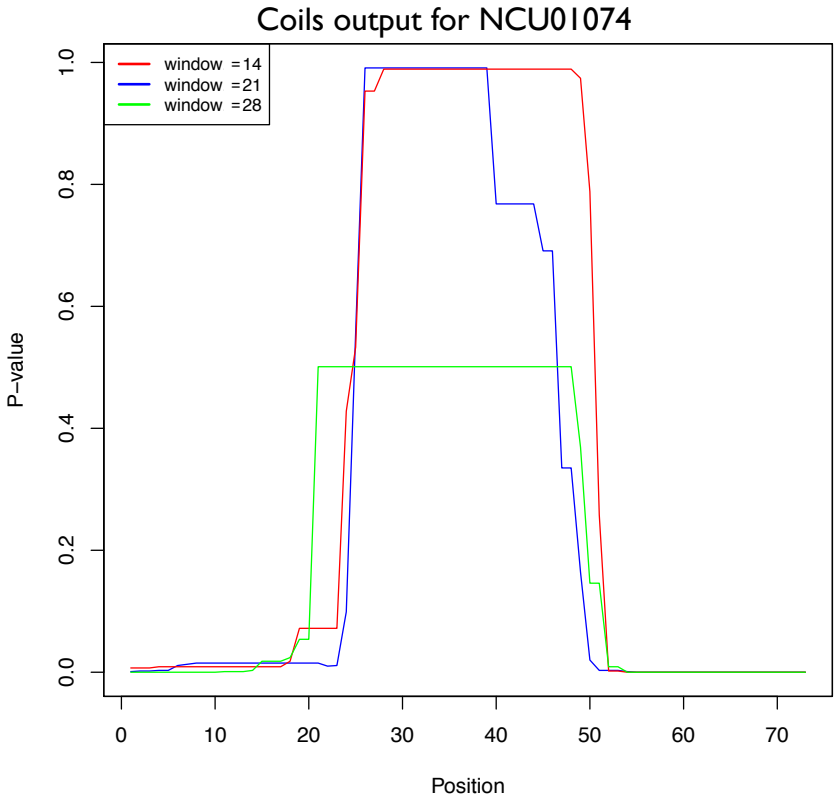


Figure 14. COILS output of NCU01074 bzip transcription factor domain.
A graph displaying the output from COILS, a program that predicts the coiled coil regions in proteins. NCU01074 was compared to a database of known parallel two stranded coiled coils and to determine the probability of each amino acid in the NCU01074 bzip transcription factor domain sequence allowing NCU01074 to adopt a coiled-coil conformation was determined. P-values for each amino acid were determined using a sliding window approach and the graph displays the data for a 14, 21, and 28 amino acid sliding window. The x-axis represents amino acid position in the input sequence and the y-axis represents the p-value from 0.0 to 1.0. In COILS, the higher

the p-value of an amino acid the greater the likelihood that the amino acid is part of a coiled-coil segment.

Interactions[1]	Comparison[2]	Genes Up/Down Regulated[3]	FunCat Level[4]	FunCat Term[5]	P-Value[6]
NcA1 vs. NcA1	Alone vs. Before	UP	LEVEL 3	01.03.01 purin nucleotide nucleoside nucleobase metabolism	8.94E-03
NcA1 vs. NcA1	Alone vs. Before	UP	LEVEL 3	10.01.09 DNA restriction or modification	8.94E-03
NcA1 vs. NcA1	Alone vs. Before	UP	LEVEL 2	42.10 nucleus	4.02E-02
NcA1 vs. NcA1	Alone vs. Before	UP	LEVEL 3	42.10.03 organization of chromosome structure	8.94E-03
NcA1 vs. NcA2	Alone vs. Before	UP	LEVEL 1	11 TRANSCRIPTION	4.26E-02
NcA1 vs. NcA2	Alone vs. Before	UP	LEVEL 3	11.02.01 rRNA synthesis	3.43E-03
NcA1 vs. NcA2	Alone vs. Before	UP	LEVEL 1	12 PROTEIN SYNTHESIS	3.68E-02
NcA1 vs. NcC	Alone vs. Before	UP	LEVEL 2	01.25 extracellular metabolism	3.38E-03
NcA1 vs. NcC	Alone vs. Before	UP	LEVEL 3	01.25.07 extracellular ester compound degradation	1.60E-02
NcA1 vs. NcC	Alone vs. Before	UP	LEVEL 1	11 TRANSCRIPTION	1.16E-03
NcA1 vs. NcC	Alone vs. Before	UP	LEVEL 2	11.04 RNA processing	1.71E-08
NcA1 vs. NcC	Alone vs. Before	UP	LEVEL 3	11.04.01 rRNA processing	2.28E-12
NcA1 vs. NcC	Alone vs. Before	UP	LEVEL 2	11.06 RNA modification	3.38E-03
NcA1 vs. NcC	Alone vs. Before	UP	LEVEL 3	11.06.01 rRNA modification	1.83E-04
NcA1 vs. NcC	Alone vs. Before	UP	LEVEL 1	12 PROTEIN SYNTHESIS	2.39E-04
NcA1 vs. NcC	Alone vs. Before	UP	LEVEL 2	12.01 ribosome biogenesis	2.82E-07
NcA1 vs. NcC	Alone vs. Before	UP	LEVEL 2	16.03 nucleic acid binding	2.20E-03
NcA1 vs. NcC	Alone vs. Before	UP	LEVEL 3	16.03.03 RNA binding	2.24E-06
NcA1 vs. NcC	Alone vs. Before	UP	LEVEL 3	20.01.07 amino acid amino acid derivatives transport	2.09E-02
NcA1 vs. NcC	Alone vs. Before	UP	LEVEL 3	20.09.18 cellular import	4.05E-02

NcA1 vs. NcC	Alone vs. Before	UP	LEVEL 3	32.01.04 pH stress response	1.60E-02
NcA1 vs. Nd	Alone vs. Before	UP	LEVEL 2	32.05 disease virulence and defense	4.47E-02
NcA1 vs. NcA1	Alone vs. Before	DOWN	LEVEL 3	20.01.27 drug toxin transport	3.48E-02
NcA1 vs. NcA1	Alone vs. Before	DOWN	LEVEL 3	32.07.05 detoxification by export	1.55E-02
NcA1 vs. NcA1	Alone vs. Before	DOWN	LEVEL 2	42.01 cell wall	4.31E-03
NcA1 vs. NcA2	Alone vs. Before	DOWN	LEVEL 4	18.02.01.01 enzyme activator	4.33E-02
NcA1 vs. NcA2	Alone vs. Before	DOWN	LEVEL 5	18.02.01.01.05 kinase activator	3.22E-02
NcA1 vs. NcA2	Alone vs. Before	DOWN	LEVEL 4	20.01.01.01 cation transport H Na K Ca2 NH4 etc	4.33E-02
NcA1 vs. NcA2	Alone vs. Before	DOWN	LEVEL 5	20.01.01.01.01 heavy metal ion transport Cu Fe3 etc	3.22E-02
NcA1 vs. NcA2	Alone vs. Before	DOWN	LEVEL 5	30.01.05.01.06 serine threonine kinase	3.22E-02
NcA1 vs. NcA2	Alone vs. Before	DOWN	LEVEL 4	30.05.01.18 transmembrane receptor protein serine threonine kinase signalling pathways	7.03E-03
NcA1 vs. NcA2	Alone vs. Before	DOWN	LEVEL 4	34.11.03.03 chemotaxis	4.33E-02
NcA1 vs. NcC	Alone vs. Before	DOWN	LEVEL 1	01 METABOLISM	4.17E-13
NcA1 vs. NcC	Alone vs. Before	DOWN	LEVEL 4	01.02.02.09 catabolism of nitrogenous compounds	2.34E-02
NcA1 vs. NcC	Alone vs. Before	DOWN	LEVEL 2	01.05 C compound and carbohydrate metabolism	1.80E-12
NcA1 vs. NcC	Alone vs. Before	DOWN	LEVEL 3	01.05.02 sugar glucoside polyol and carboxylate metabolism	2.02E-02
NcA1 vs. NcC	Alone vs. Before	DOWN	LEVEL 4	01.05.02.04 sugar glucoside polyol and carboxylate anabolism	3.42E-02
NcA1 vs. NcC	Alone vs. Before	DOWN	LEVEL 4	01.05.02.07 sugar glucoside polyol and carboxylate catabolism	6.76E-03
NcA1 vs. NcC	Alone vs. Before	DOWN	LEVEL 2	01.20 secondary metabolism	6.69E-07
NcA1 vs. NcC	Alone vs. Before	DOWN	LEVEL 3	01.20.01 metabolism of primary metabolic sugar derivatives	1.13E-03
NcA1 vs. NcC	Alone vs. Before	DOWN	LEVEL 4	01.20.17.09 metabolism of alkaloids	6.76E-03
NcA1 vs. NcA1 NcA1 vs. NcA2	Alone vs. Before	DOWN	LEVEL 3	01.06.02 membrane lipid metabolism	3.04E-02

NcA1 vs. NcA1 NcA1 vs. NcA2	Alone vs. Before	DOWN	LEVEL 4	01.06.02.01 phospholipid metabolism	2.66E-02
NcA1 vs. NcA1 NcA1 vs. NcA2	Alone vs. Before	DOWN	LEVEL 3	20.01.01 ion transport	3.04E-02
NcA1 vs. NcA1 NcA1 vs. NcA2	Alone vs. Before	DOWN	LEVEL 3	20.01.13 lipid fatty acid transport	3.04E-02
NcA1 vs. NcA1 NcA1 vs. NcA2	Alone vs. Before	DOWN	LEVEL 2	20.03 transport facilities	3.62E-02
NcA1 vs. NcA1 NcA1 vs. NcA2	Alone vs. Before	DOWN	LEVEL 3	20.03.22 transport ATPases	3.16E-02
NcA1 vs. NcA1 NcA1 vs. NcA2	Alone vs. Before	DOWN	LEVEL 3	32.01.04 pH stress response	8.60E-04
NcA1 vs. NcA1 NcA1 vs. NcA2	Alone vs. Before	DOWN	LEVEL 3	40.01.03 directional cell growth morphogenesis	3.04E-02
NcA1 vs. NcA1 NcA1 vs. NcC	Alone vs. Before	DOWN	LEVEL 2	01.06 lipid fatty acid and isoprenoid metabolism	3.48E-02
NcA1 vs. NcA1 NcA1 vs. NcC	Alone vs. Before	DOWN	LEVEL 3	20.01.03 C compound and carbohydrate transport	1.68E-02
NcA1 vs. NcA1 NcA1 vs. NcC	Alone vs. Before	DOWN	LEVEL 3	20.01.23 allantoin and allantoate transport	4.08E-04
NcA1 vs. NcA1 NcA1 vs. NcC	Alone vs. Before	DOWN	LEVEL 3	20.01.25 vitamine cofactor transport	1.27E-02
NcA1 vs. NcA1 NcA1 vs. NcC	Alone vs. Before	DOWN	LEVEL 3	20.09.18 cellular import	1.68E-02
NcA1 vs. NcA1 NcA1 vs. NcC	Alone vs. Before	DOWN	LEVEL 4	20.09.18.07 non vesicular cellular import	6.56E-04
NcA1 vs. NcA2 NcA1 vs. NcC	Alone vs. Before	DOWN	LEVEL 5	01.01.09.01.01 biosynthesis of glycine	1.90E-02
NcA1 vs. NcA2 NcA1 vs. NcC	Alone vs. Before	DOWN	LEVEL 5	01.01.09.02.01 biosynthesis of serine	1.90E-02
NcA1 vs. NcA2 NcA1 vs. NcC	Alone vs. Before	DOWN	LEVEL 3	01.20.19 metabolism of secondary products derived from glycine L serine and L alanine	2.86E-02
NcA1 vs. NcA2 NcA1 vs. NcC	Alone vs. Before	DOWN	LEVEL 4	01.20.19.01 metabolism of porphyrins	7.42E-03
NcA1 vs. NcA2 NcA1 vs. NcC	Alone vs. Before	DOWN	LEVEL 6	20.01.01.01.01.01 siderophore iron transport	4.09E-03
NcA1 vs. NcA2 NcA1 vs. NcC	Alone vs. Before	DOWN	LEVEL 3	32.05.01 resistance proteins	4.60E-02
NcA1 vs. NcA2 NcA1 vs. NcC	Alone vs. Before	DOWN	LEVEL 3	32.07.03 detoxification by modification	4.60E-02
NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 1	01 METABOLISM	1.16E-02
NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 4	01.01.03.02 metabolism of glutamate	1.44E-02
NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 5	01.01.03.02.02 degradation of glutamate	1.59E-03
NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 4	01.01.09.05 metabolism of tyrosine	1.44E-02
NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 2	01.05 C compound and carbohydrate metabolism	2.65E-02

NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 3	01.20.17 metabolism of secondary products derived from primary amino acids	4.01E-02
NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 3	16.21.07 NAD NADP binding	4.01E-02
NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 3	32.05.03 defense related proteins	4.01E-02
NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 1	41 DEVELOPMENT Systemic	4.81E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC	Alone vs. Before	DOWN	LEVEL 2	01.01 amino acid metabolism	2.62E-03
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC	Alone vs. Before	DOWN	LEVEL 3	01.01.03 assimilation of ammonia metabolism of the glutamate group	5.62E-03
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC	Alone vs. Before	DOWN	LEVEL 4	01.01.03.05 metabolism of arginine	8.79E-03
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC	Alone vs. Before	DOWN	LEVEL 5	01.01.03.05.02 degradation of arginine	2.28E-03
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC	Alone vs. Before	DOWN	LEVEL 3	01.01.11 metabolism of the pyruvate family alanine isoleucine leucine valine and D alanine	1.13E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC	Alone vs. Before	DOWN	LEVEL 4	01.01.11.02 metabolism of isoleucine	8.79E-03
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC	Alone vs. Before	DOWN	LEVEL 4	01.01.11.03 metabolism of valine	8.79E-03
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC	Alone vs. Before	DOWN	LEVEL 4	01.01.11.04 metabolism of leucine	1.41E-03
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC	Alone vs. Before	DOWN	LEVEL 5	01.01.11.04.02 degradation of leucine	2.94E-03
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC	Alone vs. Before	DOWN	LEVEL 3	16.17.01 calcium binding	2.73E-02
NcA1 vs. NcA1 NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 3	01.01.09 metabolism of the cysteine aromatic group	6.32E-03
NcA1 vs. NcA1 NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 4	01.01.09.04 metabolism of phenylalanine	4.07E-04
NcA1 vs. NcA1 NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 5	01.01.09.04.02 degradation of phenylalanine	7.51E-05
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 3	02.16.01 alcohol fermentation	4.08E-02

NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 3	32.01.01 oxidative stress response	3.96E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 1	01 METABOLISM	1.01E-04
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 5	01.01.03.02.02 degradation of glutamate	2.41E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 4	01.01.05.01 metabolism of polyamines	4.58E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 5	01.01.05.01.02 degradation of polyamines	4.04E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 4	01.01.09.04 metabolism of phenylalanine	2.37E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 5	01.01.09.04.01 biosynthesis of phenylalanine	2.41E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 4	01.01.09.05 metabolism of tyrosine	1.21E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 2	01.05 C compound and carbohydrate metabolism	2.39E-06
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 4	01.05.02.07 sugar glucoside polyol and carboxylate catabolism	2.80E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 3	01.05.06 C 2 compound and organic acid metabolism	4.54E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 4	01.05.06.07 C 2 compound and organic acid catabolism	2.80E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 4	01.05.11.07 aromate catabolism	2.80E-02

NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 5	01.05.11.07.01 aerobic aromate catabolism	2.41E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 3	01.06.06 isoprenoid metabolism	4.49E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 4	01.06.06.13 tetraterpenes carotinoids metabolism	2.80E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 2	01.20 secondary metabolism	2.11E-04
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 3	01.20.17 metabolism of secondary products derived from primary amino acids	3.08E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 4	01.20.17.09 metabolism of alkaloids	4.58E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 3	01.20.35 metabolism of secondary products derived from L phenylalanine and L tyrosine	3.98E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 4	01.20.35.01 metabolism of phenylpropanoids	2.37E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 3	01.25.03 extracellular protein degradation	4.54E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 1	02 ENERGY	1.63E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 2	02.13 respiration	1.76E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 2	02.16 fermentation	2.48E-06
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 3	02.16.01 alcohol fermentation	4.96E-02

NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 3	02.16.03 lactate fermentation	3.98E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 3	16.17.03 potassium binding	4.54E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 3	16.21.07 NAD NADP binding	3.87E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 3	20.01.03 C compound and carbohydrate transport	3.98E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 3	20.01.09 peptide transport	4.54E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 2	20.03 transport facilities	3.31E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 3	20.03.25 ABC transporters	3.98E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 4	20.09.18.07 non vesicular cellular import	4.58E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 1	32 CELL RESCUE DEFENSE AND VIRULENCE	2.79E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 2	32.05 disease virulence and defense	1.22E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 3	32.05.01 resistance proteins	3.98E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 4	32.05.01.03 chemical agent resistance	2.80E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 2	32.07 detoxification	1.23E-02

NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 2	32.10 degradation modification of foreign exogenous compounds	2.45E-02
NcA1 vs. NcA1	Before vs. After	UP	LEVEL 3	14.07.11 protein processing proteolytic	2.67E-02
NcA1 vs. NcA1	Before vs. After	UP	LEVEL 2	14.13 protein peptide degradation	3.72E-02
NcA1 vs. NcA1	Before vs. After	UP	LEVEL 3	14.13.04 lysosomal and vacuolar protein degradation	1.27E-03
NcA1 vs. NcA2	Before vs. After	UP	LEVEL 4	01.01.03.05 metabolism of arginine	1.88E-02
NcA1 vs. NcA2	Before vs. After	UP	LEVEL 5	01.01.03.05.01 biosynthesis of arginine	8.93E-03
NcA1 vs. NcA2	Before vs. After	UP	LEVEL 3	12.01.01 ribosomal proteins	7.54E-03
NcA1 vs. NcC	Before vs. After	UP	LEVEL 1	01 METABOLISM	2.10E-05
NcA1 vs. NcC	Before vs. After	UP	LEVEL 2	01.01 amino acid metabolism	6.96E-04
NcA1 vs. NcC	Before vs. After	UP	LEVEL 5	01.01.03.03.02 degradation of proline	5.60E-03
NcA1 vs. NcC	Before vs. After	UP	LEVEL 6	01.01.06.05.01.01 biosynthesis of homocysteine	1.52E-02
NcA1 vs. NcC	Before vs. After	UP	LEVEL 3	01.01.09 metabolism of the cysteine aromatic group	2.03E-02
NcA1 vs. NcC	Before vs. After	UP	LEVEL 4	01.01.09.01 metabolism of glycine	8.88E-03
NcA1 vs. NcC	Before vs. After	UP	LEVEL 5	01.01.09.01.01 biosynthesis of glycine	8.20E-04
NcA1 vs. NcC	Before vs. After	UP	LEVEL 5	01.01.09.01.02 degradation of glycine	3.33E-03
NcA1 vs. NcC	Before vs. After	UP	LEVEL 4	01.01.09.02 metabolism of serine	1.35E-02
NcA1 vs. NcC	Before vs. After	UP	LEVEL 5	01.01.09.02.01 biosynthesis of serine	3.33E-03
NcA1 vs. NcC	Before vs. After	UP	LEVEL 3	01.01.11 metabolism of the pyruvate family alanine isoleucine leucine valine and D alanine	2.03E-02
NcA1 vs. NcC	Before vs. After	UP	LEVEL 4	01.01.11.04 metabolism of leucine	1.41E-02
NcA1 vs. NcC	Before vs. After	UP	LEVEL 5	01.01.11.04.02 degradation of leucine	1.03E-03
NcA1 vs. NcC	Before vs. After	UP	LEVEL 2	01.02 nitrogen sulfur and selenium metabolism	3.84E-02
NcA1 vs. NcC	Before vs. After	UP	LEVEL 2	01.05 C compound and carbohydrate metabolism	1.06E-02
NcA1 vs. NcC	Before vs. After	UP	LEVEL 4	01.05.05.04 C 1	3.75E-

				compound anabolism	02
NcA1 vs. NcC	Before vs. After	UP	LEVEL 2	01.06 lipid fatty acid and isoprenoid metabolism	1.21E-02
NcA1 vs. NcC	Before vs. After	UP	LEVEL 2	01.07 metabolism of vitamins cofactors and prosthetic groups	1.21E-02
NcA1 vs. NcC	Before vs. After	UP	LEVEL 2	01.20 secondary metabolism	1.35E-02
NcA1 vs. NcC	Before vs. After	UP	LEVEL 1	02 ENERGY	3.75E-02
NcA1 vs. NcC	Before vs. After	UP	LEVEL 2	16.13 C compound binding	1.21E-02
NcA1 vs. NcC	Before vs. After	UP	LEVEL 2	16.21 complex cofactor cosubstrate vitamine binding	6.96E-04
NcA1 vs. NcC	Before vs. After	UP	LEVEL 3	32.01.01 oxidative stress response	4.45E-02
NcA1 vs. NcC	Before vs. After	UP	LEVEL 2	32.07 detoxification	2.57E-02
NcA1 vs. NcC	Before vs. After	UP	LEVEL 4	32.07.07.07 superoxide metabolism	2.86E-02
NcA1 vs. Nd	Before vs. After	UP	LEVEL 4	01.01.09.06 metabolism of tryptophan	2.43E-02
NcA1 vs. Nd	Before vs. After	UP	LEVEL 3	01.05.06 C 2 compound and organic acid metabolism	2.78E-02
NcA1 vs. Nd	Before vs. After	UP	LEVEL 4	01.05.06.07 C 2 compound and organic acid catabolism	2.43E-02
NcA1 vs. Nd	Before vs. After	UP	LEVEL 3	01.06.05 fatty acid metabolism	2.78E-02
NcA1 vs. NcA1 NcA1 vs. NcC	Before vs. After	UP	LEVEL 2	01.02 nitrogen sulfur and selenium metabolism	2.05E-02
NcA1 vs. NcA1 NcA1 vs. Nd	Before vs. After	UP	LEVEL 2	01.05 C compound and carbohydrate metabolism	2.27E-02
NcA1 vs. NcA1 NcA1 vs. Nd	Before vs. After	UP	LEVEL 3	01.05.03 polysaccharide metabolism	1.20E-03
NcA1 vs. NcA1 NcA1 vs. Nd	Before vs. After	UP	LEVEL 2	32.05 disease virulence and defense	2.54E-02
NcA1 vs. NcA2 NcA1 vs. NcC	Before vs. After	UP	LEVEL 1	01 METABOLISM	8.27E-08
NcA1 vs. NcA2 NcA1 vs. NcC	Before vs. After	UP	LEVEL 2	01.05 C compound and carbohydrate metabolism	3.07E-05
NcA1 vs. NcA2 NcA1 vs. NcC	Before vs. After	UP	LEVEL 4	01.05.02.07 sugar glucoside polyol and carboxylate catabolism	2.78E-02
NcA1 vs. NcA2 NcA1 vs. NcC	Before vs. After	UP	LEVEL 2	01.07 metabolism of vitamins cofactors and prosthetic groups	4.60E-02
NcA1 vs. NcA2 NcA1 vs. NcC	Before vs. After	UP	LEVEL 2	01.20 secondary metabolism	1.04E-05

NcA1 vs. NcA2 NcA1 vs. NcC	Before vs. After	UP	LEVEL 3	01.20.17 metabolism of secondary products derived from primary amino acids	2.97E-03
NcA1 vs. NcA2 NcA1 vs. NcC	Before vs. After	UP	LEVEL 4	01.20.17.09 metabolism of alkaloids	1.65E-03
NcA1 vs. NcA2 NcA1 vs. NcC	Before vs. After	UP	LEVEL 3	01.20.37 metabolism of peptide derived compounds	2.66E-02
NcA1 vs. NcA2 NcA1 vs. NcC	Before vs. After	UP	LEVEL 4	01.20.37.01 metabolism of thioredoxin glutaredoxin glutathion	1.25E-02
NcA1 vs. NcA2 NcA1 vs. NcC	Before vs. After	UP	LEVEL 2	02.16 fermentation	3.07E-05
NcA1 vs. NcA2 NcA1 vs. NcC	Before vs. After	UP	LEVEL 3	02.16.01 alcohol fermentation	4.48E-03
NcA1 vs. NcA2 NcA1 vs. NcC	Before vs. After	UP	LEVEL 2	16.21 complex cofactor cosubstrate vitamine binding	1.78E-02
NcA1 vs. NcA2 NcA1 vs. NcC	Before vs. After	UP	LEVEL 3	16.21.07 NAD NADP binding	4.48E-03
NcA1 vs. NcA2 NcA1 vs. NcC	Before vs. After	UP	LEVEL 3	32.07.03 detoxification by modification	4.53E-02
NcA1 vs. NcA2 NcA1 vs. NcC	Before vs. After	UP	LEVEL 4	32.07.07.03 glutathione conjugation reaction	8.86E-03
NcA1 vs. NcA2 NcA1 vs. NcC	Before vs. After	UP	LEVEL 4	34.11.03.13 osmosensing and response	4.61E-02
NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 3	01.01.03 assimilation of ammonia metabolism of the glutamate group	6.54E-03
NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 4	01.01.03.02 metabolism of glutamate	1.95E-03
NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 5	01.01.03.02.02 degradation of glutamate	1.45E-03
NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 3	01.01.05 metabolism of urea cycle creatine and polyamines	1.15E-02
NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 4	01.01.05.01 metabolism of polyamines	3.39E-03
NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 5	01.01.05.01.02 degradation of polyamines	1.36E-03
NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 4	01.01.09.04 metabolism of phenylalanine	5.89E-03
NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 5	01.01.09.04.01 biosynthesis of phenylalanine	1.45E-03
NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 4	01.01.09.05 metabolism of tyrosine	5.89E-03
NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 3	01.05.03 polysaccharide metabolism	4.23E-02
NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 3	01.05.06 C 2 compound and organic acid metabolism	1.03E-02

NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 4	01.05.06.07 C 2 compound and organic acid catabolism	5.24E-03
NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 3	01.06.06 isoprenoid metabolism	4.52E-02
NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 3	01.20.17 metabolism of secondary products derived from primary amino acids	6.54E-03
NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 4	01.20.17.01 metabolism of nonprotein amino acids	3.39E-03
NcA1 vs. NcA2 NcA1 vs. Nd	Before vs. After	UP	LEVEL 2	01.25 extracellular metabolism	2.55E-03
NcA1 vs. NcA2 NcA1 vs. Nd	Before vs. After	UP	LEVEL 3	01.25.01 extracellular polysaccharide degradation	2.08E-04
NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 3	02.16.01 alcohol fermentation	1.03E-02
NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 3	32.01.01 oxidative stress response	6.54E-03
NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 3	32.01.03 osmotic and salt stress response	3.13E-02
NcA1 vs. NcA2 NcA1 vs. Nd	Before vs. After	UP	LEVEL 2	32.05 disease virulence and defense	2.61E-02
NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 3	32.05.01 resistance proteins	4.52E-02
NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 3	32.05.03 defense related proteins	1.15E-02
NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 3	32.07.09 detoxification by degradation	6.54E-03
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 1	01 METABOLISM	2.47E-03
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 2	01.01 amino acid metabolism	2.03E-03
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 3	01.01.05 metabolism of urea cycle creatine and polyamines	8.64E-03
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 4	01.01.05.01 metabolism of polyamines	2.33E-02
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 3	01.01.09 metabolism of the cysteine aromatic group	8.64E-03
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 4	01.01.09.04 metabolism of phenylalanine	3.62E-02
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 4	01.01.09.05 metabolism of tyrosine	4.27E-04

NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 2	01.05 C compound and carbohydrate metabolism	1.16E-03
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 3	01.05.06 C 2 compound and organic acid metabolism	3.07E-02
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 4	01.05.06.07 C 2 compound and organic acid catabolism	3.14E-02
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 3	01.06.06 isoprenoid metabolism	1.08E-02
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 2	01.07 metabolism of vitamins cofactors and prosthetic groups	3.34E-02
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 2	01.20 secondary metabolism	7.41E-04
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 3	01.20.17 metabolism of secondary products derived from primary amino acids	1.20E-02
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 1	02 ENERGY	4.93E-02
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 2	02.16 fermentation	7.41E-04
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 3	32.01.01 oxidative stress response	2.54E-02
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 2	32.05 disease virulence and defense	3.34E-02
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 3	32.05.01 resistance proteins	3.47E-02
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 2	32.07 detoxification	2.49E-02
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 3	32.07.07 oxygen and radical detoxification	8.64E-03
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 1	34 INTERACTION WITH THE ENVIRONMENT	1.94E-02
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 2	34.11 cellular sensing and response to external stimulus	3.34E-02
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 3	34.11.03 chemoperception and response	3.07E-02

NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 2	01.25 extracellular metabolism	2.00E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 3	01.25.03 extracellular protein degradation	2.20E-03
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 3	20.01.07 amino acid amino acid derivatives transport	4.32E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 3	20.01.09 peptide transport	9.82E-03
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 3	32.05.05 virulence disease factors	1.72E-02
NcA1 vs. NcA1	Before vs. After	DOWN	LEVEL 1	01 METABOLISM	3.77E-02
NcA1 vs. NcA1	Before vs. After	DOWN	LEVEL 2	01.05 C compound and carbohydrate metabolism	1.83E-02
NcA1 vs. NcA1	Before vs. After	DOWN	LEVEL 2	01.06 lipid fatty acid and isoprenoid metabolism	4.35E-02
NcA1 vs. NcA1	Before vs. After	DOWN	LEVEL 1	02 ENERGY	3.77E-02
NcA1 vs. NcA1	Before vs. After	DOWN	LEVEL 2	02.01 glycolysis and gluconeogenesis	8.69E-04
NcA1 vs. NcA2	Before vs. After	DOWN	LEVEL 5	01.01.09.04.01 biosynthesis of phenylalanine	4.57E-02
NcA1 vs. NcA2	Before vs. After	DOWN	LEVEL 5	01.05.11.07.01 aerobic aromate catabolism	4.57E-02
NcA1 vs. NcA2	Before vs. After	DOWN	LEVEL 4	20.09.18.09 vesicular cellular import	5.00E-02
NcA1 vs. NcA2	Before vs. After	DOWN	LEVEL 5	20.09.18.09.01 endocytosis	1.25E-02
NcA1 vs. NcA2	Before vs. After	DOWN	LEVEL 1	40 CELL FATE	1.31E-02
NcA1 vs. NcA2	Before vs. After	DOWN	LEVEL 2	40.01 cell growth morphogenesis	1.39E-02
NcA1 vs. NcA2	Before vs. After	DOWN	LEVEL 1	42 BIOGENESIS OF CELLULAR COMPONENTS	9.79E-04
NcA1 vs. NcA2	Before vs. After	DOWN	LEVEL 2	42.01 cell wall	7.67E-03
NcA1 vs. NcA2	Before vs. After	DOWN	LEVEL 2	42.09 intracellular transport vesicles	3.57E-02
NcA1 vs. NcC	Before vs. After	DOWN	LEVEL 3	01.05.03 polysaccharide metabolism	2.53E-02

NcA1 vs. NcC	Before vs. After	DOWN	LEVEL 2	01.25 extracellular metabolism	9.08E-03
NcA1 vs. NcC	Before vs. After	DOWN	LEVEL 3	01.25.07 extracellular ester compound degradation	1.45E-03
NcA1 vs. NcC	Before vs. After	DOWN	LEVEL 4	20.09.18.09 vesicular cellular import	4.80E-03
NcA1 vs. NcC	Before vs. After	DOWN	LEVEL 5	20.09.18.09.01 endocytosis	3.20E-03
NcA1 vs. NcC	Before vs. After	DOWN	LEVEL 3	32.01.03 osmotic and salt stress response	4.79E-02
NcA1 vs. NcC	Before vs. After	DOWN	LEVEL 3	32.05.05 virulence disease factors	4.79E-02
NcA1 vs. NcC	Before vs. After	DOWN	LEVEL 3	34.11.03 chemoperception and response	2.53E-02
NcA1 vs. NcC	Before vs. After	DOWN	LEVEL 4	34.11.03.07 pheromone response mating type determination sex specific proteins	4.80E-03
NcA1 vs. NcC	Before vs. After	DOWN	LEVEL 3	42.04.03 actin cytoskeleton	4.79E-02
NcA1 vs. NcC	Before vs. After	DOWN	LEVEL 3	43.01.03 fungal and other eukaryotic cell type differentiation	4.79E-02
NcA1 vs. Nd	Before vs. After	DOWN	LEVEL 2	42.29 bud growth tip	4.69E-02
NcA1 vs. NcA2 NcA1 vs. NcC	Before vs. After	DOWN	LEVEL 3	40.01.03 directional cell growth morphogenesis	1.96E-02
NcA1 vs. NcA2 NcA1 vs. NcC	Before vs. After	DOWN	LEVEL 2	43.01 fungal microorganismic cell type differentiation	4.81E-02
NcA1 vs. NcA2 NcA1 vs. NcC	Before vs. After	DOWN	LEVEL 3	43.01.03 fungal and other eukaryotic cell type differentiation	1.96E-02
NcA1 vs. NcA2 NcA1 vs. NcC	Before vs. After	DOWN	LEVEL 4	43.01.03.05 budding cell polarity and filament formation	2.30E-02
NcA1 vs. NcA2 NcA1 vs. NcC	Before vs. After	DOWN	LEVEL 4	43.01.03.09 development of asco basidio or zygospore	4.29E-02
NcA1 vs. NcA2 NcA1 vs. Nd	Before vs. After	DOWN	LEVEL 4	14.07.11.01 autoproteolytic processing	2.18E-02
NcA1 vs. NcA2 NcA1 vs. Nd	Before vs. After	DOWN	LEVEL 4	18.02.01.02 enzyme inhibitor	2.18E-02
NcA1 vs. NcA2 NcA1 vs. Nd	Before vs. After	DOWN	LEVEL 5	18.02.01.02.01 GTPase inhibitor GIP	1.44E-03
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	DOWN	LEVEL 4	30.01.09.11 polyphosphoinositol mediated signal transduction	2.80E-02
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	DOWN	LEVEL 5	20.09.16.09.03 exocytosis	4.85E-02

NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	DOWN	LEVEL 2	32.05 disease virulence and defense	5.54E-03
NcA1 vs. NcA1	Alone vs. After	DOWN	LEVEL 1	01 METABOLISM	2.60E-02
NcA1 vs. NcA1	Alone vs. After	DOWN	LEVEL 4	01.01.03.03 metabolism of proline	6.43E-03
NcA1 vs. NcA1	Alone vs. After	DOWN	LEVEL 5	01.01.03.03.01 biosynthesis of proline	1.31E-03
NcA1 vs. NcA1	Alone vs. After	DOWN	LEVEL 4	01.01.05.01 metabolism of polyamines	9.56E-03
NcA1 vs. NcA1	Alone vs. After	DOWN	LEVEL 4	01.01.09.04 metabolism of phenylalanine	3.89E-03
NcA1 vs. NcA1	Alone vs. After	DOWN	LEVEL 4	01.01.09.05 metabolism of tyrosine	3.89E-03
NcA1 vs. NcA1	Alone vs. After	DOWN	LEVEL 2	01.05 C compound and carbohydrate metabolism	1.71E-02
NcA1 vs. NcA1	Alone vs. After	DOWN	LEVEL 4	01.05.06.07 C 2 compound and organic acid catabolism	1.55E-02
NcA1 vs. NcA1	Alone vs. After	DOWN	LEVEL 2	01.20 secondary metabolism	3.91E-02
NcA1 vs. NcA1	Alone vs. After	DOWN	LEVEL 1	02 ENERGY	3.07E-04
NcA1 vs. NcA1	Alone vs. After	DOWN	LEVEL 2	02.01 glycolysis and gluconeogenesis	4.85E-02
NcA1 vs. NcA1	Alone vs. After	DOWN	LEVEL 2	02.13 respiration	1.71E-02
NcA1 vs. NcA1	Alone vs. After	DOWN	LEVEL 2	02.16 fermentation	3.22E-06
NcA1 vs. NcA1	Alone vs. After	DOWN	LEVEL 2	16.13 C compound binding	4.80E-02
NcA1 vs. NcA2	Alone vs. After	DOWN	LEVEL 3	18.02.05 regulator of G protein signalling	1.96E-02
NcA1 vs. NcA2	Alone vs. After	DOWN	LEVEL 1	30 CELLULAR COMMUNICATION SIGNAL TRANSDUCTION MECHANISM	1.05E-03
NcA1 vs. NcA2	Alone vs. After	DOWN	LEVEL 2	30.01 cellular signalling	9.66E-03
NcA1 vs. NcA2	Alone vs. After	DOWN	LEVEL 1	40 CELL FATE	1.23E-03
NcA1 vs. NcA2	Alone vs. After	DOWN	LEVEL 2	40.01 cell growth morphogenesis	2.64E-04
NcA1 vs. NcA2	Alone vs. After	DOWN	LEVEL 3	40.01.03 directional cell growth morphogenesis	1.96E-02
NcA1 vs. NcA2	Alone vs. After	DOWN	LEVEL 2	42.01 cell wall	9.66E-03
NcA1 vs. Nd	Alone vs. After	DOWN	LEVEL 1	01 METABOLISM	1.35E-03
NcA1 vs. Nd	Alone vs. After	DOWN	LEVEL 2	01.05 C compound and	3.72E-

				carbohydrate metabolism	03
NcA1 vs. Nd	Alone vs. After	DOWN	LEVEL 4	01.20.17.09 metabolism of alkaloids	1.77E-02
NcA1 vs. Nd	Alone vs. After	DOWN	LEVEL 3	14.13.04 lysosomal and vacuolar protein degradation	2.33E-02
NcA1 vs. NcA1 NcA1 vs. NcA2	Alone vs. After	DOWN	LEVEL 1	01 METABOLISM	1.35E-03
NcA1 vs. NcA1 NcA1 vs. NcA2	Alone vs. After	DOWN	LEVEL 4	01.01.11.04 metabolism of leucine	4.37E-02
NcA1 vs. NcA1 NcA1 vs. NcA2	Alone vs. After	DOWN	LEVEL 5	01.01.11.04.02 degradation of leucine	3.79E-03
NcA1 vs. NcA1 NcA1 vs. NcA2	Alone vs. After	DOWN	LEVEL 2	01.05 C compound and carbohydrate metabolism	4.40E-03
NcA1 vs. NcA1 NcA1 vs. NcA2	Alone vs. After	DOWN	LEVEL 4	01.05.06.07 C 2 compound and organic acid catabolism	4.37E-02
NcA1 vs. NcA1 NcA1 vs. Nd	Alone vs. After	DOWN	LEVEL 2	01.05 C compound and carbohydrate metabolism	1.34E-02
NcA1 vs. NcA1 NcA1 vs. Nd	Alone vs. After	DOWN	LEVEL 1	02 ENERGY	3.23E-02
NcA1 vs. NcA1 NcA1 vs. Nd	Alone vs. After	DOWN	LEVEL 2	02.01 glycolysis and gluconeogenesis	4.66E-02
NcA1 vs. NcA1 NcA1 vs. Nd	Alone vs. After	DOWN	LEVEL 2	02.16 fermentation	2.37E-04
NcA1 vs. NcA1 NcA1 vs. Nd	Alone vs. After	DOWN	LEVEL 3	02.16.01 alcohol fermentation	1.83E-04
NcA1 vs. NcA1 NcA1 vs. Nd	Alone vs. After	DOWN	LEVEL 2	16.17 metal binding	3.25E-03
NcA1 vs. NcA1 NcA1 vs. Nd	Alone vs. After	DOWN	LEVEL 3	16.17.09 heavy metal binding Cu Fe Zn	1.71E-03
NcA1 vs. NcA2 NcA1 vs. Nd	Alone vs. After	DOWN	LEVEL 4	14.07.11.01 autoproteolytic processing	3.05E-02
NcA1 vs. NcA2 NcA1 vs. Nd	Alone vs. After	DOWN	LEVEL 2	16.09 lipid binding	4.05E-02
NcA1 vs. NcA2 NcA1 vs. Nd	Alone vs. After	DOWN	LEVEL 4	20.01.03.01 sugar transport	4.52E-02
NcA1 vs. NcA2 NcA1 vs. Nd	Alone vs. After	DOWN	LEVEL 4	20.09.18.07 non vesicular cellular import	4.52E-02
NcA1 vs. NcA2 NcA1 vs. Nd	Alone vs. After	DOWN	LEVEL 3	30.01.09 second messenger mediated signal transduction	3.86E-02
NcA1 vs. NcA2 NcA1 vs. Nd	Alone vs. After	DOWN	LEVEL 4	30.01.09.11 polyphosphoinositol mediated signal transduction	4.52E-02
NcA1 vs. NcA2 NcA1 vs. Nd	Alone vs. After	DOWN	LEVEL 4	32.05.01.03 chemical agent resistance	4.52E-02
NcA1 vs. NcA2 NcA1 vs. Nd	Alone vs. After	DOWN	LEVEL 3	34.11.12 perception of nutrients and nutritional adaptation	4.05E-02

NcA1 vs. NcA2 NcA1 vs. Nd	Alone vs. After	DOWN	LEVEL 4	43.01.03.09 development of asco basidio or zygospore	4.52E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC	Alone vs. After	DOWN	LEVEL 2	20.03 transport facilities	1.41E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Alone vs. After	DOWN	LEVEL 4	32.05.01.03 chemical agent resistance	1.07E-02

Supplemental Table 1. FunCat functional enrichment of shared and unique genes between four interactions.
[1] Interaction with unique genes or interactions with shared genes.
[2] Comparisons observed: alone versus before, before versus after, alone versus after.
[3] Gene regulation being observed: upregulated or downregulated genes
[4] FunCat hierarchy level. The higher the level the more specific the FunCat category, e.g. “01 - Metabolism” is level one and “01.01.11.04.02 degradation of leucine” is level 5.
[5] FunCat term significantly enriched for genes shared between interactions or unique to interactions.
[6] Adjusted p-value of FunCat enrichment, the smaller the adjusted p-value the more significant.

Interactions[1]	Expression Pattern[2]	FunCat Level[3]	FunCat Term[4]
NcA1 vs. NcA1	NNN	LEVEL 3	20.09.18 cellular import
NcA1 vs. NcA1	NNN	LEVEL 5	01.01.09.01.01 biosynthesis of glycine
NcA1 vs. NcA1	NNN	LEVEL 5	01.01.09.02.01 biosynthesis of serine
NcA1 vs. NcA1	NNN	LEVEL 6	20.01.01.01.01.01 siderophore iron transport
NcA1 vs. NcA1	NND	LEVEL 2	02.16 fermentation
NcA1 vs. NcA1	NND	LEVEL 3	01.20.05 metabolism of acetic acid derivatives
NcA1 vs. NcA1	NND	LEVEL 3	02.16.01 alcohol fermentation
NcA1 vs. NcA1	NND	LEVEL 4	01.20.05.11 metabolism of polyketides
NcA1 vs. NcA1	DDD	LEVEL 1	02 ENERGY
NcA1 vs. NcA1	NDN	LEVEL 2	01.06 lipid fatty acid and isoprenoid metabolism
NcA1 vs. NcA1	UNN	LEVEL 3	01.01.11 metabolism of the pyruvate family alanine isoleucine leucine valine and D alanine
NcA1 vs. NcA1	UNN	LEVEL 4	01.01.06.04 metabolism of threonine
NcA1 vs. NcA1	UNN	LEVEåL 4	01.01.06.05 metabolism of methionine
NcA1 vs. NcA1	UNN	LEVEL 4	01.01.06.06 metabolism of lysine
NcA1 vs. NcA1	UNN	LEVEL 4	01.01.09.02 metabolism of serine
NcA1 vs. NcA1	UNN	LEVEL 5	01.01.06.04.01 biosynthesis of threonine

NcA1 vs. NcA1	UNN	LEVEL 5	01.01.06.06.01 biosynthesis of lysine
NcA1 vs. NcA1	NDD	LEVEL 2	02.01 glycolysis and gluconeogenesis
NcA1 vs. NcA1	NDD	LEVEL 3	01.03.01 purin nucleotide nucleoside nucleobase metabolism
NcA1 vs. NcA1	DUN	LEVEL 3	14.13.04 lysosomal and vacuolar protein degradation
NcA1 vs. NcA1	DUN	LEVEL 3	20.01.09 peptide transport
NcA1 vs. NcA1	DNN	LEVEL 3	20.01.23 allantoin and allantoate transport
NcA1 vs. NcA1	DNN	LEVEL 3	20.01.27 drug toxin transport
NcA1 vs. NcA1	DNN	LEVEL 3	32.01.03 osmotic and salt stress response
NcA1 vs. NcA1	DNN	LEVEL 3	32.07.05 detoxification by export
NcA1 vs. NcA1	DNN	LEVEL 5	01.01.09.05.02 degradation of tyrosine
NcA1 vs. NcA1	NUU	LEVEL 3	01.05.03 polysaccharide metabolism
NcA1 vs. NcA1	UDN	LEVEL 2	16.17 metal binding
NcA1 vs. NcA1	UDN	LEVEL 2	16.21 complex cofactor cosubstrate vitamine binding
NcA1 vs. NcA1	UDN	LEVEL 3	16.17.09 heavy metal binding Cu Fe Zn
NcA1 vs. NcA1	UDN	LEVEL 3	16.21.07 NAD NADP binding
NcA1 vs. NcA1	DND	LEVEL 1	01 METABOLISM
NcA1 vs. NcA1	DND	LEVEL 2	01.01 amino acid metabolism
NcA1 vs. NcA1	DND	LEVEL 2	01.05 C compound and carbohydrate metabolism
NcA1 vs. NcA1	DND	LEVEL 3	01.05.03 polysaccharide metabolism
NcA1 vs. NcA1	DND	LEVEL 3	01.05.06 C 2 compound and organic acid metabolism
NcA1 vs. NcA1	DND	LEVEL 3	02.16.01 alcohol fermentation
NcA1 vs. NcA1	DND	LEVEL 3	16.17.03 potassium binding
NcA1 vs. NcA1	DND	LEVEL 3	20.01.03 C compound and carbohydrate transport
NcA1 vs. NcA1	DND	LEVEL 3	20.03.25 ABC transporters
NcA1 vs. NcA1	DND	LEVEL 3	32.01.04 pH stress response
NcA1 vs. NcA1	DND	LEVEL 3	32.05.01 resistance proteins
NcA1 vs. NcA1	DND	LEVEL 4	01.01.05.01 metabolism of polyamines
NcA1 vs. NcA1	DND	LEVEL 4	01.01.09.04 metabolism of phenylalanine
NcA1 vs. NcA1	DND	LEVEL 4	01.01.09.05 metabolism of tyrosine
NcA1 vs. NcA1	DND	LEVEL 4	01.05.06.07 C 2 compound and organic acid catabolism
NcA1 vs. NcA1	DND	LEVEL 4	01.06.06.05 sesquiterpenes metabolism
NcA1 vs. NcA1	DND	LEVEL 4	01.06.06.13 tetraterpenes carotinoids metabolism
NcA1 vs. NcA1	DND	LEVEL 4	01.20.35.01 metabolism of phenylpropanoids
NcA1 vs. NcA1	NUN	LEVEL 2	32.05 disease virulence and defense
NcA1 vs. NcA1	NUN	LEVEL 3	01.05.02 sugar glucoside polyol and carboxylate metabolism
NcA1 vs. NcA2	NNN	LEVEL 4	20.09.18.07 non vesicular cellular import
NcA1 vs. NcA2	NND	LEVEL 5	30.01.05.01.03 MAPKKK cascade
NcA1 vs. NcA2	DDD	LEVEL 3	20.01.03 C compound and carbohydrate transport

NcA1 vs. NcA2	NDN	LEVEL 1	40 CELL FATE
NcA1 vs. NcA2	NDN	LEVEL 1	42 BIOGENESIS OF CELLULAR COMPONENTS
NcA1 vs. NcA2	NDN	LEVEL 2	02.25 oxidation of fatty acids
NcA1 vs. NcA2	NDN	LEVEL 2	34.05 cell motility
NcA1 vs. NcA2	NDN	LEVEL 2	42.09 intracellular transport vesicles
NcA1 vs. NcA2	NDN	LEVEL 3	20.09.18 cellular import
NcA1 vs. NcA2	NDN	LEVEL 3	32.01.03 osmotic and salt stress response
NcA1 vs. NcA2	NDN	LEVEL 3	34.05.01 cell migration
NcA1 vs. NcA2	NDN	LEVEL 4	14.13.04.02 vacuolar protein degradation
NcA1 vs. NcA2	DUD	LEVEL 3	01.05.02 sugar glucoside polyol and carboxylate metabolism
NcA1 vs. NcA2	DUD	LEVEL 4	01.05.02.07 sugar glucoside polyol and carboxylate catabolism
NcA1 vs. NcA2	DUD	LEVEL 4	34.11.03.13 osmosensing and response
NcA1 vs. NcA2	UNU	LEVEL 3	01.01.06 metabolism of the aspartate family
NcA1 vs. NcA2	NDD	LEVEL 2	40.01 cell growth morphogenesis
NcA1 vs. NcA2	NDD	LEVEL 2	42.01 cell wall
NcA1 vs. NcA2	DUN	LEVEL 3	16.17.09 heavy metal binding Cu Fe Zn
NcA1 vs. NcA2	DNN	LEVEL 5	01.01.03.03.02 degradation of proline
NcA1 vs. NcA2	DNN	LEVEL 5	01.01.09.02.02 degradation of serine
NcA1 vs. NcA2	DNN	LEVEL 6	20.01.01.01.01.01 siderophore iron transport
NcA1 vs. NcA2	NUN	LEVEL 1	01 METABOLISM
NcA1 vs. NcA2	NUN	LEVEL 3	01.20.17 metabolism of secondary products derived from primary amino acids
NcA1 vs. NcA2	NUN	LEVEL 3	12.01.01 ribosomal proteins
NcA1 vs. NcA2	NUN	LEVEL 4	01.01.03.05 metabolism of arginine
NcA1 vs. NcA2	NUN	LEVEL 4	01.20.17.01 metabolism of nonprotein amino acids
NcA1 vs. NcA2	NUN	LEVEL 5	01.01.03.05.01 biosynthesis of arginine
NcA1 vs. NcC	NNN	LEVEL 1	30 CELLULAR COMMUNICATION SIGNAL TRANSDUCTION MECHANISM
NcA1 vs. NcC	NNN	LEVEL 1	40 CELL FATE
NcA1 vs. NcC	NNN	LEVEL 1	42 BIOGENESIS OF CELLULAR COMPONENTS
NcA1 vs. NcC	NNN	LEVEL 2	30.01 cellular signalling
NcA1 vs. NcC	NNN	LEVEL 2	40.01 cell growth morphogenesis
NcA1 vs. NcC	NNN	LEVEL 2	42.01 cell wall
NcA1 vs. NcC	NNN	LEVEL 3	14.13.04 lysosomal and vacuolar protein degradation
NcA1 vs. NcC	NNN	LEVEL 3	20.01.13 lipid fatty acid transport
NcA1 vs. NcC	NNN	LEVEL 3	32.07.05 detoxification by export
NcA1 vs. NcC	NNN	LEVEL 6	01.05.11.07.01.03 meta cleavage
NcA1 vs. NcC	NDN	LEVEL 2	42.01 cell wall
NcA1 vs. NcC	NDN	LEVEL 3	30.01.09 second messenger mediated signal transduction
NcA1 vs. NcC	NDN	LEVEL 3	34.11.03 chemoperception and response
NcA1 vs. NcC	NDN	LEVEL 3	40.01.03 directional cell growth

			morphogenesis
NcA1 vs. NcC	NDN	LEVEL 3	43.01.03 fungal and other eukaryotic cell type differentiation
NcA1 vs. NcC	NDN	LEVEL 4	20.09.07.25 vesicle formation
NcA1 vs. NcC	NDN	LEVEL 4	30.01.09.11 polyphosphoinositol mediated signal transduction
NcA1 vs. NcC	NDN	LEVEL 4	34.11.03.03 chemotaxis
NcA1 vs. NcC	NDN	LEVEL 4	40.01.03.01 regulation of directional cell growth
NcA1 vs. NcC	DUD	LEVEL 3	20.03.25 ABC transporters
NcA1 vs. NcC	DUD	LEVEL 3	32.05.01 resistance proteins
NcA1 vs. NcC	UNN	LEVEL 1	11 TRANSCRIPTION
NcA1 vs. NcC	UNN	LEVEL 1	12 PROTEIN SYNTHESIS
NcA1 vs. NcC	UNN	LEVEL 2	11.06 RNA modification
NcA1 vs. NcC	UNN	LEVEL 2	12.01 ribosome biogenesis
NcA1 vs. NcC	UNN	LEVEL 2	16.03 nucleic acid binding
NcA1 vs. NcC	UNN	LEVEL 3	11.06.01 rRNA modification
NcA1 vs. NcC	UNN	LEVEL 3	16.03.03 RNA binding
NcA1 vs. NcC	UNN	LEVEL 3	20.01.07 amino acid amino acid derivatives transport
NcA1 vs. NcC	UNN	LEVEL 4	20.09.18.07 non vesicular cellular import
NcA1 vs. NcC	DUN	LEVEL 1	02 ENERGY
NcA1 vs. NcC	DUN	LEVEL 2	01.01 amino acid metabolism
NcA1 vs. NcC	DUN	LEVEL 2	01.02 nitrogen sulfur and selenium metabolism
NcA1 vs. NcC	DUN	LEVEL 2	01.06 lipid fatty acid and isoprenoid metabolism
NcA1 vs. NcC	DUN	LEVEL 2	02.45 energy conversion and regeneration
NcA1 vs. NcC	DUN	LEVEL 2	16.13 C compound binding
NcA1 vs. NcC	DUN	LEVEL 2	16.21 complex cofactor cosubstrate vitamine binding
NcA1 vs. NcC	DUN	LEVEL 3	01.01.03 assimilation of ammonia metabolism of the glutamate group
NcA1 vs. NcC	DUN	LEVEL 3	01.01.11 metabolism of the pyruvate family alanine isoleucine leucine valine and D alanine
NcA1 vs. NcC	DUN	LEVEL 3	01.02.02 nitrogen metabolism
NcA1 vs. NcC	DUN	LEVEL 3	01.05.02 sugar glucoside polyol and carboxylate metabolism
NcA1 vs. NcC	DUN	LEVEL 3	01.05.05 C 1 compound metabolism
NcA1 vs. NcC	DUN	LEVEL 3	01.07.01 biosynthesis of vitamins cofactors and prosthetic groups
NcA1 vs. NcC	DUN	LEVEL 3	01.20.01 metabolism of primary metabolic sugar derivatives
NcA1 vs. NcC	DUN	LEVEL 3	01.20.05 metabolism of acetic acid derivatives
NcA1 vs. NcC	DUN	LEVEL 3	01.20.35 metabolism of secondary products derived from L phenylalanine and L tyrosine
NcA1 vs. NcC	DUN	LEVEL 3	01.20.37 metabolism of peptide derived compounds

NcA1 vs. NcC	DUN	LEVEL 3	16.17.03 potassium binding
NcA1 vs. NcC	DUN	LEVEL 3	16.21.07 NAD NADP binding
NcA1 vs. NcC	DUN	LEVEL 3	20.01.15 electron transport
NcA1 vs. NcC	DUN	LEVEL 3	20.01.23 allantoin and allantoate transport
NcA1 vs. NcC	DUN	LEVEL 3	20.01.25 vitamine cofactor transport
NcA1 vs. NcC	DUN	LEVEL 3	32.01.03 osmotic and salt stress response
NcA1 vs. NcC	DUN	LEVEL 4	01.01.09.01 metabolism of glycine
NcA1 vs. NcC	DUN	LEVEL 4	01.01.09.02 metabolism of serine
NcA1 vs. NcC	DUN	LEVEL 4	01.01.11.04 metabolism of leucine
NcA1 vs. NcC	DUN	LEVEL 4	01.02.02.09 catabolism of nitrogenous compounds
NcA1 vs. NcC	DUN	LEVEL 4	01.05.02.07 sugar glucoside polyol and carboxylate catabolism
NcA1 vs. NcC	DUN	LEVEL 4	01.05.05.07 C 1 compound catabolism
NcA1 vs. NcC	DUN	LEVEL 4	01.05.11.07 aromate catabolism
NcA1 vs. NcC	DUN	LEVEL 4	01.06.06.13 tetraterpenes carotinoids metabolism
NcA1 vs. NcC	DUN	LEVEL 4	01.20.01.09 metabolism of aminoglycoside antibiotics
NcA1 vs. NcC	DUN	LEVEL 4	01.20.35.01 metabolism of phenylpropanoids
NcA1 vs. NcC	DUN	LEVEL 4	01.20.37.01 metabolism of thioredoxin glutaredoxin glutathion
NcA1 vs. NcC	DUN	LEVEL 4	32.07.07.07 superoxide metabolism
NcA1 vs. NcC	DUN	LEVEL 5	01.01.03.03.02 degradation of proline
NcA1 vs. NcC	DUN	LEVEL 5	01.01.05.01.01 biosynthesis of polyamines
NcA1 vs. NcC	DUN	LEVEL 5	01.01.09.02.01 biosynthesis of serine
NcA1 vs. NcC	DUN	LEVEL 5	01.01.09.04.02 degradation of phenylalanine
NcA1 vs. NcC	DUN	LEVEL 5	01.01.11.04.02 degradation of leucine
NcA1 vs. NcC	DUN	LEVEL 6	01.01.06.05.01.01 biosynthesis of homocysteine
NcA1 vs. NcC	DNN	LEVEL 2	01.05 C compound and carbohydrate metabolism
NcA1 vs. NcC	DNN	LEVEL 2	01.06 lipid fatty acid and isoprenoid metabolism
NcA1 vs. NcC	DNN	LEVEL 2	01.20 secondary metabolism
NcA1 vs. NcC	DNN	LEVEL 4	01.20.17.09 metabolism of alkaloids
NcA1 vs. NcC	UDN	LEVEL 1	32 CELL RESCUE DEFENSE AND VIRULENCE
NcA1 vs. NcC	UDN	LEVEL 2	01.25 extracellular metabolism
NcA1 vs. NcC	UDN	LEVEL 2	32.10 degradation modification of foreign exogenous compounds
NcA1 vs. NcC	UDN	LEVEL 2	34.05 cell motility
NcA1 vs. NcC	UDN	LEVEL 3	01.25.07 extracellular ester compound degradation
NcA1 vs. NcC	UDN	LEVEL 3	32.01.03 osmotic and salt stress response
NcA1 vs. NcC	UDN	LEVEL 3	32.01.04 pH stress response
NcA1 vs. NcC	UDN	LEVEL 3	32.05.05 virulence disease factors
NcA1 vs. NcC	UDN	LEVEL 3	34.05.01 cell migration

NcA1 vs. NcC	UDN	LEVEL 5	20.09.16.09.03 exocytosis
NcA1 vs. NcC	UDN	LEVEL 5	20.09.18.09.01 endocytosis
NcA1 vs. NcC	UDN	LEVEL 5	30.01.05.05.01 small GTPase mediated signal transduction
NcA1 vs. NcC	NUN	LEVEL 3	34.01.03 homeostasis of anions
NcA1 vs. NcC	NUN	LEVEL 4	20.01.01.07 anion transport
NcA1 vs. Nd	NND	LEVEL 2	42.01 cell wall
NcA1 vs. Nd	NND	LEVEL 4	01.06.02.01 phospholipid metabolism
NcA1 vs. Nd	NDN	LEVEL 1	30 CELLULAR COMMUNICATION SIGNAL TRANSDUCTION MECHANISM
NcA1 vs. Nd	NDN	LEVEL 2	30.01 cellular signalling
NcA1 vs. Nd	NDN	LEVEL 2	34.07 cell adhesion
NcA1 vs. Nd	NDN	LEVEL 2	42.04 cytoskeleton structural proteins
NcA1 vs. Nd	NDN	LEVEL 2	42.29 bud growth tip
NcA1 vs. Nd	NDN	LEVEL 4	20.09.16.09 vesicular cellular export
NcA1 vs. Nd	NDN	LEVEL 4	40.01.03.03 guidance of longitudinal cell extension e g pollen tube guidance axonal pathfinding
NcA1 vs. Nd	NDN	LEVEL 5	18.02.01.01.05 kinase activator
NcA1 vs. Nd	NDD	LEVEL 1	01 METABOLISM
NcA1 vs. Nd	NDD	LEVEL 4	01.05.02.07 sugar glucoside polyol and carboxylate catabolism
NcA1 vs. Nd	NDD	LEVEL 4	14.07.11.01 autoproteolytic processing
NcA1 vs. Nd	NDD	LEVEL 4	20.01.03.01 sugar transport
NcA1 vs. Nd	NDD	LEVEL 4	20.03.02.03 antiporter
NcA1 vs. Nd	DNN	LEVEL 4	01.01.09.04 metabolism of phenylalanine
NcA1 vs. Nd	DNN	LEVEL 4	01.20.37.01 metabolism of thioredoxin glutaredoxin glutathion
NcA1 vs. Nd	DNN	LEVEL 4	34.11.03.07 pheromone response mating type determination sex specific proteins
NcA1 vs. Nd	DNN	LEVEL 5	01.01.09.04.01 biosynthesis of phenylalanine
NcA1 vs. Nd	UDD	LEVEL 2	32.05 disease virulence and defense
NcA1 vs. Nd	UDN	LEVEL 3	01.05.02 sugar glucoside polyol and carboxylate metabolism
NcA1 vs. Nd	UDN	LEVEL 3	34.11.03 chemoperception and response
NcA1 vs. Nd	UDN	LEVEL 4	01.05.02.07 sugar glucoside polyol and carboxylate catabolism
NcA1 vs. Nd	UDN	LEVEL 4	34.11.03.13 osmosensing and response
NcA1 vs. Nd	DND	LEVEL 1	32 CELL RESCUE DEFENSE AND VIRULENCE
NcA1 vs. Nd	DND	LEVEL 4	01.20.17.09 metabolism of alkaloids
NcA1 vs. Nd	DND	LEVEL 5	01.01.03.02.02 degradation of glutamate
NcA1 vs. Nd	NUN	LEVEL 3	01.06.05 fatty acid metabolism
NcA1 vs. NcA1 NcA1 vs. NcA2	NNN	LEVEL 3	01.20.01 metabolism of primary metabolic sugar derivatives
NcA1 vs. NcA1 NcA1 vs. Nd	NNN	LEVEL 2	01.06 lipid fatty acid and isoprenoid metabolism

NcA1 vs. NcA1 NcA1 vs. Nd	NND	LEVEL 2	01.05 C compound and carbohydrate metabolism
NcA1 vs. NcA1 NcA1 vs. Nd	DUD	LEVEL 2	01.25 extracellular metabolism
NcA1 vs. NcA1 NcA1 vs. Nd	DUD	LEVEL 3	01.25.03 extracellular protein degradation
NcA1 vs. NcA1 NcA1 vs. Nd	UNN	LEVEL 3	01.01.06 metabolism of the aspartate family
NcA1 vs. NcA1 NcA1 vs. Nd	DNN	LEVEL 3	20.01.03 C compound and carbohydrate transport
NcA1 vs. NcA1 NcA1 vs. Nd	DNN	LEVEL 4	20.09.18.07 non vesicular cellular import
NcA1 vs. NcA1 NcA1 vs. Nd	DNN	LEVEL 5	01.01.09.04.02 degradation of phenylalanine
NcA1 vs. NcA1 NcA1 vs. Nd	NUU	LEVEL 2	01.05 C compound and carbohydrate metabolism
NcA1 vs. NcA1 NcA1 vs. Nd	DND	LEVEL 1	02 ENERGY
NcA1 vs. NcA1 NcA1 vs. Nd	DND	LEVEL 2	02.16 fermentation
NcA1 vs. NcA1 NcA1 vs. Nd	DND	LEVEL 4	32.05.01.03 chemical agent resistance
NcA1 vs. NcA1 NcA1 vs. Nd	NUN	LEVEL 3	01.05.03 polysaccharide metabolism
NcA1 vs. NcA1 NcA1 vs. Nd	NUN	LEVEL 3	32.05.05 virulence disease factors
NcA1 vs. NcA2 NcA1 vs. NcC	NNN	LEVEL 3	01.05.03 polysaccharide metabolism
NcA1 vs. NcA2 NcA1 vs. NcC	NDN	LEVEL 2	40.01 cell growth morphogenesis
NcA1 vs. NcA2 NcA1 vs. NcC	DUN	LEVEL 2	01.07 metabolism of vitamins cofactors and prosthetic groups
NcA1 vs. NcA2 NcA1 vs. NcC	DUN	LEVEL 3	01.05.11 aromate metabolism
NcA1 vs. NcA2 NcA1 vs. NcC	DUN	LEVEL 3	32.07.03 detoxification by modification
NcA1 vs. NcA2 NcA1 vs. NcC	DUN	LEVEL 4	01.20.17.09 metabolism of alkaloids
NcA1 vs. NcA2 NcA1 vs. NcC	DUN	LEVEL 4	32.07.07.03 glutathione conjugation reaction
NcA1 vs. NcA2 NcA1 vs. NcC	DUN	LEVEL 4	32.07.07.05 peroxidase reaction
NcA1 vs. NcA2 NcA1 vs. NcC	DUN	LEVEL 5	01.01.09.01.01 biosynthesis of glycine
NcA1 vs. NcA2 NcA1 vs. Nd	NDN	LEVEL 3	42.04.03 actin cytoskeleton

NcA1 vs. NcA2 NcA1 vs. Nd	NDN	LEVEL 4	18.02.01.02 enzyme inhibitor
NcA1 vs. NcA2 NcA1 vs. Nd	NDN	LEVEL 5	18.02.01.02.01 GTPase inhibitor GIP
NcA1 vs. NcA2 NcA1 vs. Nd	NDD	LEVEL 2	01.05 C compound and carbohydrate metabolism
NcA1 vs. NcA2 NcA1 vs. Nd	NUN	LEVEL 3	01.01.03 assimilation of ammonia metabolism of the glutamate group
NcA1 vs. NcC NcA1 vs. Nd	NDN	LEVEL 2	43.01 fungal microorganismic cell type differentiation
NcA1 vs. NcC NcA1 vs. Nd	NDN	LEVEL 3	10.03.03 cytokinesis cell division septum formation and hydrolysis
NcA1 vs. NcC NcA1 vs. Nd	NDN	LEVEL 4	30.01.05.05 G protein mediated signal transduction
NcA1 vs. NcC NcA1 vs. Nd	NDN	LEVEL 5	20.09.16.09.03 exocytosis
NcA1 vs. NcC NcA1 vs. Nd	UNN	LEVEL 2	11.04 RNA processing
NcA1 vs. NcC NcA1 vs. Nd	UNN	LEVEL 3	11.04.01 rRNA processing
NcA1 vs. NcC NcA1 vs. Nd	DUN	LEVEL 2	32.05 disease virulence and defense
NcA1 vs. NcC NcA1 vs. Nd	DUN	LEVEL 3	01.01.05 metabolism of urea cycle creatine and polyamines
NcA1 vs. NcC NcA1 vs. Nd	DUN	LEVEL 3	01.01.09 metabolism of the cysteine aromatic group
NcA1 vs. NcC NcA1 vs. Nd	DUN	LEVEL 3	01.05.06 C 2 compound and organic acid metabolism
NcA1 vs. NcC NcA1 vs. Nd	DUN	LEVEL 3	01.20.29 metabolism of secondary products derived from L glutamic acid L proline and L ornithine
NcA1 vs. NcC NcA1 vs. Nd	DUN	LEVEL 3	01.20.31 metabolism of secondary products derived from L lysine L arginine and L histidine
NcA1 vs. NcC NcA1 vs. Nd	DUN	LEVEL 3	32.05.03 defense related proteins
NcA1 vs. NcC NcA1 vs. Nd	DUN	LEVEL 3	32.07.09 detoxification by degradation
NcA1 vs. NcC NcA1 vs. Nd	DUN	LEVEL 4	01.01.03.02 metabolism of glutamate
NcA1 vs. NcC NcA1 vs. Nd	DUN	LEVEL 4	01.01.03.03 metabolism of proline
NcA1 vs. NcC NcA1 vs. Nd	DUN	LEVEL 4	01.01.05.01 metabolism of polyamines
NcA1 vs. NcC NcA1 vs. Nd	DUN	LEVEL 4	01.05.06.07 C 2 compound and organic acid catabolism
NcA1 vs. NcC NcA1 vs. Nd	DUN	LEVEL 4	01.20.17.01 metabolism of nonprotein amino acids

NcA1 vs. NcC NcA1 vs. Nd	DUN	LEVEL 5	01.01.03.02.02 degradation of glutamate
NcA1 vs. NcC NcA1 vs. Nd	DUN	LEVEL 5	01.01.03.03.01 biosynthesis of proline
NcA1 vs. NcC NcA1 vs. Nd	DUN	LEVEL 5	01.01.05.01.02 degradation of polyamines
NcA1 vs. NcC NcA1 vs. Nd	DUN	LEVEL 5	01.01.09.04.01 biosynthesis of phenylalanine
NcA1 vs. NcC NcA1 vs. Nd	DNN	LEVEL 1	01 METABOLISM
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC	DUN	LEVEL 5	01.05.11.07.01 aerobic aromate catabolism
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC	UDN	LEVEL 3	01.05.03 polysaccharide metabolism
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. Nd	NNN	LEVEL 1	01 METABOLISM
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. Nd	NNN	LEVEL 2	01.20 secondary metabolism
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	NDN	LEVEL 4	20.09.18.09 vesicular cellular import
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	NDN	LEVEL 4	43.01.03.05 budding cell polarity and filament formation
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	NDN	LEVEL 5	20.09.18.09.01 endocytosis
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	NDN	LEVEL 5	30.01.05.05.01 small GTPase mediated signal transduction
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	UNN	LEVEL 3	11.02.01 rRNA synthesis
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	DUN	LEVEL 1	01 METABOLISM
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	DUN	LEVEL 2	01.05 C compound and carbohydrate metabolism
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	DUN	LEVEL 2	01.20 secondary metabolism

NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	DUN	LEVEL 2	02.16 fermentation
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	DUN	LEVEL 2	32.07 detoxification
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	DUN	LEVEL 3	01.06.06 isoprenoid metabolism
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	DUN	LEVEL 3	01.20.17 metabolism of secondary products derived from primary amino acids
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	DUN	LEVEL 3	02.16.01 alcohol fermentation
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	DUN	LEVEL 3	32.01.01 oxidative stress response
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	DUN	LEVEL 3	32.07.07 oxygen and radical detoxification
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	DUN	LEVEL 4	01.01.09.04 metabolism of phenylalanine
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	DUN	LEVEL 4	01.01.09.05 metabolism of tyrosine
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	NNN	LEVEL 2	01.05 C compound and carbohydrate metabolism

Supplemental Table 2. FunCat functional enrichment categories shared and unique to expression patterns found in four interactions.

[1] Interaction with unique genes or interactions with shared expression patterns and enriched FunCat terms.

[2] Expression pattern shared by interaction/s.

[3] FunCat hierarchy level. The higher the level the more specific the FunCat category, e.g. “01 - Metabolism” is level one and “01.01.11.04.02 degradation of leucine” is level 5.

[4] FunCat term significantly enriched (adjusted p-value<0.05) for genes shared between interactions or unique to interactions.

CHAPTER THREE

Differential gene expression in *Neurospora crassa* when it encounters *Penicillium chrysogenum*

Christopher Francisco Villalta

Abstract

In nature, fungi routinely encounter different fungi. Studies of the response of fungi to encounters with other fungi have grown to include assays of gene expression. Previously, we have studied the responses of *Neurospora crassa* when it encounters fungi from the same population, from different populations, and from different *Neurospora* species. Here we expand those studies to a much more distantly related fungus, *Penicillium chrysogenum*. We used RNAseq to study the transcription response of *N. crassa* in encounters with *P. chrysogenum*. We have studied encounters with both young and old *P. chrysogenum* colonies because this fungus secretes an antifungal protein, PAF, as it ages. Our five transcription profiles of *N. crassa* include it growing alone and encountering young or old *P. chrysogenum* shortly before-contact and shortly after-contact. We made six comparisons of the five profiles; for encounters with both young and old *P. chrysogenum*, alone versus before-contact, before-contact versus after-contact, and alone versus after-contact. The general response to before-contact with young or old *P. chrysogenum* was downregulation of many genes, as had been seen previously for encounters within the genus *Neurospora*. After-contact, upregulation was seen and the response was much larger with old *P. chrysogenum*, where PAF was encountered. Sets of genes with shared changes in transcription were identified for all encounters *between N. crassa* and fungi with different genotypes, for encounters with young and old *P. chrysogenum* and for encounters with just old *P. chrysogenum*. We chose genes from these three sets related to the cell wall and cell membrane structure, signaling pathways, cell membrane transport proteins, transcriptional regulators, and virulence factors. Our transcriptome data provides a full genome wide characterization of gene expression in a fungus inhibited by PAF and genes of interest found in our study will provide a promising next step for over expression and deletion mutant studies that can help us fully understand how growth is inhibited by PAF producing *P. chrysogenum.*

Introduction

Fungi are important tools for understanding how life functions at the cellular level (Davis 2000; Davis and Perkins 2002), how organisms behave with other organism in the wild (Boddy 2000; Rayner 1991), and for fighting disease (Fleming 1929; Frisvad et al. 2004). Our study attempts to observe how *Neurospora crassa* gene expression changes when it comes into contact with a distantly related fungal competitor, such as *Penicillium chrysogenum,* a fungus it could encounter in the wild, because both saprobes have a cosmopolitan distribution (Henk et al. 2011; Turner et al. 2001). We cultured *N. crassa* with a 72 hour old *P. chrysogenum* colony that inhibits growth and a 24 hour old *P. chrysogenum* colony that does not inhibit growth. The interaction was ecologically relevant because we have collected Sordariomycetes (*N. discreta*) and Eurotiomycetes

(*Aspergillus niger*) growing together in nature after a fire in the Lake Tahoe region of California in 2007. We wanted to recreate the interaction in the lab to better understand how the fungi interact in the wild. We had previously observed *Neurospora* growth inhibited when exposed to 72 hour old colonies of *Aspergillus* and *Penicillium*, but not younger colonies, which *Neurospora* grows over with no apparent effect. The pattern of inhibition was consistent with two anti fungal proteins Anafp in *A. niger* and PAF (*Penicillium* anti fungal protein) in *P. chrysogenum*, part of a family of anti fungal proteins found in Eurotiomycetes (Marx et al. 2008). Anti fungal proteins do not inhibit growth in animal cells or bacteria (Marx 2004), have no known toxic effects when exposed to mammalian cells (Henrietta et al. 2005), and appear to act synergistically with commonly used anti fungal drugs (Galgoczy et al. 2008). Eurotiomycetes produce the protein in a time dependent manner related to environmental cues or the age of the hyphae with anti fungal protein transcription and growth inhibition increasing after 48 hours of growth (Meyer and Stahl 2002). The growth inhibition qualities of Anafp and PAF were found to decrease in the presence of high extracellular concentrations of salt compounds (Marx 2004), e.g., high concentrations of $CaCl_2$ (Binder et al. 2010) were shown to suppress PAF induced growth inhibition of *N. crassa*. The same study also found an increase in intracellular Ca^{2+} plays an important role in PAF growth inhibition of *N. crassa* and when Ca^{2+}, in the media, was chelated PAF growth inhibition of *N. crassa* was suppressed (Binder et al. 2010). While we know PAF and other anti fungal proteins interfere with the homoeostasis of ions like Ca^{2+} in susceptible mycelia, more research is needed to determine all the pathways being disrupted by anti fungal proteins and PAF in particular.

We decided it would be important to study *N. crassa* interacting with an anti fungal protein producing fungus and chose *P. chrysogenum Wisconsin* because the progenitor of the *P. chrysogenum* strain used in the production of penicillin has its genome sequenced (van den Berg et al. 2008). Using *P. chrysogenum Wisconsin* is advantageous because there is more information available about the fungus in comparison to the *A. niger* isolate we collected with *N. discreta* from nature. *P. chrysogenum Wisconsin* produces PAF and inhibits growth in the same manner as *A. niger* and other anti fungal protein producing fungi. No one yet understands how exactly PAF and the other anti fungal proteins initially inhibit fungal growth. There is evidence that the anti fungal proteins interact with specific cell membrane proteins and cause the upregulation or downregulation of a signaling pathway, but the first initial protein PAF interacts with is still unknown (Binder et al. 2010; Marx et al. 2008). We have a decent understanding of what appears to be happening to susceptible fungi when their growth is inhibited. There is an increased ion permeability across the cell membrane causing a hyperpolarization of the cell membrane, but ions are not leaking nonspecifically, and the mycelia are not being lysed (Marx et al. 2008). PAF appears to cause an increase of ions in the media near the affected mycelia (Marx et al. 2008). Anti fungal proteins also lead to an increase in reactive oxygen species (ROS) and mitoptosis with eventual programmed cell death (PCD) occurring (Marx et al. 2008). None of the studies looking at PAF and the other anti fungal proteins has involved a genome wide expression

analysis of a susceptible fungus exposed to PAF. Observing the genes that are significantly differentially expressed during growth inhibition by PAF producing *P. chrysogenum* will help find the genes whose expression is disrupted by PAF and pathways that are important during the growth inhibition of fungi susceptible to antifungal proteins.

We modeled our experiments on the research in Chapter 2 where we measured gene expression in *N. crassa* upon initial contact with other fungi using differences in gene expression between *N. crassa* growing alone, *N. crassa* before contact with another fungus, and within an hour after contact between mycelia. *Neurospora* is an excellent model organism because the *Neurospora* community has a wealth of transcription data (Hutchinson et al. 2009; Kasuga and Glass 2008; Kasuga et al. 2005; Tian et al. 2011; Videira et al. 2009) including previous interaction data between *N. crassa* and other *Neurospora* (Chapter 2), a full gene knockout library (Colot et al. 2006), a good understanding of the phylogenetic relationship between *Neurospora* and other fungi (Dettman et al. 2006; Ellison et al. 2011; James et al. 2006; Menkis et al. 2009; Villalta et al. 2009), and more than 60 years of cell biology research to help understand the cellular processes being affected by PAF. We modeled our experiments after the previous study in order to directly compare *N. crassa* interacting with other *Neurospora* and *P. chrysogenum*. Having datasets from Chapter 2 available allowed us to exclude genes that were differentially expressed when *N. crassa* interacts with any fungus, with a fungus of different genotype, and with *P. chrysogenum* colonies from genes only differentially expressed during inhibition of growth by PAF producing *P. chrysogenum*. We compared new expression profiles with the previous data and characterized the interactions between *N. crassa* with the young and old colonies of *P. chrysogenum.* Our study will provide a model and baseline for future research involving important genes that we find significantly upregulated or downregulated.

We anticipated that gene expression profiles of *N. crassa* interacting with young *P. chrysogenum* colonies would show the least amount of differential gene expression of the six interactions because growth was not being inhibited. The differentially expressed genes will be those that are always significantly differentially expressed when *N. crassa* comes into contact with any Ascomycete. In the interaction between *N. crassa* and the 72 hour old *P. chrysogenum* colony there will be a large amount of differentially expressed genes because many changes in cellular signaling and metabolism are occurring to inhibit growth in *Neurospora*. There is a strong likelihood of our expression data supporting the upregulation of ROS metabolism and melanin synthesis related genes like in the interactions involving *Neurospora* of different genotype because *Neurospora* is coming into contact with another fungus in both interactions with young and old *P. chrysogenum* and previous studies found evidence for ROS production when growth was inhibited by PAF (Marx et al. 2008).The production of ROS and possible melanin synthesis could be a reaction *Neurospora* has during interactions with any fungus of a different genotype. We expect to find a functional enrichment for genes related to PCD in *Neurospora* when it comes into contact with the older growth inhibiting

P. chrysogenum colony (Marx et al. 2008). There will be several proteins significantly differentially expressed related to transport across, signaling across, and maintenance of the cell membrane since the cell membrane is the point of contact between N*. crassa* and PAF, which affects its permeability to certain ions and growth. We expect to find significantly differentially expressed genes related to the cell wall since normal fungal growth is related to the regulation of genes and pathways involved in cell wall maintenance and synthesis. The overall trend we expect is PAF affecting gene expression in proteins that are important to several signaling pathways involved in mycelial growth and many of the genes we find significantly differentially expressed will not have an affect on cell growth alone, but will cumulatively be responsible for the growth inhibition phenotype we see in *N. crassa* encountering PAF producing *P. chrysogenum*.

Our study is a first look at gene expression in *Neurospora* when growth is inhibited by PAF. We expect it to give us a better idea of how fungi interact in the wild, of what is occurring on a cellular level by understanding how signaling pathways affected by PAF are interrelated, and we think it is a promising experimental model that can easily be expanded for the further study of anti fungal proteins. Our model and research is important because it will provide insight into how anti fungal proteins affect susceptible cells and can help in determining whether anti fungal proteins are a viable option in treating fungal pathogens of plants and animals, including humans.

Methods

Strains utilized and culture conditions

We obtained wild type *Neurospora crassa* FGSC 2489 (NcA1) (McCluskey et al. 2010) from the Caribbean population of the NcA clade and *Penicillium chrysogenum Wisconsin* 54-1255 ATCC 28089™ (PcW). To study fungal-fungal interactions, we followed the Neurospora large plate protocol (http://www.yale.edu/townsend/Links/ffdatabase/downloads.html) (Kasuga and Glass 2008). *Neurospora* and *Penicillium* from stock cultures were inoculated into slants of Vogel's medium (Vogel 1956) with 1% sucrose and incubated at 30°C for three days followed by five days at 25°C in constant light to suppress synchronous gene expression associated with circadian rhythms (Kasuga and Glass 2008). NcA1 and PcW conidia were collected from the slant by vortexing the culture with 1 mL of ddH_2O to give a concentration of approximately 5 x 10^6 NcA1 conidia/mL and undiluted PcW conidia. Large, 23 cm x 23 cm, plastic culture plates of Bird's medium (Metzenberg 2004) were overlaid with cellophane. To prepare mycelium for experiments, the suspension of conidia were evenly distributed across culture plates by shaking 5 mm glass beads across the plate for 10 seconds, retrieving the beads, and incubating the plates at 25 °C in constant light for 24 hours. The study consisted of two experimental conditions, in the first condition we placed cellophane strips (0.5cm x 22cm) cut from a monolayer of PcW on one side of a new large plate of Bird's media overlaid with cellophane and incubated the colony for 48 hours before we placed a second strip of cellophane with NcA1 on the other side of the plate (Table 1). In the second condition a

strip of cellophane with NcA1 and PcW were placed on opposite sides of a fresh large plate of Bird's media overlaid with cellophane at the same time (Table 1). Mycelium from NcA1 was collected at two different times from replicate plates, once before mycelial contact (24 hours) and within an hour of contact with young PcW or PAF produced by old PcW (~27 hours) (Figure 1). Whether growth of NcA1 was being inhibited or not was determined by preliminary experiments and by measuring the growth rate of NcA1 every hour, once 24 hours had passed, and waiting for a decrease in growth. Collection at 24 hours involved removing a strip of mycelium (1 cm x 22 cm) that was 0 – 3 hours old from the growth front (Chapter 2). Approximately three hours later, within an hour of contact with young PcW or PAF produced by old PcW, a 1cm x 22cm strip of mycelia from NcA1 was collected, but 0.5cm away from the zone of contact with young PcW to avoid contamination (Chapter 2).

RNA extraction and RNAseq library construction

To extract RNA, mycelium in each 1 cm x 7 cm strip was broken in 1 mL of TRIzol (Invitrogen Life Technologies) using a MiniBeadBeater and Zirconia/Silica beads (0.2 g, 0.5 mm diameter, Biospec products), twice for 30 seconds at maximum speed (Kasuga et al. 2005). The total RNA was extracted following a protocol adapted from the TRIzol manufacturer's protocol, in which, following the gentle shaking of incubating samples, the samples were further disrupted in chloroform using a MiniBeadBeater (Kasuga and Glass 2008). A 1 μL sample of the total RNA was electrophoresed on a 1.5% agarose gel at 150 mA and quantified using a Nanodrop ND-1000 Spectrophotometer (ThermoScientific). We used the RNeasy Mini Kit (Qiagen) to clean total RNA of cell debris and fragments. Messenger RNA (mRNA) was purified from the total RNA, fragmented, synthesized into cDNA, and processed into RNAseq libraries following the Illumina mRNA Sequencing sample preparation guide (September 2009 version). We quantified concentration of cDNA libraries with the Qubit Fluorometer (Invitrogen Life Technologies). Libraries were then sent to the University of California, Berkeley QB3 Functional Genomics Laboratory where insert size was determined (~200bp), and where DNA concentration was again measured using the Bioanalyzer 2000 (Agilent). Libraries were sequenced on individual lanes from single ends to 76 base pairs (bp) at the University of California, Berkeley QB3 Vincent J. Coates Genomics Sequencing Laboratory using the Illumina Genome Analyzer platforms and sequencing technology.

RNAseq data utilized from interactions between Neurospora

We used the 24 RNAseq libraries from Chapter 2 that included bioreplicates of before and after-contact conditions between *Neurospora crassa* 2489 (NcA1) colony in a self-self interaction with another NcA1 colony, in a interspecific interaction with a *Neurospora* colony of a different genotype from the same population (NcA2), in a interpopulation interaction with a *Neurospora* colony from a different population within the *N. crassa* species (NcC), and in a interspecific interaction with a *Neurospora* from a different species (*N. discreta*) within the same genus (Nd) all chosen based on their position in the *Neurospora* phylogeny (Table 1). The libraries also included three bioreplicates of NcA1 growing alone (Table 1).

Mapping of libraries and processing of samples
Sequenced reads from libraries were mapped to the NcA1 (*N. crassa* FGSC 2489) genome (Galagan et al. 2003) with TopHat v1.3.1 (Trapnell et al. 2009) parameters set to two splice mismatches, minimum intron length of 40, a maximum intron length of 200, and three threads.

The total amount of raw non-normalized read counts mapping to each gene in NcA1 was calculated using a Perl script and coverage information (.wig) from Tophat and gene coordinates from the NcA1 version 10 genome annotation (.gff3). For each comparison of transcription for a pair of conditions, raw read counts for the three bioreplicates from each of the two conditions were compiled into a dataset. Genes with no read counts in any of the six libraries were removed. Individual genes were normalized by the upper-quartile (75^{th} percentile) specific to their library (Bullard et al. 2010). To determine if transcription profile variation was lower within conditions than between conditions, as expected. We first employed MA plots of the pairwise difference in gene expression against the level of gene expression for libraries within conditions and across conditions (Figure 2) (Cleveland and Delvin 1988). We also fit LOESS lines was to the coordinates in each plot and we used a Pearson's chi square test (chitest in R) to determine if our y-coordinates from the LOESS line fit the zero y-axis with a sum of the critical values having a p-value greater than 0.05. Our expectation was that there would not be significant expression differences between the majority of genes and therefore the LOESS line should fit the zero y-axis. Library sequencing error or mapping errors would cause a significant deviation from the zero y-axis (Figure 2).

In the second, we used box plots to evaluate bioreplicate variation within condition compared to variation among all conditions. To make the box plots, for each gene we log transformed the normalized read counts and calculated the median of the log transformed read counts for the three libraries in each condition (condition median) and for all six libraries (all median), together. To compare differences in interquartile ranges (IQR), for each gene we plotted the differences between, the normalized and log transformed read counts, and the median for that condition (condition median difference), and the difference between the counts for both conditions and the median for the counts for both conditions (all median difference) using R v2.12.1 (RDevelopmentCoreTeam 2011) (Figure 3).

Differential expression analysis
Methods of assessing differential gene expression assume a negative binomial distribution of gene expression for genes with at least five counts (Bullard et al. 2009). To determine if our data for each experimental condition followed a negative binomial distribution, we compared observed data to a negative binomial distribution simulated using rnbinom in R given the observed number of genes, mean read counts, and dispersion as calculated using edgeR in R Bioconductor (Robinson et al. 2009). The experimental read counts and simulated read counts were fit separately to a negative

binomial distribution using the glm.nb package in R and p-values were collected for how well each gene fit a negative binomial distribution. To attempt to reject the null hypothesis of no difference, we used a Pearson's chi square test (chitest) with the p-values from the fit of the observed and simulated data.

To analyze differences in expression we used two negative binomial models in R Bioconductor, DESeq and edgeR (Figure 4) (Anders and Huber 2010). We excluded genes where counts were zero in all libraries. For genes where counts for just some libraries were zero, the zero values were increased to one (Anders and Huber 2010; Bullard et al. 2009; Robinson et al. 2009). Instead of using the default settings in DESeq and edgeR counts were normalized using the upper quartile and we used tag wise dispersion in edgeR. To avoid raising the false discovery rate, p-values were adjusted for multiple hypotheses using the Benjamini and Hochberg method in R (Benjamini and Hochberg 1995).

To find genes that were significantly (adjusted p-value < 0.05) differentially expressed, we used DESeq and edgeR, in combination and filtered for genes significantly differentially expressed more than 1.5 fold between conditions to produce one gene list. In our comparison between NcA1 growing alone in comparison to NcA1 growing with PcW before or after-contact we used read counts from the libraries where NcA1 was growing alone in Chapter 2 (CV265, CV310, CV314).

Coding expression differences between experimental conditions

Gene expression levels for NcA1 alone, and for NcA1 before and after encountering a second mycelium in the four interactions from Chapter 2 in addition to the two interactions from this study were compared in three ways, alone versus before-contact, before-contact versus after-contact, and alone versus after-contact. Where expression levels showed a significant difference, they were coded as either D (down) or U (up). Where there was no significant difference, they were coded N (none). Thus, each gene was assigned a three-letter code, for example, DUN representing differential expression in the order alone versus before-contact, before-contact versus after-contact and alone versus after-contact. A fourth possible code X, was available for conflicting results between edgeR and DESeq, but we had no conflicting results.

The patterns of expression represented by the three-letter codes were used to compare transcription across the six comparisons by bar chart of pattern frequency (Figure 5), by similarity in gene patterns (Figure 6), and by distance among conditions based on shared gene patterns (Figure 7). The bar graph was based on the proportion of three-letter codes for genes that were present in DESeq plus edgeR and 1.5 fold. To compare expression differences for each gene across the six interactions, we collected all the genes that were significantly differentially expressed in DESeq or edgeR and greater than 1.5 fold in each interaction to determine their three-letter expression code. The list of three-letter significant expression codes for each interaction were assigned a single character, akin to abbreviations for each codon of an amino acid, saved as an alignment in fasta format, and visualized using Jalview 2.6.1 (Figure 6) (Clamp et al. 2004).

To estimate the distances among the gene expression patterns for the six encounters we converted the alignment from fasta format to Phylip format using trimAL version 1.2 revision 59 (Capella-Gutierrez et al. 2009), made a distance matrix using Distmat from the EMBOSS 3.6.0 package (Rice et al. 2000), made an unrooted neighbor-joining tree with Neighbor from the Phylip 3.68 package (Felsenstein 1989), and visualized the tree with Mesquite version 2.74 (Figure 7) (Maddison and Maddison 2010). Bootstrap support for the internal branches was based on 100 data sets resampled with replacement using Phylip 3.68.

Functional Enrichment

To determine if the collection of genes showing the same expression patterns during the fungal interactions was enriched for specific functions, we compared our data to functional categories determined for NcA1 in FunCat version 2.1 (Ruepp et al. 2004) from MIPS (Mewes et al. 2004) using phyper (Fury et al. 2006; Johnson et al. 1992) in R and a p-value less than 0.05 as the threshold for significance after Benjamini and Hochberg correction for multiple hypothesis testing. The more overlap there was between our set of genes of interest and a known set of genes with a shared known functional relationship the more significant the p-value and the smaller the likelihood that the overlap was a result of chance.

Results

Observations of NcA1 growing and interacting with PcW

Our experimental conditions involved NcA1 interacting with young PcW that did not inhibit growth of NcA1 mycelia and old PcW that did inhibit growth (Figure1). The NcA1 colonies had a linear growth rate of 2.2 +/- 0.2 mm/hr and the linear growth rate for PcW colonies was negligible in comparison to NcA1, but more mycelial mass was present in the three day old PcW colonies in comparison to one day old PcW colonies (Figure 1). The growth rate of NcA1 was not affected, until growth was halted by PAF excreted from old PcW. Young PcW did not affect the growth rate of NcA1 and if growth was allowed to continue after initial contact, NcA1 grew over the young PcW colony and continued to the other side of the culture plate. In plates with old PcW, NcA1 did not grow inside the zone of inhibition surrounding the old PcW colony (Figure 1). During growth inhibition by PAF NcA1 growing with old PcW had less aerial mycelia present at the hyphal growth front compared to NcA1 growing with young PcW. The hyphae of NcA1 being inhibited by PAF did not appear lysed or physically damaged upon microscopic examination.

RNAseq library sequencing and read mapping

Twelve RNAseq libraries were sequenced and included three biological replicates of before (24 hours) and after-contact (~27 hours) of NcA1 interacting with young PcW and old PcW (Table 2). The 12 lanes of PcW interaction data contained an average of 26,381,761 reads and an average of 23,949,281 (91%) reads mapped back to the NcA1 genome. In addition, we included sequencing data from the self-self, intrapopulation,

interpopulation, and intraspecific interactions between *Neurospora* collected in Chapter 2 under similar growth conditions. Out of the 9,907 annotated genes in the NcA1 genome 9,516 or 96% were expressed in the 12 RNAseq libraries. When the 24 RNAseq libraries from *the Neurospora* interactions were included with our RNAseq data of NcA1 interacting with PcW there was expression found for 9,589 genes or 97% of all genes found in NcA1.

Testing consistency among bioreplicates with MA plots and median difference box plots. Gene expression variation among bioreplicates within a condition was lower than that between conditions as judged from MA plots (Figure 2). Our expectation was while we would find differential expression between conditions in *Neurospora*, the majority of genes would be similarly expressed and LOESS lines fit to comparisons of libraries among and between conditions would not significantly deviate from the zero y-axis. We fit LOESS lines to all the points in the MA plot and determined that our data did not deviate significantly from the zero y-axis (Pearson's Chi square test, p-value>0.05), which could be evidence of a sequencing or mapping error in one of the libraries (Figure 2).

Similarly, comparison of IQR calculated for variation among bioreplicates within a condition showed variation to be less than those calculated for comparisons between conditions (Figure 3, Table 3).

Genes found differently expressed using DESeq and edgeR
For all five experimental conditions, we found no significant difference (p-value>0.05) using Pearson's chi-square test between the probabilities from a generalized linear model that our observed transcription data fit a negative binomial distribution and the probabilities that data simulated to represent a negative binomial distribution fit that same distribution.

We could use DESeq and edgeR, based on a negative binomial distribution, with confidence for differential expression analyses. To compare three condition 1 bioreplicates to three condition 2 bioreplicates. From both differential expression analyses we collected genes that were significantly differentially expressed (adjusted p-value <0.05) and expressed greater than 1.5fold (Table 4). As in Chapter 2 our analyses show that DESeq recognized more significantly differentially expressed genes than edgeR but missed some that were recognized by edgeR, and that almost all of the genes recognized by the two methods had at least a 1.5 fold change in expression (Figure 4). For our comparative analyses, we considered all genes found to have significant differential expression by either DESeq or edgeR and a change in expression of 1.5 fold or greater (Chapter 2, Tian et al. 2011). Significantly differentially expressed genes were evenly distributed amongst genes found lowly and highly expressed as displayed in smear and MA plots (Figure 4).

The most apparent difference when we compared NcA1 interacting with young PcW and old PcW was that there were six times more genes (1,277 to 211) being differentially expressed between interactions (Figure 8a-b). The trend of more gene downregulation than upregulation was similar to what we saw in all four interactions between *Neurospora* in Chapter 2. NcA1 growing with young PcW shared a majority of its downregulated genes with the interaction between NcA1 and old PcW. The majority of the differentially expressed genes in NcA1 growing with old PcW appeared unique to the interaction. When NcA1 growing alone was compared to NcA1 before-contact with young PcW because NcA1 never stops growing even after-contact with young PcW (Table?). The result of the interaction between NcA1 and young PcW was different from what we observed when NcA1 encountered old PcW, which stops growing upon contact with PAF and the interruption of growth that takes place in the four *Neurospora* interactions in Chapter 2 (Table?). The 190 downregulated genes shared by both young and old interactions between NcA1 and PcW were significantly enriched for the FunCat terms “C-compound and carbohydrate metabolism,” “Polysaccharide metabolism,” “Extracellular protein degradation,” and “ABC transporters” appear to be a subset of the larger response seen when NcA1 comes into contact with an older PcW (Supplemental Table 2). Old PcW had a large effect on NcA1 gene expression and metabolism (Supplemental Table 2) than young PcW as evidenced by the 1,087 genes uniquely differentially expressed in the interaction between NcA1 and old PcW.

When gene expression in NcA1 before-contact with PcW was compared to after-contact with PcW, we found that NcA1 interacting with old PcW not only had more genes significantly differentially expressed, but had five times (814 genes to 168 genes) more genes being upregulated than in NcA1 encountering a young PcW (Figure 8c-d). The interaction between NcA1 and young PcW shared the majority (155 out of 168) of its upregulated genes with NcA1 versus old PcW, and these genes are a subset of those upregulated in NcA1 when encountering old PcW. Among the 155 shared genes we found 15 significantly enriched FunCat terms of which, nine were related to “Metabolism,” three to “Energy,” two to “Protein with binding function or cofactor requirement,” and one to “Cell rescue, defense, and virulence.” Of the nine “Metabolism” FunCat terms six were related to melanin synthesis: “Metabolism,” “Metabolism of phenylalanine,” Metabolism of tyrosine,” “Secondary metabolism,” “Metabolism of secondary products derived from primary amino acids,” “Metabolism of phenylpropanoids.” Contact between NcA1 with either young or old PcW causes an upregulation of genes related to melanin synthesis between NcA1 growing before-contact and after-contact. The increase in genes upregulated is similar to what we saw for all four *Neurospora* interactions and the increase in expression of genes related to melanin synthesis was similar to what we saw in nonself interactions between *Neurospora.*

When NcA1 growing alone was compared to NcA1 after-contact with PcW, we found no significant upregulation in either interactions between NcA1 and PcW, very few genes were significantly downregulated in NcA1 versus young PcW (6 genes) and old PcW (2

genes), with no overlap between interactions. There was little to no change in expression between alone versus after-contact because genes that were downregulated in alone versus before-contact were upregulated in before versus after-contact resulting in no net change in gene expression. Similar gene expression patterns were seen in the interactions between *Neurospora* isolates (Chapter 2) except that there was a significant amount of downregulated genes when NcA1 growing alone was compared to after-contact.

Comparing expression between Neurospora encountering other Neurospora and PcW
We determined genes and cellular processes that were downregulated in the alone versus before-contact comparison and were upregulated in the before versus after comparison in all six interactions and between nonself and PcW (Figure 9). All four *Neurospora* (NcA1, NcA2, NcC, Nd) interactions shared 372 significantly downregulated genes in alone versus before-contact (Figure 9a) and in Chapter 2, 72 significantly downregulated genes were specific to the three nonself *Neurospora* (NcA2, NcC, Nd) interactions (Figure 9b). There was a significant overlap of 173 downregulated genes between interactions involving NcA1 with PcW and those involving the four interactions between *Neurospora*. The 173 genes were significantly enriched for two FunCat terms "Extracellular protein degradation" and "ABC transporters" (Supplemental Table 3). We found that NcA1 interacting with old PcW and all the *Neurospora* interactions shared more downregulated genes in common (184 genes) than they did with NcA1 versus young PcW (Figure 9a). The 184 genes were significantly enriched for 35 FunCat terms and 17 were related to "Metabolism," five to "Energy," three to "Protein with binding function or cofactor requirement," two to "Cellular transport, transport facilitation, and transport routes," and eight to "Cell rescue, defense, and virulence." It appears that NcA1 versus old PcW and all *Neurospora* interactions have more cellular processes in common with 35 FunCat terms enriched in comparison to two FunCat terms shared by all interactions. The interactions excluding NcA1 versus young PcW have more in common because in all five of the other interactions the growth of *Neurospora* appears to stop or slow down because of contact with PAF or contact with another mycelium as opposed to the interaction between NcA1 and young PcW where NcA1 just continues to grow.

In Chapter 2 nonself *Neurospora* interactions were more similar to each other than to the self-self (NcA1 versus NcA1) interaction, which had the lowest amount of differential expression occurring among the four interactions. We compared downregulated genes (72 genes) that were only shared between nonself *Neurospora* interactions in Chapter 2 to NcA1 versus young and old PcW. Only four downregulated genes were shared between all four interactions while a majority (61 gene) of the nonself *Neurospora* interaction genes were shared with NcA1 versus old PcW (Fig 9b). The 61 genes were only significantly enriched for one FunCat term, "alcohol fermentation" (Supplemental Table 4).

Next we compared genes upregulated in NcA1 in before-contact compared to after-contact (Figure 9c). There were 33 genes upregulated shared between all *Neurospora* interactions when before-contact was compared to after-contact. There were 10 upregulated genes shared by all interactions between before versus after-contact, but they were not enriched for any FunCat terms. NcA1 versus old PcW and all *Neurospora* shared 18 genes that were significantly enriched for “Alcohol fermentation.”

When we compared the 66 genes upregulated only in nonself *Neurospora* interactions from Chapter 2 in before-contact versus after-contact, we found that 29 of those genes were also upregulated in the interactions between NcA1 with young and old PcW (Figure 9d). The 29 genes were enriched in FunCat for seven terms related to “Metabolism,” one related to “Energy,” and one related to “Cellular transport, transport facilitation, and transport routes.” We found that six of the “Metabolism” FunCat terms were related to melanin synthesis: “Amino acid metabolism,” “Metabolism of urea cycle creatine and polyamines,” “Metabolism of cysteine aromatic group,” “Metabolism of phenylalanine,” “Metabolism of tyrosine,” “Secondary metabolism,” and “Metabolism of secondary products derived from primary amino acids.” It appears that all five interactions between NcA1 and nonself (NcA1, NcA2, NcC, Nd, young and old PcW) elicit an upregulation of melanin synthesis genes between before versus after-contact. There were 20 upregulated genes shared between NcA1 versus old PcW and non-NcA1 *Neurospora* enriched for four FunCat terms related to “Metabolism,” two related to “Energy,” and one related to “Cell rescue, defense, and virulence.”

Expression pattern analysis

From the above results it is clear that gene expression is changing throughout the interactions and that it would be useful to look at the pattern of gene expression before-contact, during contact and after-contact.

To compare patterns of changes in gene expression, we coded significant changes as down (D) or up (U) and used N for no significant change for the three comparisons of gene expression: alone versus before-contact, before-contact versus after-contact and alone versus after-contact. Of the 27 combinations of D, U and N, seven would be impossible to observe (NUD, NDU, DNU, DDU, UND, UUN, and UUD), no gene was seen with a UDU pattern and we did not consider unchanged genes (NNN). To see the proportion of genes within each of the 18 remaining trends, bar graphs were made of the trends (Figure 5). Bar graphs were used to judge the similarity of patterns in the six different interactions. As in Chapter 2 having a cutoff of genes differentially expressed 1.5 fold or greater did not change the proportions of gene expression patterns in NcA1 versus young PcW and old PcW. NcA1 interacting with young PcW and old PcW both had a lower percentage of genes with the DND expression pattern than the *Neurospora* interactions. NcA1 versus young PcW had the largest percentage of genes with the NUN expression pattern and NcA1 interacting with old PcW had the largest percentage of genes with an expression pattern of DUN. Both interactions between NcA1 and PcW

did not look very similar to any of the four interactions between *Neurospora* or to each other (Figure 5).

When genes from each interaction were aligned, with the inclusion of NNN, we were able to view the differences and similarities between interactions (Figure 6). The NNN expression pattern continued to be the dominant pattern over the 18 expression patterns (Chapter 2). NcA1 versus old PcW (1,390) had the third most differentially expressed genes after NcA1 versus NcA2 (1,655 genes) and NcA1 versus NcC (1,638 genes). NcA1 versus young PcW had the least amount of genes significantly differentially expressed with 286 genes. NcA1 interacting with NcA1 and young PcW shared 1,993 genes with an NNN expression pattern, but did not share many other genes with specific expression patterns in common (13 or less than 1%). NcA1 interacting with NcC and old PcW shared genes with the same DNN (256 genes), DUN (251 genes), and NNN (746 genes) expression patterns. NcA1 interacting with old PcW did not appear very similar to the other interactions where NcA1 was grown with NcA1, NcA2, and Nd (Figure 6). We also constructed a second alignment of just the interactions between NcA1 and PcW that displays how very different the interactions were and their few similarities (Figure 6b).

A distance tree was made from the ordered expression profiles, NcA1 versus young PcW and NcA1 versus NcA1 (self-self) were separated by the shortest distance (Figure 7). NcA1 versus old PcW grouped closest to NcA1 versus NcC (interpopulation) while NcA1 versus NcA2 (intrapopulation) and NcA1 versus Nd (interspecific) shared the shortest distance between each other. NcA1 interacting with young PcW and old PcW were not very similar in expression patterns of specific genes (Figs 6, 7) but shared some similarities in the collective pattern of gene regulation (Figure 5). These trends emerge because there were massive changes in gene expression occurring in NcA1 upon contact with old PcW in comparison to young PcW with significantly less change in gene expression (Figure 6b).

Expression pattern functional category enrichment

Significant enrichment for FunCat terms was assessed in each group of genes sharing the same pattern of expression for any of the six interactions, e.g., genes in NcA1 versus old PcW with a DUN expression trend (841 genes) were significantly enriched for the FunCat term “Metabolism of tyrosine” (adjusted p-value = 0.002) due to the presence of nine genes related to tyrosine metabolism. To see how significantly enriched FunCat terms for each pattern were shared across the four interactions we again used Venn diagrams (Figure 9) (Supplemental Table 5).

We grouped expression patterns and corresponding FunCat terms to compare what was shared between NcA1 interacting with both young and old PcW, all *Neurospora* interactions, and NcA1 versus nonself *Neurospora* interactions (Supplemental Table 5). We did not find any expression patterns with significantly enriched FunCat terms shared by all six interactions, but there was an overlap of nine FunCat terms between NcA1

versus old PcW and non-NcA1 *Neurospora*. The nine FunCat terms were significantly enriched for the DUN expression pattern and included five terms related to melanin synthesis: “Metabolism,” “Secondary metabolism,” “Metabolism of secondary products derived from primary amino acids,” “Metabolism of phenylalanine,” and “Metabolism of tyrosine.” There was also one FunCat term, “Detoxification,” related to ROS metabolism between NcA1 interacting with old PcW and non-NcA1 *Neurospora*. When we looked at significantly enriched FunCat terms unique to NcA1 versus old PcW we found the DUN expression pattern was significantly enriched for one more FunCat term “detoxification by modification,” which is related to ROS metabolism.

NcA1 interacting with young PcW was significantly enriched for the FunCat terms “metabolism of secondary products derived from primary amino acids,” “metabolism of phenylalanine,” “metabolism of tyrosine” related to melanin synthesis and “oxygen and radical detoxification” related to ROS metabolism in the group of genes with an NUN expression pattern. As noted earlier there was not a large downregulation of genes between NcA1 growing alone and before-contact in NcA1 when interacting with young PcW in comparison to the other four nonself interactions, resulting in genes with a NUN expression pattern, instead of the DUN. When we compared the 46 significantly enriched FunCat terms for DUN in NcA1 versus old PcW and the 31 enriched FunCat terms with an NUN in NcA1 versus young PcW we found that 17 of the 31 (55%) FunCat terms overlapped. Six of the FunCat terms that overlapped were related to melanin synthesis: “Metabolism,” “Secondary metabolism,” “C2 compound and organic acid metabolism,” “Metabolism of secondary products derived from primary amino acids,” “Metabolism of phenylalanine,” and “Metabolism of tyrosine.” While genes related to melanin synthesis are not significantly differentially expressed between alone versus before-contact, when NcA1 encounters a young PcW, as in all other nonself interactions genes related to melanin synthesis are still significantly upregulated upon contact with the other fungus.

There were 2,102 gene and expression pattern pairs out of 2,814 genes from the alignment (Figure 6) unique to NcA1 when encountering old PcW. When these genes were grouped by expression pattern there were a total of 45 enriched FunCat terms for specific expression patterns (Table 5). Among the 45 FunCat terms 20 had a DUN expression pattern and 10 of those FunCat terms were related to “Metabolism,” one to “Fermentation,” six to “Cellular transport, transport facilitation, and transport routes,” two to “Cell rescue, virulence, and defense,” and one to “Homeostasis.” Of the six FunCat terms related to “Cellular transport, transport facilitation, and transport routes” two of the terms “non-vesicular cellular import “and “non-vesicular cellular import” were important because one of the hypothesized modes of growth inhibition by PAF is related to the increase in import of K^+ and Ca^{2+} in sensitive fungi (Marx et al. 2008). The FunCat term related to homeostasis, “homeostasis of metal ions Na K Ca etc” is important evidence that upon contact with PAF produced by old PcW, NcA1 is undergoing an upregulation of genes related to metal ion homeostasis to counter balance the influx of Ca^{2+} ions (Binder et al. 2010). We found in NcA1 versus young PcW the expression trend NNN

was significantly enriched for “non-vesicular cellular import” and is evidence that there was a significant amount of genes that showed no change in expression and indirectly no change in the amount of K^+ and Ca^{2+} being imported into mycelia, which corresponds to no PAF induced growth inhibition occurring.

There were 15 FunCat trends unique to NcA1 interacting with old PcW for the NDN expression pattern that included nine terms related to aerobic respiration: “Energy,” “Tricarboxylic acid pathway, citrate cycle, Krebs cycle, TCA cycle,” “Electron transport and membrane associated energy conservation,” “Accessory proteins of electron transport and membrane associated energy conservation,” “Respiration,” “Aerobic respiration,” “Transported compounds substrates,” “Electron transport,” and “Mitochondrion” (Table 5). The downregulation of genes related to respiration and mitochondrial genes after-contact with PAF secreted by old PcW could be a result of the degradation of mitochondria or because of another method of PAF growth inhibition that prevents mitochondria from functioning correctly. The downregulation of mitochondrial genes could be responsible for the significant enrichment of FunCat terms related to fermentation in genes with a DUN expression pattern in NcA1 interacting with old PcW.

Neurospora knockout candidates

We collected significantly upregulated or downregulated genes in NcA1 versus old PcW whose expression was significantly affected by PAF induced growth inhibition between before and after-contact (Table 6). We found 19 genes of interest involved in signaling, secondary metabolism, transcription, stability of the cell membrane, transport, and gene regulation using FunCat, the annotation information on the Broad *Neurospora* website (http://www.broadinstitute.org/annotation/genome/neurospora/MultiHome.html), matching *Neurospora* genes to annotated homologs using the *Saccharomyces* genome database and NCBI BLAST (Table 7)(Altschul et al. 1997). The 19 genes were divided into four groups, upregulated genes shared by all nonself interactions, upregulated genes shared by interactions between NcA1 and PcW, genes upregulated only in NcA1 when interacting with old PcW, and genes downregulated only in NcA1 when interacting with old PcW.

The first group included three genes; two of the genes were upregulated during NcA1 versus old PcW, young PcW, NcA2, and NcC. Both genes appear important to NcA1 during contact with a fungus of a different genotype. The genes were NCU04197, a homolog of the *Aspergillus* virulence factor CipC (Bauer et al. 2009) and NCU04415, a stress response and nuclear envelope protein. The third gene, NCU02175, was upregulated in NcA1 versus old PcW, young PcW, and Nd. NCU02175 is a phospholipase, important to cell signaling.

The second group contained five genes upregulated in interactions between NcA1 and PcW. Two of the genes encode cell wall proteins (NCU07569 and NCU07817), two genes encode cell membrane proteins (NCU05649 and NCU07938), and one an

endochitinase (NCU04554). All five genes had a DUN expression pattern in NcA1 when interacting with young and old PcW.

The third group contained seven genes that were only upregulated when NcA1 encountered old PcW and included a monooxygenase involved in detoxification (NCU00955), a zinc finger transcription factor (NCU01209), a cysteine protease involved in Ca^{2+} signaling (NCU03355), a multidrug resistance associated transporter (NCU04161), a cytoplasmic protein that binds to lipids (NCU07572), a protein with a PAS domain that may be involved in signaling (NCU06390), and a protein involved in the response to dsRNA (NCU07257). We found a significantly upregulated monoxygenase (NCU00955) in NcA1 possibly related to the increased presence of ROS and an attempt at detoxification. The monoxygenase had an expression trend of DUN, which was the same expression trend enriched for ROS metabolism and response to stress in NcA1 when interacting with old PcW. The upregulated zinc finger transcription factor had a DUN expression trend and could be important in the regulation of the other genes that share the same expression pattern. The last gene in this group NCU07257, is an F box domain protein that appears to be involved in the response to double stranded RNA and is a stress response protein. The protein could be a response to dsRNA resulting from the induction of apoptosis in mycelia as a result of cell death/growth inhibition occurring in NcA1 because of PAF.

The last group of contained four genes that were significantly downregulated between before-contact compared to after-contact only in NcA1 versus old PcW. The genes included a RNA II transcriptional regulator (NCU05944), a protein with a helicase domain (NCU06306), an integral membrane protein involved in signal transduction (NCU06839) and a protein involved in hyphae formation (NCU08038). The putative helicase (NCU06306) could be important because its downregulation may signal that DNA is not being modified for transcription. The significant downregulation of NCU05944, is evidence for the downregulation of transcription because the RNA II transcriptional regulator is important to the formation of a regulation mediator complex that initializes transcription. NCU06839 is a protease and an integral membrane protein involved in signal transduction and its downregulation could result in the downregulation of a signaling pathway related to growth. All three proteins, thus far mentioned, had the same expression trend, NDN, and appear to play a role in growth inhibition. NCU08038 is a homolog to gEgh 16, a protein first described in *Erysiphe graminis* (Justesen et al. 1996), and was significantly upregulated between alone versus before-contact but then downregulated in NcA1 versus old PcW in before versus after-contact (UDN), but was upregulated between alone versus before-contact and then displayed no change (UNN) or no change at all in expression in the other interactions (NNN). In obligate fungal plant pathogens gEgh 16 homologs are significantly upregulated in appresoria during infection and is considered a virulence factor (Xue et al. 2002). In FunCat, NCU08038 is grouped with genes important to “hyphae formation” in *Neurospora* and its downregulation during contact with old PcW could be playing a role in the growth inhibition caused by PAF.

Discussion

We investigated gene expression in *N. crassa* represented by NcA1 during contact with a one day old *P. chrysogenum* colony that does not inhibit growth and a three day old *P. chrysogenum* colony that produces PAF in high enough concentrations to inhibit growth in NcA1. As initially expected, fewer significantly differentially expressed genes were present in NcA1 versus young PcW compared to NcA1 versus old PcW. In our study we determined gene expression changes resulting from an encounter with any fungus, from encountering nonself, and from encountering PAF producing PcW.

Trend of downregulation between alone versus before-contact

Both interactions between NcA1 and PcW shared a trend of downregulation relative to NcA1 growing alone, but NcA1 interacting with old PcW (1,171 genes) had many more downregulated genes than NcA1 interacting with young PcW (201 genes). The majority downregulated genes in NcA1 versus young PcW overlapped with NcA1 versus old PcW, while most of the downregulated genes in NcA1 versus old PcW were unique to the interaction (Figure 8). The shift in transcription occurred before hyphal contact and NcA1 must be reacting to changes in the environment caused by another fungus being present, similar to what we observed in all *Neurospora* interactions in Chapter 2. The 190 downregulated genes shared between both NcA1 and PcW interactions were enriched in FunCat for “C-compound and carbohydrate metabolism,” “Polysaccharide metabolism,” “Extracellular protein degradation,” and “ABC transporters.” There were few genes unique to NcA1 versus young PcW with no enrichment, but NcA1 versus old PcW had 25 FunCat terms related to “Metabolism”. As in the interactions between *Neurospora* the downregulation of metabolism, to varying degrees, is an important response to growing with another fungus. It appears that NcA1 does detect the presence of young PcW, hence the overlap of several genes with old PcW. The interaction between NcA1 and young PcW lacks the large trend in gene downregulation as seen in NcA1 versus old PcW and all four *Neurospora* interactions, because young PcW does not elicit a big response, possibly because it is a distant relative and is not yet producing PAF.

The highest levels of gene upregulation occurred between before versus after-contact

Upon contact between NcA1 with young PcW and old PcW we saw an upregulation of genes occurring; this contrasts with the comparison of alone versus before-contact. NcA1 interacting with young PcW had 168 upregulated genes compared to 5 downregulated genes while NcA1 versus old PcW had 814 upregulated genes in comparison to 110 downregulated genes. NcA1 versus young PcW shared a majority of its significantly upregulated genes (155 genes) with NcA1 versus old PcW, but had five times less genes being upregulated. The shared genes were significantly enriched for six FunCat terms related to melanin synthesis. Upregulation of genes related to melanin synthesis are a result of NcA1 coming into contact with young PcW or PAF produced by old PcW. The 155 genes shared between both interactions with PcW are in response to encountering another fungus independent of PAF inhibition and is similar to the

upregulation of melanin synthesis genes observed in nonself interactions between *Neurospora*. As in downregulated genes during alone versus before-contact there was a large group of genes uniquely upregulated in NcA1 interacting with old PcW (659 genes) in comparison to the interaction with young PcW. The upregulation in several of these genes is likely related to NcA1 growth being inhibited by PAF. During before versus after-contact in the *Neurospora* interactions the highest amount of gene upregulation occurred in the *Neurospora* interpopulation interaction (NcA1 versus NcC, 459 genes), which was half as many genes as were significantly upregulated in NcA1 interacting with old PcW.

Neurospora growing alone versus after-contact showed little upregulation
The most important observation about alone versus before-contact is that there are no upregulated genes and only a few downregulated genes in NcA1 when interacting with young PcW (6 genes) and old PcW (2 genes). Comparisons of gene expression between alone versus after-contact displayed that gene expression is dynamic between NcA1 growing alone, before-contact, and after-contact. Between alone versus before-contact genes are downregulated, but several of those genes are then upregulated between before versus after-contact, thus causing little change in expression between alone versus after. The results were similar to what we saw in the interactions between NcA1 versus all *Neurospora*, except there was more down regulation occurring between alone versus after in the *Neurospora* interactions. The difference in downregulation could be because the *Neurospora* interactions are intragenus while the interactions between NcA1 and PcW are with fungi from a distant order.

NcA1 versus PcWy has fewer genes enriched for down regulation of metabolism
There were 372 downregulated genes in common between all four *Neurospora* interactions in NcA1 growing alone versus before-contact. The genes are downregulated in all *Neurospora* interactions and we decided to test how many of those genes were shared with NcA1 interacting with young and old PcW. We found 173 of the genes were shared between all six interactions, but were only enriched for two FunCat terms “Extracellular protein degradation” and “ABC transporters”. The 173 genes are not only downregulated when NcA1 comes into contact with other *Neurospora*, but distantly related Eurotiomycetes as well. NcA1 interacting with old PcW and all four *Neurospora* shared 184 genes enriched for 35 FunCat terms and more than half were related to the downregulation of metabolism (17 FunCat terms). There was more enrichment for metabolism and a stronger response in NcA1 when encountering old PcW and in all the *Neurospora* interactions in comparison to NcA1 encountering young PcW. Of the 72 downregulated genes in NcA1 versus non-NcA1 *Neurospora,* 61 of the downregulated genes were shared with NcA1 when interacting with old PcW and were enriched for “Alcohol fermentation.”

Melanin synthesis genes upregulated in interactions between different genotypes
There were 33 upregulated genes shared by all *Neurospora* interactions and nonself *Neurospora* interactions shared 67 upregulated genes when expression was compared

between before versus after-contact. The interactions between NcA1 and PcW shared 10 upregulated genes with the four *Neurospora* interactions, but they were not enriched for any FunCat terms. There were 18 upregulated genes shared between interactions with NcA1 and old PcW that were shared with all *Neurospora* enriched for “alcohol fermentation” which was interesting because “alcohol fermentation” was downregulated in alone versus before for the same five interactions. It appears that the decrease in alcohol fermentation before-contact could be related to the decrease in metabolism, but the increase after-contact may be because growth decreases, resulting in a decrease in respiration.

When we compared upregulated genes in interactions between nonself *Neurospora* interactions and interactions between NcA1 and PcW we found that all five interactions between NcA1 and nonself fungi shared 29 upregulated genes that were enriched for seven FunCat terms. More importantly six of the seven FunCat terms were related to melanin synthesis. We found evidence that when *Neurospora* encounters fungi of a different genotype such as closely related *Neurospora* and distantly related *Penicillium* that a common response is the upregulation of genes involved in melanin synthesis. The seventh FunCat term we found enriched and related to fungi of different genotypes coming into contact was “ABC transporters”. ABC transporters could play a role in the export or import of solutes, such as secondary metabolites or metal ions, which could be secreted into the media by other fungi during interactions.

Genes shared between nonself *Neurospora* interactions in Chapter 2 were enriched for FunCat terms related to ROS metabolism, but within the 29 genes shared by all five interactions there was no enrichment for FunCat terms related to ROS metabolism. When we looked at enriched FunCat terms for upregulated genes unique to NcA1 interacting with young PcW we found enrichment for “oxygen and radical detoxification” and in NcA1 interacting with old PcW we found enrichment for “detoxification by modification”. NcA1 interacting with PcW and nonself *Neurospora* may experience different levels and types of ROS metabolism since NcA1 when encountering old PcW is being exposed to PAF while NcA1 encountering young PcW does not stop growing upon contact, which are both different from NcA1 encountering nonself *Neurospora* where hyphal fusion and heterokaryon incompatibility is very likely occurring (Table 1).

There were 20 genes shared between NcA1 interacting with old PcW and nonself *Neurospora* interactions that were enriched for 7 FunCat terms, 4 were related to carbon metabolism, 2 to energy, and 1 to “disease virulence and defense.” The FunCat terms related to energy and metabolism were not shared with NcA1 interacting with young PcW because the interaction did not have as large global downregulation followed by a upregulation of a subset of metabolism and energy genes.

Analysis of Expression patterns found differences in gene regulation of melanin synthesis and evidence of ROS

When we characterized groups of genes based on their expression patterns from the alignment (Figure 6) we found 17 FunCat terms enriched for groups of genes with a specific expression that were shared with nonself *Neurospora* interactions (Supplemental Table 4). When we compared the 17 FunCat terms to the 83 significantly enriched FunCat terms with different expression patterns in NcA1 interacting with old PcW we found an overlap of nine FunCat terms that had a DUN expression pattern. Five of the nine enriched terms were related to melanin synthesis. There was no overlap of FunCat terms with a DUN expression pattern related to melanin synthesis that was shared by interactions between NcA1 growing with young PcW, old PcW and nonself *Neurospora*. This was surprising because we had found enrichment for melanin synthesis in our comparison of upregulated genes between before versus after-contact in all five interactions.

The reason we found no enrichment for FunCat trends related to melanin synthesis in genes with a DUN expression pattern in NcA1 interacting with young PcW is because the interaction does not have a large amount of gene downregulation occurring between alone versus before-contact. As a result many of the FunCat terms enriched in genes with a DUN expression pattern instead were enriched in gene with that were NUN (Figure 7, 8). In fact when we compared the 46 enriched FunCat terms with a DUN in NcA1 interacting with old PcW and the 31 enriched FunCat terms with an NUN in NcA1 interacting with young PcW we found that 17 of the 31 (55%) FunCat terms overlapped. Five of those 17 FunCat terms were the same as the terms we found related to melanin synthesis that had a DUN pattern in NcA1 during interactions with old PcW and nonself *Neurospora*. While genes related to melanin synthesis are upregulated in NcA1 when it comes into contact with PcW and nonself *Neurospora*, the genes in NcA1 versus young PcW related to melanin synthesis have a different pattern of expression, NUN, than the other interactions.

When we searched for potential FunCat terms related to ROS metabolism we found one FunCat term, “Detoxification,” between NcA1 interacting with old PcW and the nonself *Neurospora* interactions. Separately in FunCat terms unique to NcA1 encountering old PcW we found genes with DUN significantly enriched for “detoxification by modification,” which is related to ROS metabolism. In the interaction between NcA1 and young PcW we found genes with a NUN expression pattern enriched for “oxygen and radical detoxification” related to ROS metabolism just as we had with melanin synthesis genes. While there might be an increase in ROS metabolism when NcA1 encounters PcW there does not appear to be as large of an increase in gene expression related to ROS metabolism as when NcA1 encounters a *Neurospora* of different genotype.

Growth inhibition by PAF leads to upregulation of cellular import and a downregulation of respiration

Of the 2,814 (Figure 6) genes in our alignment those that shared the same expression pattern between NcA1 interacting with old PcW with any of the other interactions were removed so that we were left with 2,102 (814 differentially expressed, 1,288 NNN)

genes with an expression pattern unique to NcA1 when encountering old PcW that were enriched for 45 FunCat terms (Table 5).

Of the 15 enriched FunCat terms with a DUN expression pattern in NcA1 interacting with old PcW (Table 5) we found “Metabolism,” “Secondary metabolism,” and “Metabolism of phenylpropanoids.” There were still genes present that were related to secondary metabolism in our interaction even after the exclusion of shared genes which could mean that during encounters between NcA1 and old PcW there may be more genes related to melanin synthesis upregulated than in the other interactions or another secondary metabolic process is being upregulated. Two FunCat terms were significantly enriched for “Cellular import” and “Non vesicular cellular import,” which is important because the enrichment corresponds to previous research on growth inhibition caused by PAF where researchers found an increase in the import of K^+ and Ca^{2+}, which led to a hyperpolarization of membranes at the hyphal tip exposed to PAF (Binder et al. 2010; Marx et al. 2008). Finding genes related to non vesicular cellular import is relevant because there is evidence that PAF is imported into the cell through a non endocytosis mediated mechanism (Marx et al. 2008). Additionally in interactions between NcA1 and old PcW we also found enrichment for “homeostasis of metal ions Na K Ca etc,” which we think is related to the increase in metal ions and results in genes being upregulated to counterbalance the increase in Ca^{2+} in NcA1 (Binder et al. 2010).

In the NDN expression pattern where genes are downregulated in NcA1 between before and after-contact, there were nine enriched FunCat terms related to aerobic respiration and mitochondria: “Energy,” “Tricarboxylic acid pathway, citrate cycle, Krebs cycle, TCA cycle,” “Electron transport and membrane associated energy conservation,” “Accessory proteins of electron transport and membrane associated energy conservation,” “Respiration,” “Aerobic respiration,” “Transported compounds substrates,” “Electron transport,” and “Mitochondrion” (Table 5). In relation the DUN expression pattern was enriched for “heterofermentative pathway and fermentation of other saccharides,” which inversely was upregulated between before and after-contact. Upon contact with PAF we found there was downregulation of genes related to respiration and mitochondria creating a need for an upregulation of genes related to other energy pathways such as fermentation. We do not know the exact reason for a downregulation in respiration and further investigation is needed to determine how PAF is preventing respiration, which could be occurring because of the change in Ca^{2+} concentration in the cytoplasm or through disruption of a signaling pathway (Binder et al. 2010; Marx et al. 2008). The downregulation of respiration and mitochondrial genes to the extent we saw in NcA1 when interacting with old PcW appears to be specific to this interaction and is likely playing a role in the slow down of metabolism and the inhibition of growth in NcA1 when exposed to PAF.

Gene candidates for future studies to characterize PAF growth inhibition
An important purpose of our study was to find significantly differentially expressed genes in the interaction between NcA1 and old PcW between before versus after-

contact when the effects of PAF are visible. We wanted to find gene that when deleted or over expressed in mutants could have an observable phenotypic difference from wild type NcA1, such as no growth inhibition when exposed to PAF, a larger amount of growth inhibition when exposed to PAF, or significantly different expression profiles that could be studied in further detail to provide a better characterization of growth inhibition by PAF.

We found 19 genes of interest in four categories: genes significantly upregulated in interactions between NcA1 with PcW and nonself *Neurospora*, genes significantly upregulated in interactions between NcA1 and PcW, and genes significantly upregulated and downregulated only in interactions between NcA1 and old PcW (Table 6).

Out of the 15 significantly upregulated genes in NcA1 when interacting with old PcW, five of the genes (NCU04415, NCU04554, NCU07569, NCU07938, NCU07572) had homologs in *S. cerevisiae* that when over expressed lead to a decrease in growth rate. The upregulation of these genes between before and after-contact could play a cumulative role in the inhibition of growth in NcA1 when exposed to PAF produced by old PcW. Two of the genes, one encoding an endochitinase and the other involved in the synthesis of O-glycosylated cell wall protein, are involved in cell wall construction and maintenance. A third protein NCU07817 is a non-anchored cell wall protein. The endochitinase was previously found upregulated in *Neurospora* during heterokaryon incompatibility and exposure to phytosphingosine, which both caused PCD (Hutchinson et al. 2009; Videira et al. 2009). The endochitinase could be playing a similar role when NcA1 encounters PAF and could be evidence for PCD (Kaiserer et al. 2003; Marx et al. 2008). In Hutchinson et al. 2009 NCU04554 appeared to play a role in cell death during heterokaryon incompatibility, but no difference was found in the amount of cell death that occurred when the gene was deleted. It would be interesting to test an NCU04554 over expression mutant for a decrease in growth rate as seen in the *S. cerevisiae* CTS2 over expression mutant (Table 7). PAF could be causing NcA1 to break down its own cell walls with an increase in endochitinase (NCU04554) expression and NcA1 could be responding through the increased production of other cell wall proteins such as ncw-3 (NCU07817) and the PLC1 (NCU07569) homolog to maintain cell wall stability at the expense of decreasing growth rate.

One of the five genes with an *S. cerevisiae* homolog was NCU07572 a lipid binding cytoplasmic protein with a Pleckstrin homology (PH) domain related to YHR131C in *S. cerevisiae*. NCU07572 could play a role in growth inhibition caused by old PcW because its homolog, YHR131C, in *S. cerevisiae* leads to cell cycle arrest and growth inhibition.

Three genes, NCU07938, NCU05649, and NCU04161 were involved with transport across the cell membrane and were upregulated between before versus after-contact in NcA1 when interacting with old PcW. NCU07938 is involved in dityrosine transport, and could be related to the increase in tyrosine metabolism important in the production of

melanin. There was FunCat enrichment for detoxification in NcA1 when interacting with old PcW and NcA1 could be oxidizing dityrosine as a method of detoxification. NCU05649 is a homolog to RTA1 in *S. cerevisiae*, a protein responsible for 7-aminocholesterol resistance and is involved in the transport of lipids across the cell membrane. The RTA1 homolog could be pumping out lipids produced by old PcW detrimental to NcA1 or lipid byproducts left over from PCD (Marx et al. 2008). NCU04161 is an ABC transporter that could be important to NcA1 when it encounters fungi of a different genotype such as PcW. It would be interesting to determine if the protein is transporting a secondary metabolite like PAF across the NcA1 cell membrane.

There were three genes upregulated (NCU02175, NCU03355, and NCU06390) and one downregulated (NCU06839) involved in cell signaling pathways. We found a phosphoinositide phospholipase C (NCU02175) involved in signaling pathways in a Ca^{2+} dependent manner (Finn et al. 2006) that was upregulated in NcA1 versus old PcW, young PcW, and Nd. The gene is involved in the response to nonself fungi and not specifically to NcA1 interacting with PcW. NCU02175 is important because it was upregulated in *Neurospora* during heterokaryon incompatibility resulting in PCD (Hutchinson et al. 2009) just as in fungi exposed to PAF (Marx et al. 2008). NCU03355, a calpain-5 family cysteine protease putatively involved in Ca^{2+} signaling was significantly upregulated in NcA1 when interacting with old PcW. The cysteine protease may be affected by the increase in cytoplasm Ca^{2+} found in *Neurospora* exposed to PAF (Binder et al. 2010). NCU02175 and NCU03355 are both involved in Ca^{2+} signaling and have the same DUN expression pattern in NcA1 when interacting with old PcW. Both genes would make good candidates for single and double knockout experiments to determine if they are involved in the same pathway and play a role in PAF growth inhibition.

NCU06839, an integral membrane protein belonging to the Rhomboid protein family and involved in signal transduction was downregulated in NcA1 versus old PcW. The protein could be at the start of a signaling transduction pathway and it would be interesting to see how over expression and deletion mutants play a role in PAF induced growth inhibition.

We found three regulatory genes of interest in NcA1 when interacting with old PcW, one that was significantly upregulated (NCU01209) and two that were significantly downregulated (NCU05944, NCU06306). The first gene NCU01209 is a zinc finger transcription factor that has not been studied (Colot et al. 2006; Tian et al. 2011). It would be interesting to see if the NcA1 NCU01209 knockout has the same growth inhibition phenotype as wild type NcA1 when grown with old PcW. RNAseq could be done on ΔNCU01209 strains to determine differences in gene expression between wild type and mutants. ChipSeq could be done with wild type NcA1 to find what sequences bind to the transcription factor, find new genes that are regulated by NCU01209, and determine if expression of these genes are affected ΔNCU01209. The other two regulatory genes were, a homolog to MED7 (NCU05944) involved in a transcription

regulatory complex and a helicase (NCU06306) with an SNF2 domain that could be involved in chromatin conformational changes or transcriptional regulation, were significantly downregulated in NcA1 during PAF growth inhibition.

We found two genes considered virulence factors in pathogenic fungi that played an important role in NcA1 versus old PcW. The first gene NCU04197 is a homolog to CipC in *A. nidulans* and was upregulated when NcA1 came into contact with PcW and nonself *Neurospora*. The gene was found previously upregulated in fungi when exposed to antimicrobials and is upregulated during infection in pathogenic fungi (Bauer et al. 2009; Shimizu et al. 2009; Steen et al. 2003). NCU04197 could be important to *Neurospora* and other fungi when interacting with other organisms such as a host, during intragenus interactions between *Neurospora*, or during interactions with a fungus like *Penicillium* that releases an assortment of antimicrobial products. CipC appears to be a stress response protein important to interactions between fungi and other organisms that behaves as a virulence factor in some fungi. CipC would be interesting to further study and will help better understand how all fungi interact with the world around them. The second virulence gene, NCU08038 was significantly downregulated when NcA1 came into contact with old PcW, but was upregulated in the other interactions where growth was not being inhibited by PAF. Its homolog, gEgh 16, is a virulence factor in plant pathogenic fungi and is highly upregulated in appresoria, but in *Neurospora* is an important gene in hyphae formation and growth. It appears that PAF is causing a downregulation of NCU08038, which plays a role in growth inhibition. Understanding how NCU08038 is affected by PAF, what genes it interacts with, and is co-regulated with in *Neurospora* could provide insight into other genes important to virulence in plant pathogens that are co regulated with gEgh 16 during the infection of plants. Just like CipC, gEgh 16 is another example of how the term virulence can be transient depending on the fungus and the type of interaction taking place.

The last gene NCU00955 is a monooxygense important to detoxification in NcA1 that was upregulated during interactions with old PcW. MIPS described the gene as an oxidoreductase involved in the reduction of O_2, which could be helpful during times of oxidative stress.

Interactions between NcA1 and PcW different from Neurospora interactions
The interactions between NcA1 and PcW were different from the interactions between *Neurospora* (Chapter 2) because NcA1 when grown with young PcW continued to grow after-contact was made with young PcW and because NcA1 when grown in old PcW had growth inhibited by PAF before mycelial contact was made with old PcW. Both interactions were different from the interactions between *Neurospora* where NcA1 kept growing until it came into contact with the mycelium of the other colonies, after which it ceased polar growth. The distribution of expression patterns in our bar graphs for NcA1 when interacting with young PcW and old PcW displayed a distribution that did not look very similar to each other or the *Neurospora* congeneric interactions (Figure 5). As displayed in the neighbor joining tree (Figure 7) we found that NcA1 interacting with

young PcW and the self-self *Neurospora* interaction shared the most genes with the same expression patterns in common while NcA1 interacting with old PcW and the interpopulation *Neurospora* interaction had the most in common. Taken as a whole the gene expression patterns in NcA1 interacting with PcW were very unique because NcA1 interacting with old PcW had the most unique genes that were significantly differentially expressed and NcA1 interacting with young PcW had the fewest, of which a significant portion were shared with the interaction with old PcW (Figure 6a, 8).

Future characterization of melanin production and ROS metabolism
In the interactions between NcA1 and young PcW and old PcW we found evidence for melanin synthesis as in interactions between NcA1 and *Neurospora* of a different genotype (Chapter 2). In Chapter 2 the interpopulation interaction between NcA1 and NcC showed production of melanin on L-DOPA plates (Chun and Madhani 2010). Our experimental protocol did not work well on L-DOPA plates as in Chapter 2 because the timing of the interactions was changed as NcA1 and PcW grew at a decreased growth rate, which appeared to slow the production of PAF in PcW. It would be interesting to modify L-DOPA plates to recreate growth inhibition conditions similar to those on Birds media or use another method of melanin detection to determine if NcA1 is creating melanin during interactions with PcW.

It would also be interesting to test production of ROS in wild type interactions and deletion mutant of the monoxygenase (NCU00955) to determine if the deletion has an effect on ROS by observing levels of superoxide and peroxide production with (http://www.fgsc.net/fgn37/munkres1.html) (Silar 2005). We could determine if there are increased levels of ROS in NcA1 when interacting with PcW that corresponds to the enriched FunCat terms related to ROS metabolism and melanin synthesis. There is evidence the increase in melanin synthesis and ROS metabolism are related to each other because during infection melanin synthesis is increased in pathogenic fungi to protect against host production of ROS (Casadevall et al. 2000; Langfelder et al. 2003).

Mutants that have a phenotype different from wild type NcA1 could be studied using the same RNAseq experimental protocols as our study in order to directly compare data between mutants and wild type, which would expand our dataset and allow for a higher degree of statistical power during future analyses.

Summary of Discussion
Research on anti fungal protein induced growth inhibition in susceptible fungi is not new and the effects of PAF on *Neurospora* Ca^{2+} homeostasis was studied by Binder et al. 2010. Researchers are still unsure of all the specific genes and pathways affected by anti fungal protein because most of the research has involved studying anti fungal protein production in Eurotiomycetes and how solutes like Ca^{2+}, Mg^{2+}, and K^{+} affect growth inhibition in susceptible fungi. Our study is the first to look at how PAF secretion in *P. chrysogenum* affects gene expression on a genome wide scale in the susceptible fungus, *N. crassa*. Both interactions between NcA1 and PcW displayed evidence of an

upregulation of genes related to melanin synthesis, but there was not a strong signal for ROS metabolism.

Our study has provided an expression baseline of how a fungus behaves when exposed to an anti fungal protein. Our observations, experimental model, and candidates genes of interest will be useful for future knockout and over expression experiments, which will lead to a better understanding of pathways involved in PAF growth inhibition and help find what part of the mycelium PAF initially interacts with. Understanding how *Neurospora* growth is inhibited by *Penicillium* is important because it will give us insight into how *Neurospora* interacts with other fungi in nature, will help us better understand what genes and pathways are important to mycelial growth in all fungi, and will be important in determining how to use the anti fungal properties of PAF as a tool to inhibit fungal growth in plants and animals, including humans.

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Fungus 1	Fungus 2[1]	Relation to NcA1	Result of Interaction[2]
NcA1 (Neurospora crassa subclade A 2489)	**young PcW** (*P. chrysogenum* Wisconsin)[3]	Different species, different class	Continuation of growth across plate even after mycelial contact.
NcA1	**old PcW** (*P. chrysogenum* Wisconsin)[4]	Different species, different class	Growth inhibition before contact because of antifungal peptide (PAF)
Interactions from Chapter 2			
NcA1*	**No Fungus**	-	Growth across culture plate.
NcA1*	**NcA1**	Same genotype	Interruption of growth upon mycelial contact.
NcA1*	**NcA2** (Neurospora crassa subclade A D115)	Different genotype, same population	Interruption of growth upon mycelial contact.
NcA1*	**NcC** (Neurospora crassa subclade C D107)	Different population, same species	Interruption of growth upon mycelial contact.
NcA1*	**Nd** (Neurospora discreta 8579)	Different species, same genus	Interruption of growth upon mycelial contact.

Table 1. Fungal interactions observed and relationships of fungi involved.
[1]All fungi are matA.
[2] Result of NcA1 growing alone or interaction between NcA1 and another fungus.
[3] A 24 hour old *Neurospora* colony no antifungal protein (PAF) secretion.
[4] A 72 hour old *P. chrysogenum* colony that secretes antifungal protein (PAF).
- Interactions from Chapter 2.

Interaction[1]	Library ID[2,3]	Raw Reads[4]	Accepted Reads[5]	Percent Mapped[6]
NcA1 vs. young-PcW-B	CV171	13,094,590	11,836,063	90%
NcA1 vs. young-PcW-B	CV176	27,712,583	25,745,083	93%
NcA1 vs. young-PcW-B	CV183	23,988,612	22,028,567	92%
NcA1 vs. young-PcW-A	CV207	16,773,249	14,016,517	84%
NcA1 vs. young-PcW-A	CV213	24,605,919	20,606,007	84%
NcA1 vs. young-PcW-A	CV221	25,596,147	23,084,349	90%

NcA1 vs. old-PcW-B	CV351	24,534,912	22,834,882	93%
NcA1 vs. old-PcW-B	CV356	29,341,628	26,204,108	89%
NcA1 vs. old-PcW-B	CV369	30,738,694	28,549,209	93%
NcA1 vs. old-PcW-A	CV285	33,804,424	31,287,063	93%
NcA1 vs. old-PcW-A	CV289	35,251,946	32,285,696	92%
NcA1 vs. old-PcW-A	CV295	31,138,427	28,913,829	93%

Table 2. RNAseq libraries sequenced and analyzed.
[1] Condition we sampled in each library, where "B" denotes before contact between two fungi, "A" denotes after contact between fungi, and the label "alone" refers to NcA1 growing alone.
[2] Sample identification.
[3] All RNAseq libraries are from NcA1.
[4] The number of 76 bp reads collected for each sample from one sequencing lane in the genome analyzer.
[5] The number of reads that mapped to the NcA1 genome using Tophat.
[6] The percentage of reads that mapped back to the NcA1 genome.

		Condition 1 Range of Median Difference		**Condition 2 Range of Median Difference**	
Condition 1[1]	**Condition 2[2]**	**Bioreplicates[3]**	**Complete[4]**	**Bioreplicates**	**Complete**
NcA1 vs. old-PcW-B	**NcA1 vs. young-PcW-A**	0.12	0.29	0.18	0.31
NcA1	**NcA1 vs. young-PcW-B**	0.20	0.38	0.12	0.25
NcA1	**NcA1 vs. young-PcW-A**	0.20	0.35	0.18	0.29
NcA1 vs. old-PcW-B	**NcA1 vs. old-PcW-A**	0.08	0.15	0.18	0.36
NcA1	**NcA1 vs. old-PcW-B**	0.20	0.38	0.08	0.15
NcA1	**NcA1 vs. old-PcW-A**	0.20	0.31	0.18	0.30

Table 3. Interquartile Range (IQR) of median differences calculated from median within conditions and among all conditions.
[1] The first condition in the comparison (Three bioreplicates).
[2] Second condition in the comparison (Three bioreplicates).

[3] The IQR calculated from the difference between log transformed normalized read counts (three each) and median of three log transformed normalized read counts from the same condition for each gene.
[4] The IQR calculated from the difference between log transformed normalized read counts (three each) and median of six log transformed normalized read counts from the same condition 1 and condition 2 for each gene.

Comparisons[1]		Significant (<0.05 adj. p-value)[2]		Significant (<0.05 adj. p-value and >1.5 fold)[3]		
Condition 1	**Condition 2**	**Up-regulated**	**Down-regulated**	**Up-regulated**	**Down-regulated**	**Total Genes[4]**
NcA1	**NcA1 vs. young-PcW-B**	10	201	10	201	9377
NcA1 vs. young-PcW-B	**NcA1 vs. young-PcW-A**	168	6	168	6	9235
NcA1	**NcA1 vs. young-PcW-A**	0	6	0	6	9375
NcA1	**NcA1 vs. old-PcW-B**	155	1324	106	1171	9377
NcA1 vs. old-PcW-B	**NcA1 vs. old-PcW-A**	941	146	814	111	9233
NcA1	**NcA1 vs. old-PcW-A**	0	2	0	2	9375

Table 4. Genes found significantly differentially expressed and greater than 1.5 fold.
[1] Two conditions compared to determine differential expression using DESeq and edgeR.
[2] Genes found significantly upregulated or downregulated (adjusted p-value <0.05) in DESeq or edgeR.
[3] Genes found significantly upregulated or downregulated (adjusted p-value <0.05) in DESeq or edgeR.
[4] Total amount of genes found expressed in among the six libraries in each comparison.

Expression Pattern[1]	FunCat Level[2]	FunCat term exclusive to NcA1 vs. old-PcW (Adjusted p-value < 0.05)[3]	P-Value[4]
DNN	LEVEL 4	01.01.06.04 metabolism of threonine	1.60E-02
DNN	LEVEL 5	01.01.06.04.02 degradation of threonine	5.01E-03
DNN	LEVEL 4	01.01.09.02 metabolism of serine	3.73E-03
DNN	LEVEL 5	01.01.09.02.01 biosynthesis of serine	5.01E-03
DNN	LEVEL 4	01.06.06.05 sesquiterpenes metabolism	3.73E-03
DUN	LEVEL 1	01 METABOLISM	6.10E-03

DUN	LEVEL 2	01.05 C compound and carbohydrate metabolism	8.43E-04
DUN	LEVEL 4	01.05.02.01 nucleotide sugar metabolism	3.50E-02
DUN	LEVEL 2	01.20 secondary metabolism	5.85E-03
DUN	LEVEL 4	01.20.35.01 metabolism of phenylpropanoids	3.50E-02
DUN	LEVEL 4	02.16.03.03 heterofermentative pathway and fermentaton of other saccharides	1.57E-02
DUN	LEVEL 6	20.01.01.01.01.01 siderophore iron transport	2.23E-02
DUN	LEVEL 3	20.01.03 C compound and carbohydrate transport	2.40E-03
DUN	LEVEL 4	20.01.03.01 sugar transport	1.62E-02
DUN	LEVEL 2	20.03 transport facilities	5.85E-03
DUN	LEVEL 3	20.09.18 cellular import	6.18E-04
DUN	LEVEL 4	20.09.18.07 non vesicular cellular import	1.82E-05
DUN	LEVEL 2	32.05 disease virulence and defense	2.98E-02
DUN	LEVEL 4	32.05.01.03 chemical agent resistance	3.50E-02
DUN	LEVEL 4	34.01.01.01 homeostasis of metal ions Na K Ca etc	3.50E-02
NDN	LEVEL 4	01.05.02.04 sugar glucoside polyol and carboxylate anabolism	1.18E-02
NDN	LEVEL 4	01.05.02.07 sugar glucoside polyol and carboxylate catabolism	4.36E-02
NDN	LEVEL 3	01.05.06 C 2 compound and organic acid metabolism	2.38E-02
NDN	LEVEL 4	01.05.06.07 C 2 compound and organic acid catabolism	1.18E-02
NDN	LEVEL 1	02 ENERGY	3.98E-02
NDN	LEVEL 2	02.10 tricarboxylic acid pathway citrate cycle Krebs cycle TCA cycle	1.77E-03
NDN	LEVEL 2	02.11 electron transport and membrane associated energy conservation	2.16E-03
NDN	LEVEL 3	02.11.05 accessory proteins of electron transport and membrane associated energy conservation	3.92E-04
NDN	LEVEL 2	02.13 respiration	4.67E-03
NDN	LEVEL 3	02.13.03 aerobic respiration	2.69E-02
NDN	LEVEL 3	16.21.08 Fe S binding	2.49E-03
NDN	LEVEL 2	20.01 transported compounds substrates	1.38E-02
NDN	LEVEL 3	20.01.15 electron transport	1.14E-02
NDN	LEVEL 2	42.16 mitochondrion	3.95E-02
NNN	LEVEL 1	01 METABOLISM	1.21E-03
NNN	LEVEL 2	01.05 C compound and carbohydrate metabolism	3.52E-02
NNN	LEVEL 6	01.05.11.07.01.03 meta cleavage	3.89E-02
NNN	LEVEL 2	01.06 lipid fatty acid and isoprenoid metabolism	3.52E-02
NNN	LEVEL 1	30 CELLULAR COMMUNICATION SIGNAL TRANSDUCTION MECHANISM	2.32E-02
NNN	LEVEL 1	40 CELL FATE	1.21E-03
NNN	LEVEL 2	40.01 cell growth morphogenesis	8.93E-03
NNN	LEVEL 2	42.01 cell wall	3.61E-02
NUN	LEVEL 4	34.11.03.07 pheromone response mating type determination sex specific proteins	3.85E-02
UDN	LEVEL 1	01 METABOLISM	1.00E-02
UDN	LEVEL 4	01.06.06.13 tetraterpenes carotinoids metabolism	2.03E-04

Table 5. FunCat terms specific to interaction between NcA1 and old PcW.
1Expression patterns with enriched FunCat term. Expression patterns, e.g., DUN where "D" refers to downregulation between before versus alone, "U" refers to upregulation between before versus after, and "N" refers to no change in gene expression between alone versus after.2
[2] FunCat hierarchy level. The higher the level the more specific the FunCat category, e.g. "01 - Metabolism" is level one and "01.20.35.01 metabolism of phenylpropanoids" is level 4.
[3] FunCat term significantly enriched (adjusted p-value<0.05) for genes with the shared expression pattern (Column 1) in NcA1 versus old PcW.
[4] Adjusted p-value of FunCat enrichment, the smaller the adjusted p-value the more significant.

Gene[1]	**Before vs. After** (Significantly upregulated or downregulated greater than 1.5 fold)					
	NcA1 vs. old-PcW[2]	**NcA1 vs. young-PcW**	**NcA1 vs. NcA1**	**NcA1 vs. NcA2**	**NcA1 vs. NcC**	**NcA1 vs. Nd**
NCU04197	**U[4]**	**U**	N	**U**	**U**	N
NCU04415	**U**	**U**	N	**U**	**U**	N
NCU02175	**U**	**U**	N	N	N	**U**
NCU04554	**U**	**U**	N	N	N	N
NCU05649	**U**	**U**	N	N	N	N
NCU07569	**U**	**U**	N	N	N	N
NCU07817	**U**	**U**	N	N	N	N
NCU07938	**U**	**U**	N	N	N	N
NCU00955	**U**	N	N	N	N	N
NCU01209	**U**	N	N	N	N	N
NCU03355	**U**	N	N	N	N	N
NCU04161	**U**	N	N	N	N	N
NCU07572	**U**	N	N	N	N	N
NCU06390	**U**	N	N	N	N	N
NCU07257	**U**	N	N	N	N	N
NCU05944	**D**	N	N	N	N	N
NCU06306	**D**	N	N	N	N	N
NCU06839	**D**	N	N	N	N	N
NCU08038	**D**	N	N	N	N	N

Table 6. Wild type expression between before-contact versus after-contact in knockout candidates.
[1] Candidate genes for future knockout experiments.

[2] Observed gene expression in two interactions between NcA1 and PcW and the four interactions between *Neurospora*.
[3] Genes were either significantly (adjusted p-value<0.05, >1.5 fold,) upregulated (U) or downregulated (D) or had no significant change in gene expression (N).

Gene[1]	Function[2]
NCU04197	CipC, Antibiotic response protein, protein related to pathogenesis
NCU04415	Putative stress response nuclear envelope protein. *S. cerevisiae* homolog, MSC1, when over expressed leads to a decrease in growth rate.
NCU02175	Phosphoinositide phospholipase C, important in lipid signaling pathways in a Ca^{2+} dependent manner.
NCU04554	Endochitinase. *S. cerevisiae* CTS2 homolog shows decreased growth when over expressed.
NCU05649	RTA1 domain-containing protein, membrane protein, involved in 7-aminocholesterol resistance in *S. cerevisiae* homolog, RTA1.
NCU07569	Homolog in *S. cerevisiae* an O-glycosylated protein required for cell wall stability; attached to the cell wall via beta-1,3-glucan. In *S. cerevisiae* homolog PLC1 there is a decrease in growth during over expression.
NCU07817	ncw-3, non-anchored cell wall protein-3, carbohydrate binding
NCU07938	Solute transporter, multidrug resistance transporter. *S. cerevisiae* homolog QDR1 when over expressed leads to decreased growth rate.
NCU00955	Monooxygenase involved in detoxification
NCU01209	Transcription factor, zinc finger Zn2Cys6, DNA-Binding
NCU03355	calpain-5, calpain family cysteine protease, possibly involved in Ca^{2+} signaling.
NCU04161	Multidrug resistance-associated protein 5, ABC transporter, *S. cerevisiae* homolog YOR1.
NCU07572	Contains a Pleckstrin homology (PH) domain. Related to YHR131C in *S. cerevisiae* that also has a PH domain, binds to lipids, and localizes to cytoplasm, which leads to the arrest of cell cycle when over expressed
NCU06390	PAS fold, PAS super family
NCU07257	F-box domain-containing protein, involved in response to double stranded RNA.
NCU05944	Homolog to *S. cerevisiae* protein MED7, a member of RNA polymerase II transcriptional regulation mediator complex, and involved in mRNA regulation/transcription synthesis regulation.
NCU06306	Helicase conserved C-terminal domain and SNF2 family N-terminal domain.
NCU06839	Rhomboid family protein, eukaryotic integral membrane protein domain, protease involved in signaling transduction
NCU08038	gEgh 16 homolog, involved in hyphae formation in *Neurospora*, important to virulence and appressoria formation in plant pathogens.

Table 7. Knockout Candidates and their putative functions.
[1] Knockout candidate genes.
[2] Gene annotation information collected from the Broad *Neurospora* website, NCBI Blast, Pfam, and the *Saccharomyces* genome database.

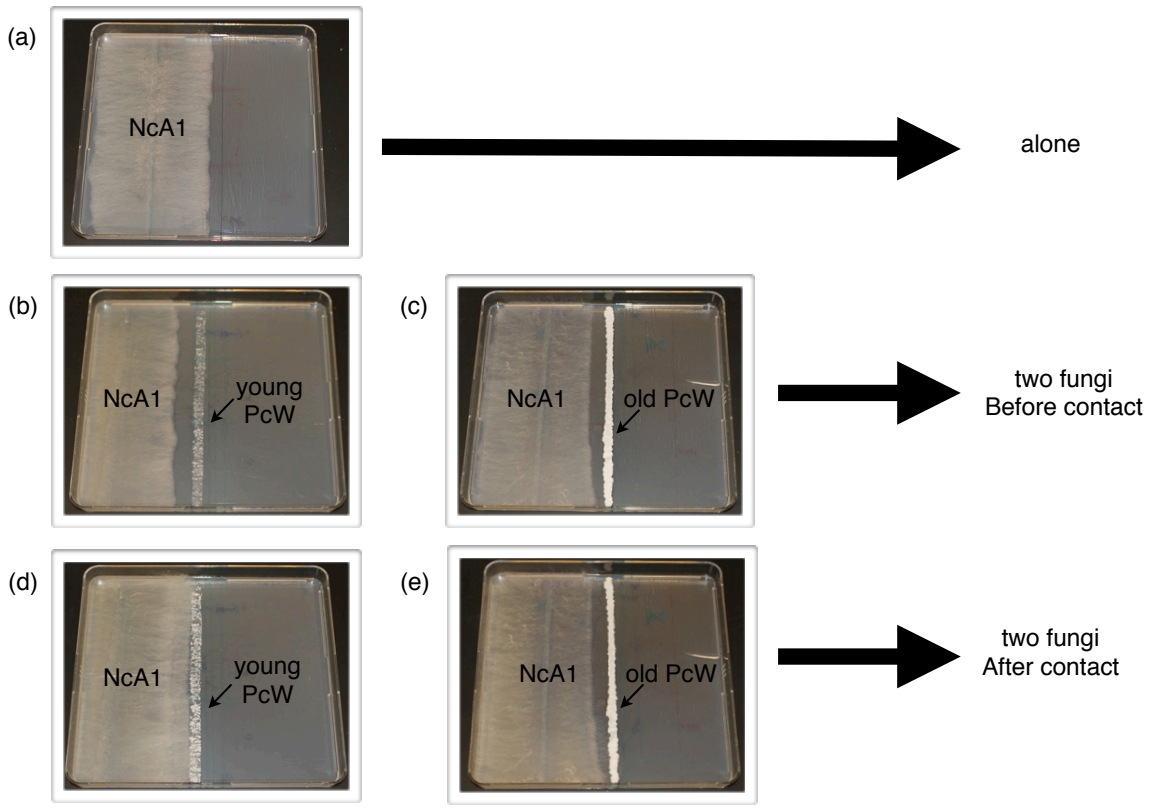

Figure 1. The three points where NcA1 mycelium was collected to gather expression data for our study of NcA1 interacting with young PcW and old PcW.
(a) The first collection point was NcA1 growing alone, the second point was during NcA1 growth with (b) young-PcW and (c) old-PcW, but before contact, and the last expression point was after mycelia contact between NcA1 with (d) young PcW and growth inhibition by (e) old PcW.

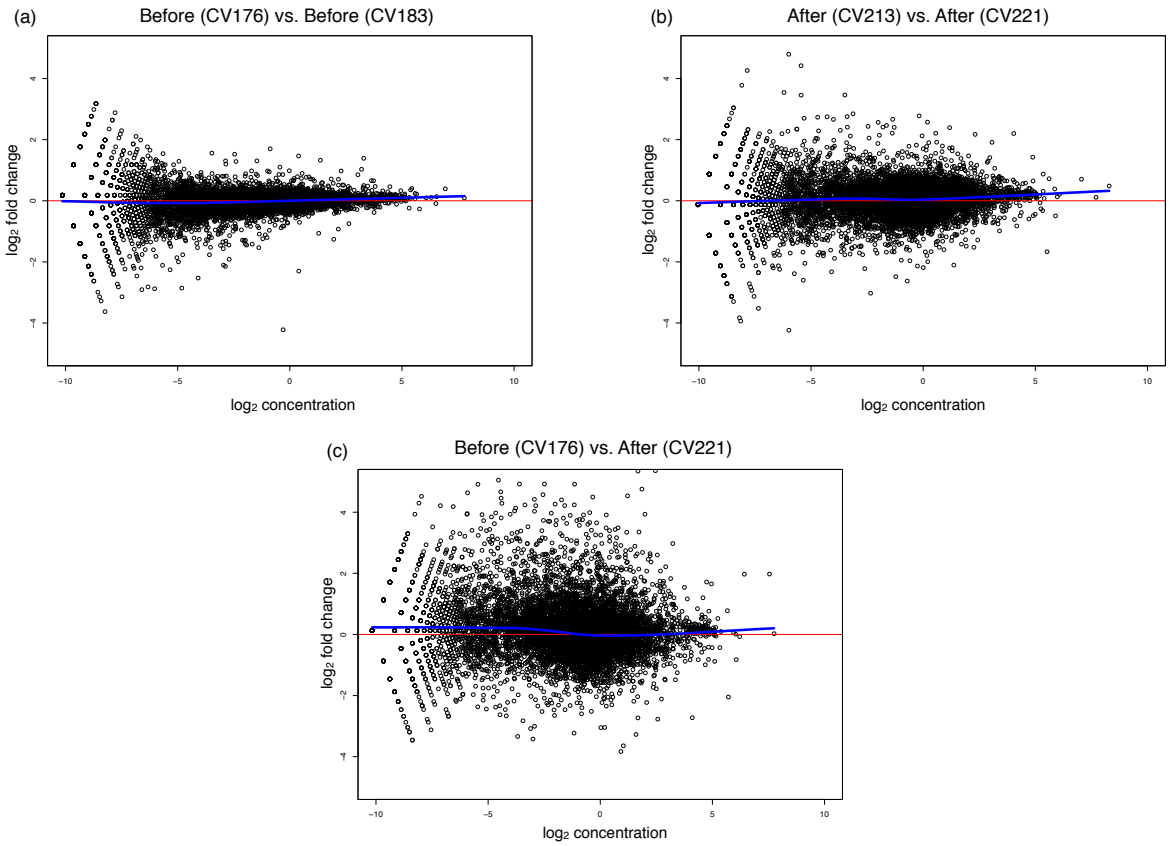

Figure 2. Pairwise comparison between bioreplicates within condition and between conditions with MA plots.
(a) MA plot of two before-contact bioreplicates, RNAseq libraries CV176 and CV183. (b) MA plot of two after-contact bioreplicates, RNAseq libraries CV213 and CV221. (c) A comparison between one before-contact (CV176) and one after-contact (CV221) RNAseq library, The red line demarcates the zero y-axis and the blue line is a Loess line fit to the data, that does not significantly deviate from the zero y-axis. Libraries were from NcA1 versus young PcW.

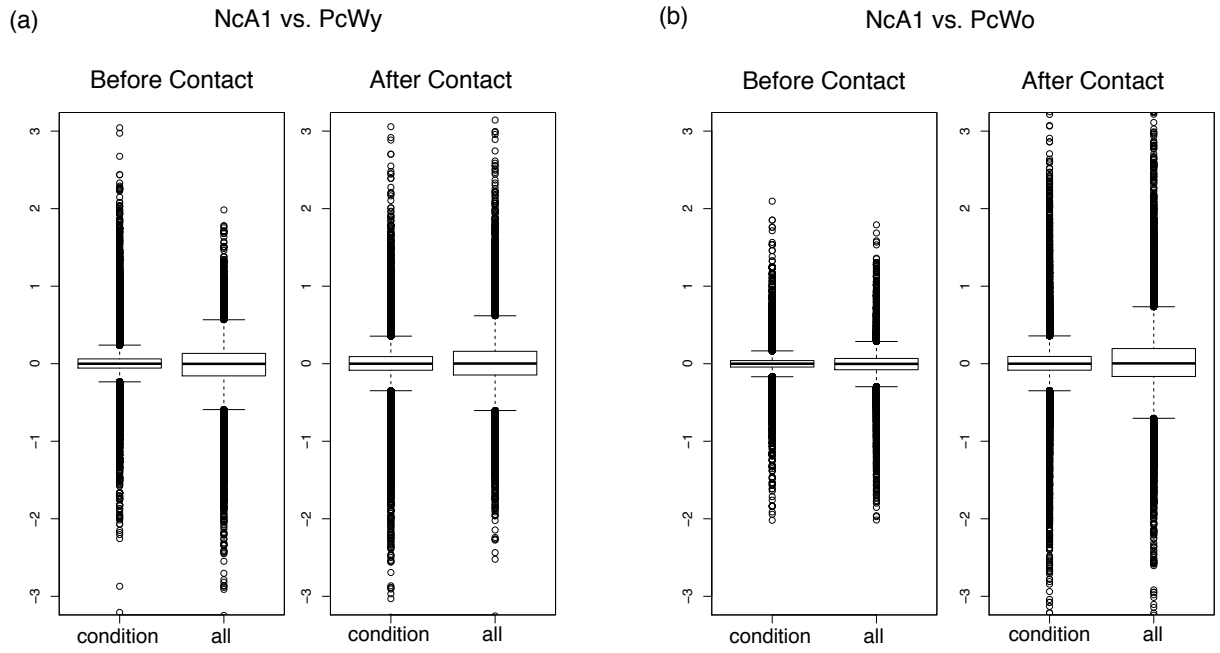

Figure 3. Box plots of log transformed median differences.
Box plots displaying the log transformed median differences for each gene calculated from the difference between the three log transformed read counts (before-contact and after-contact) with the log transformed median of the three “condition” bioreplicates (before-contact and after-contact) and “all” six libraries for the interactions of (a) NcA1 versus young PcW and (b) NcA1 versus old PcW. The box plots are composed of the median (center thick black line), the first and third quartile (bottom and top of the box), and the upper and lower whiskers (the paddles above and below the hinges separated by a dashed vertical line). The open circles above and below the whiskers are outliers that do not fall within the 95% confidence intervals of data.

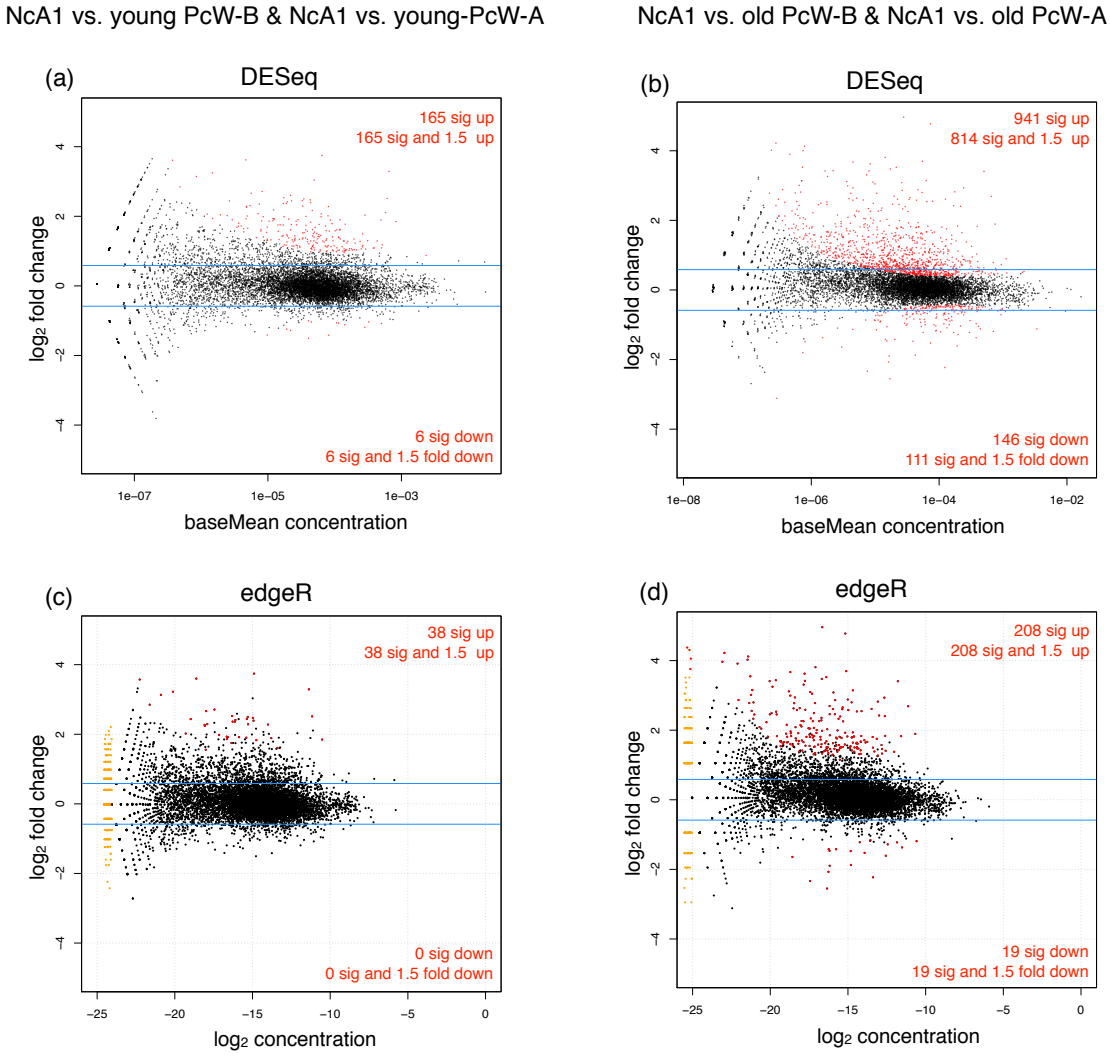

Figure 4. MA and Smear plots from DESeq and edgeR.
Plots graphing the mean expression for each gene, from before and after mycelia contact for NcA1 versus NcA2 on the x-axis and the $\log_2$ fold change in expression between before versus after mycelia contact for each gene. The light blue lines in both plots demarcate the boundary for 1.5 fold differential expression. Points colored in red were genes found significantly (adjusted p-value <0.05) differently expressed. Numbers at the top and bottom right of the plots are the total number of genes found significantly differentially expressed and greater than 1.5 fold. (a,c) The left plot from edgeR is called a “smear plot” and is analogous to MA plots. In the smear plots genes that have zero counts for each bioreplicate in at least one condition. (b,d) The MA plot to the right was made using DESeq and the x-axis displays the same information as (a,c) except instead of being displayed as $\log_2$ concentration DESeq displays it as the “baseMean”.

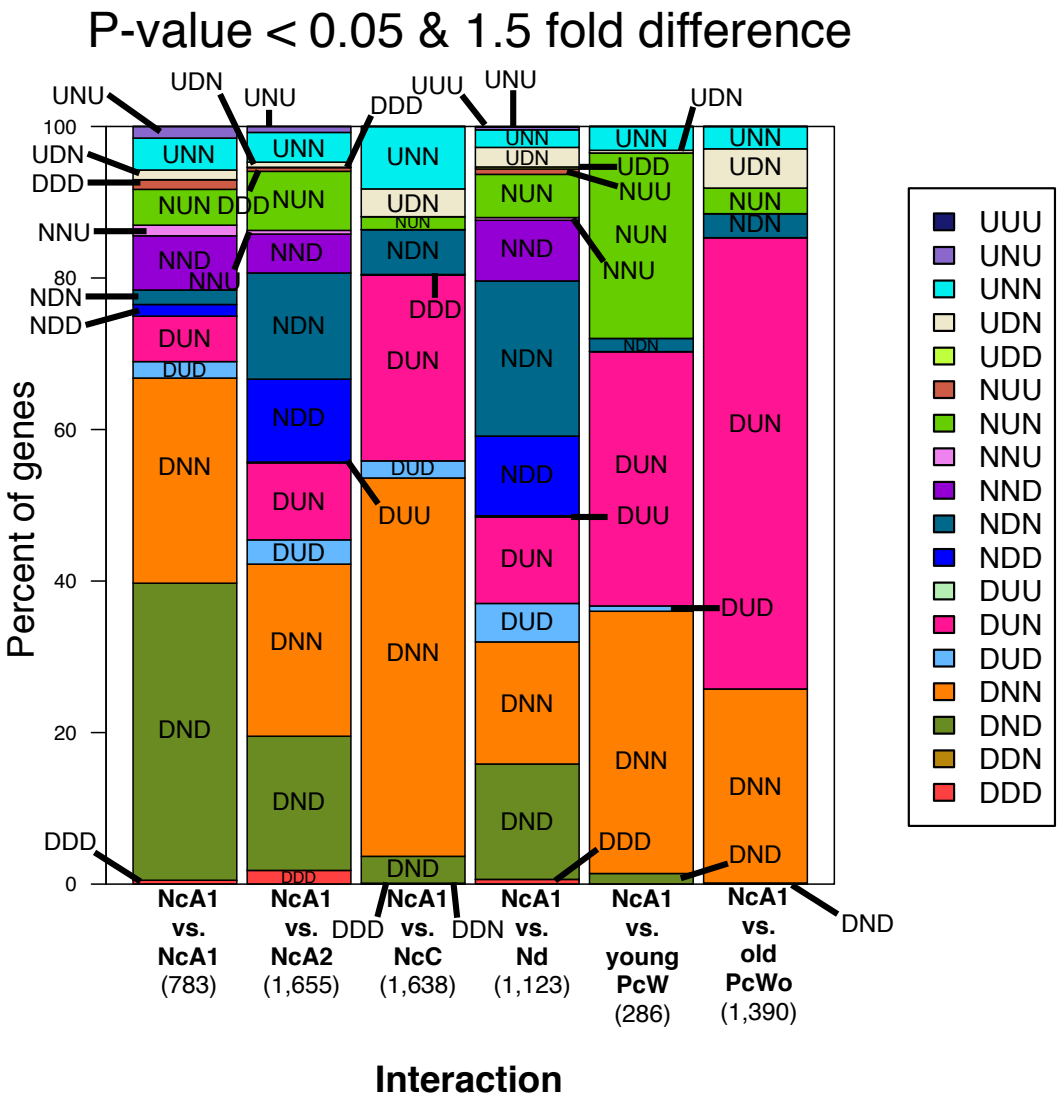

Figure 5. Bar charts of expression pattern proportions.
Bar charts displaying the genes that were found significantly expressed (adjusted p-value <0.05) and with a greater than 1.5 fold differential expression in the three comparisons for each interaction in DESeq and edgeR. Each bar chart has six vertical bars with labels of the interactions they represent on the x-axis and the total number of genes differentially expressed in parentheses. All six bars are split into subsections with different colors representing 1 of 18 expression patterns. The size of the subsection on the y-axis represents the percent of genes that fall into the expression pattern category for each interaction out of all the significant genes for each interaction. A key is present on the left of (a) and (b) that matches the color of each subsection to a specific expression trend where “U” signifies a gene is significantly upregulated, “D” signifies a

gene is significantly downregulated, and “N” signifies a gene was not significantly differentially expressed.

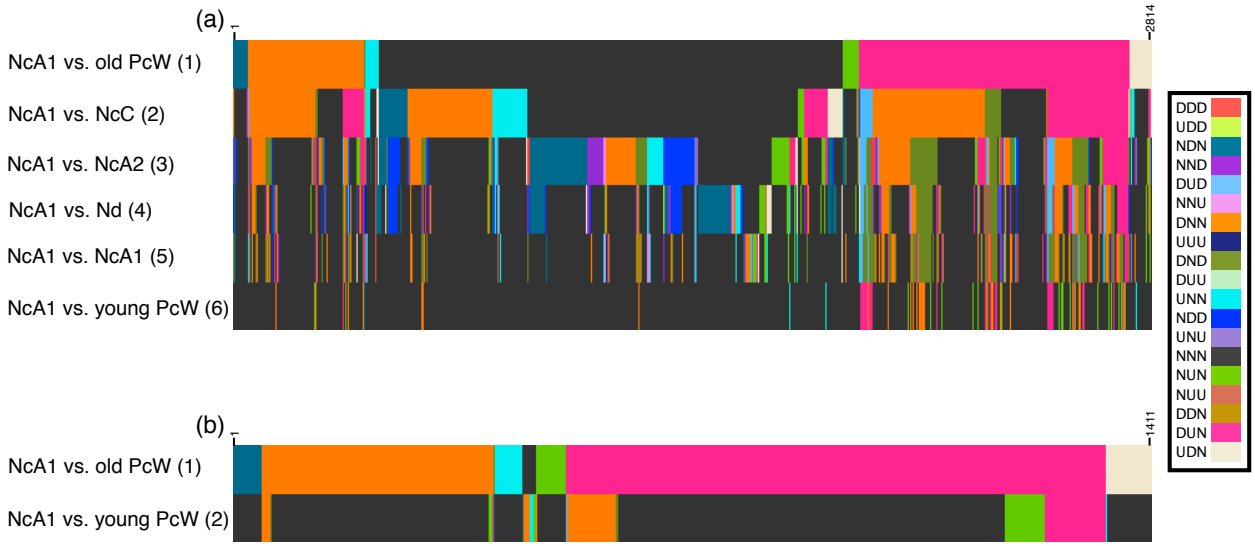

Figure 6. Six ordered expression profiles 2,814 genes that were significant differentially expressed.
Two ordered expression profiles (a) made from 2,814 genes found significantly differentially expressed more than 1.5 fold in at least one of six interactions and (b) a list of 1,411 genes found significantly differentially expressed more than 1.5 fold in the interaction between NcA1 versus young PcW or NcA1 versus old PcW. The colors in each expression profile represent different expression patterns for each gene and the key on the upper right hand side of the figure displays what expression pattern is denoted by each color. The letter “U” in the key represents genes that are significantly upregulated, the letter “D” in the key represents genes that are significantly downregulated, and the letter “N” represents genes that were found not be significantly differentially expressed. The alignment on the top (a) was hierarchically sorted starting with NcA1 interacting with old PcW (1) and following the order of the numbers in parentheses next to each interaction label on the left side of the alignments. The expression profiles were made in Jalview.

(a)

Distance Matrix

	NcA1 vs. NcA1	NcA1 vs. NcA2	NcA1 vs. NcC	NcA1 vs. Nd	NcA1 vs. young-PcW	NcA1 vs. old-PcW
NcA1 vs. NcA1	0.00	52.24	59.56	38.13	28.71	53.98
NcA1 vs. NcA2	52.24	0.00	70.75	52.84	58.71	72.17
NcA1 vs. NcC	59.56	70.75	0.00	64.11	40.19	62.19
NcA1 vs. Nd	38.13	52.84	64.11	0.00	55.76	53.55
NcA1 vs. young-PcW	28.71	58.71	40.19	55.76	0.00	46.30
NcA1 vs. old-PcW	53.98	72.17	62.19	53.55	46.30	0.00

(b)

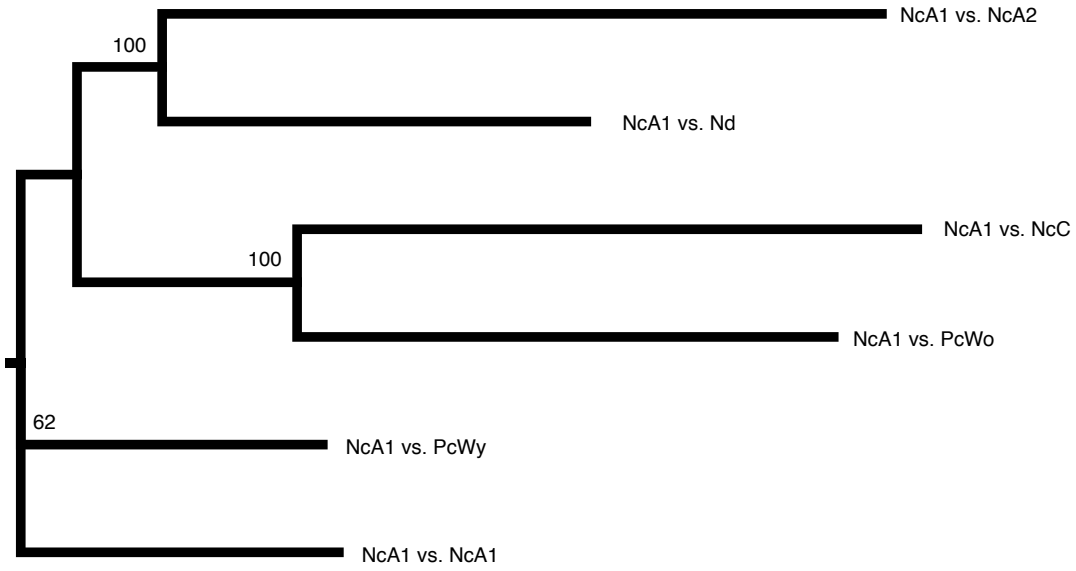

Figure 7. Distance matrix and neighbor joining tree of relationship between four expression pattern profiles.
(a) An uncorrected distance matrix compiled in Distmat from the expression pattern profile data of the six interactions in figure 6a. (b) A neighbor-joining tree produced in Phylip using the distance matrix data from (a). The tree was unrooted and the numbers present at the different nodes are the bootstrap support out of 100 percent for each clade.

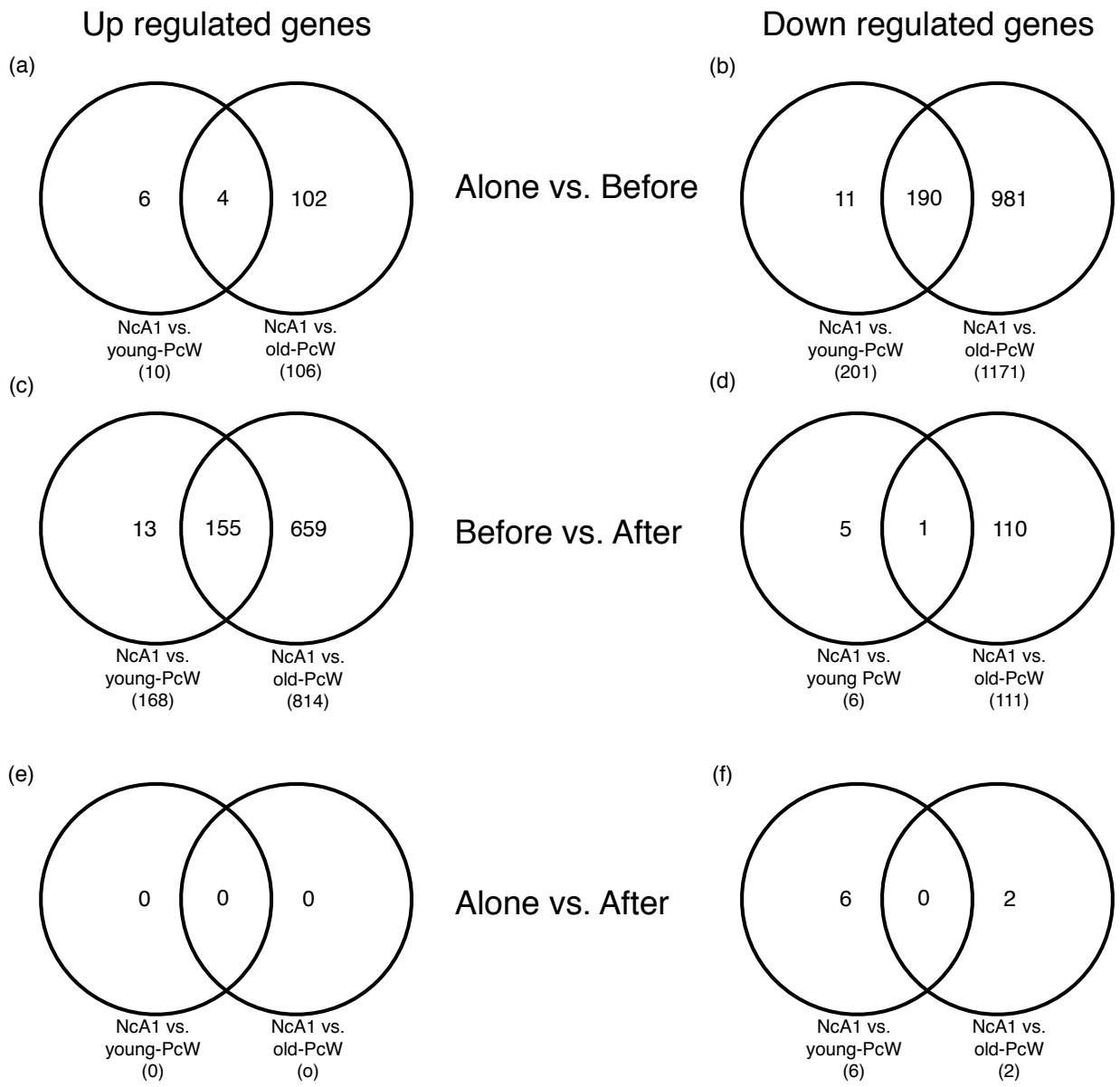

Figure 8. Comparisons of differential gene expression between NcA1 with young and old PcW.

Six two set Venn diagrams comparing the number of genes significantly upregulated or downregulated more than 1.5 fold during the three different comparisons made for NcA1 versus young-PcW and NcA1 versus old-PcW. The three comparisons included: (a-b) NcA1 growing alone compared to NcA1 growing with PcW before contact of mycelia, (c-d) NcA1 growing with PcW before mycelia contact compared to after mycelia contact, and (e-f) NcA1 growing alone compared to NcA1 after contact with PcW mycelia.

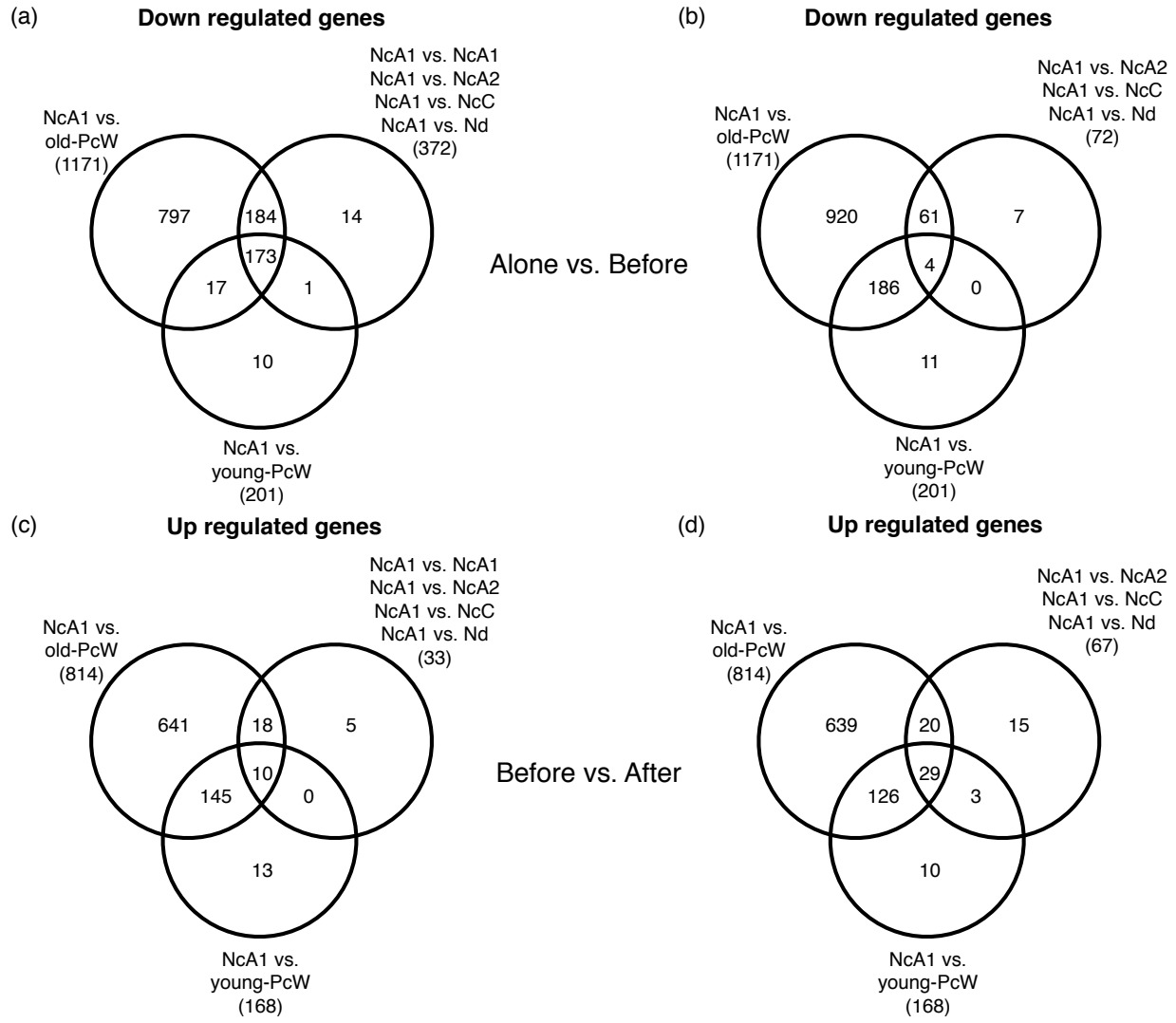

Figure 9. Comparisons of gene expression between NcA1 versus PcW interactions and interactions between *Neurospora*.
Three set Venn diagrams comparing significantly differentially expressed genes from NcA1 versus young-PcW and NcA1 versus old-PcW with genes shared between NcA1 versus all *Neurospora* (NcA1, NcA2, NcC, and Nd) and NcA1 versus nonself (NcA2, NcC, and Nd) *Neurospora*. (a-b)Significantly downregulated genes between alone versus before and (c-d) significantly upregulated genes between before versus after were compared between interactions.

Interactions	Comparison	Genes Up/Down Regulated	FunCat Level	FunCat Term	P-Value
NcA1 vs. old-PcW	Alone vs. Before	UP	LEVEL 1	01 METABOLISM	8.53E-04
NcA1 vs. old-PcW	Alone vs. Before	UP	LEVEL 3	01.01.11 metabolism of the pyruvate family alanine isoleucine leucine valine and D alanine	3.19E-02
NcA1 vs. old-PcW	Alone vs. Before	UP	LEVEL 4	01.01.11.02 metabolism of isoleucine	2.79E-02
NcA1 vs. old-PcW	Alone vs. Before	UP	LEVEL 5	01.01.11.02.01 biosynthesis of isoleucine	1.02E-02
NcA1 vs. old-PcW	Alone vs. Before	UP	LEVEL 4	01.01.11.03 metabolism of valine	2.79E-02
NcA1 vs. old-PcW	Alone vs. Before	UP	LEVEL 5	01.01.11.03.01 biosynthesis of valine	1.02E-02
NcA1 vs. old-PcW	Alone vs. Before	UP	LEVEL 4	01.01.11.04 metabolism of leucine	5.22E-04
NcA1 vs. old-PcW	Alone vs. Before	UP	LEVEL 5	01.01.11.04.01 biosynthesis of leucine	3.20E-05
NcA1 vs. old-PcW	Alone vs. Before	UP	LEVEL 5	01.01.11.04.02 degradation of leucine	1.02E-02
NcA1 vs. old-PcW	Alone vs. Before	UP	LEVEL 2	01.02 nitrogen sulfur and selenium metabolism	2.42E-02
NcA1 vs. old-PcW	Alone vs. Before	UP	LEVEL 3	01.02.02 nitrogen metabolism	4.21E-02
NcA1 vs. old-PcW	Alone vs. Before	UP	LEVEL 4	01.06.06.13 tetraterpenes carotinoids metabolism	1.50E-03
NcA1 vs. old-PcW	Alone vs. Before	UP	LEVEL 4	20.09.18.07 non vesicular cellular import	2.79E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 1	01 METABOLISM	1.35E-12
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 5	01.01.03.02.02 degradation of glutamate	8.77E-04
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 5	01.01.05.01.02 degradation of polyamines	1.45E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 5	01.01.06.04.02 degradation of threonine	3.13E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	01.01.09 metabolism of the cysteine aromatic group	1.16E-02

NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 5	01.01.09.02.02 degradation of serine	4.87E-03
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 4	01.01.09.04 metabolism of phenylalanine	3.03E-03
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 5	01.01.09.04.01 biosynthesis of phenylalanine	4.87E-03
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 5	01.01.09.04.02 degradation of phenylalanine	3.13E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 4	01.01.09.05 metabolism of tyrosine	3.19E-03
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 2	01.05 C compound and carbohydrate metabolism	7.71E-14
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	01.05.02 sugar glucoside polyol and carboxylate metabolism	4.82E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	01.05.06 C 2 compound and organic acid metabolism	4.39E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 4	01.05.06.07 C 2 compound and organic acid catabolism	4.21E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	01.05.11 aromate metabolism	3.62E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 4	01.05.11.07 aromate catabolism	4.21E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 5	01.05.11.07.01 aerobic aromate catabolism	2.20E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 2	01.06 lipid fatty acid and isoprenoid metabolism	2.57E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 2	01.20 secondary metabolism	3.38E-13
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	01.20.01 metabolism of primary metabolic sugar derivatives	6.22E-03
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 4	01.20.01.09 metabolism of aminoglycoside antibiotics	4.21E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	01.20.05 metabolism of acetic acid derivatives	3.07E-03
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	01.20.17 metabolism of secondary products derived from primary amino acids	6.04E-05
NcA1 vs. old-	Alone vs. Before	DOWN	LEVEL 4	01.20.17.09	5.13E-05

PcW				metabolism of alkaloids	
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	01.20.29 metabolism of secondary products derived from L glutamic acid L proline and L ornithine	1.68E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 2	02.16 fermentation	8.15E-06
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	02.16.01 alcohol fermentation	1.68E-03
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	02.16.03 lactate fermentation	2.00E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 4	02.16.03.03 heterofermentative pathway and fermentaton of other saccharides	4.21E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 2	16.21 complex cofactor cosubstrate vitamine binding	9.70E-04
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	16.21.07 NAD NADP binding	9.83E-06
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 6	20.01.01.01.01.01 siderophore iron transport	9.04E-04
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	20.01.03 C compound and carbohydrate transport	3.63E-03
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	20.01.23 allantoin and allantoate transport	5.64E-03
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	20.01.25 vitamine cofactor transport	1.16E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	20.09.18 cellular import	1.56E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 4	20.09.18.07 non vesicular cellular import	7.31E-05
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	32.01.01 oxidative stress response	1.16E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	32.01.04 pH stress response	1.68E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 2	32.05 disease virulence and defense	2.46E-03
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	32.05.03 defense related proteins	1.68E-03
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	32.05.05 virulence disease factors	2.43E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	32.07.03 detoxification by modification	1.68E-02

NcA1 vs. young-PcW NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 2	01.05 C compound and carbohydrate metabolism	3.28E-02
NcA1 vs. young-PcW NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	01.05.03 polysaccharide metabolism	3.61E-03
NcA1 vs. young-PcW NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	01.25.03 extracellular protein degradation	2.87E-02
NcA1 vs. young-PcW NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	20.03.25 ABC transporters	3.64E-02
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 1	01 METABOLISM	2.80E-04
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 2	01.05 C compound and carbohydrate metabolism	2.27E-08
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 2	01.20 secondary metabolism	1.62E-05
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 3	01.20.01 metabolism of primary metabolic sugar derivatives	9.29E-03
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 3	16.21.07 NAD NADP binding	2.36E-02
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 6	20.01.01.01.01.01 siderophore iron transport	1.52E-04
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 3	20.01.03 C compound and carbohydrate transport	1.59E-05
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 4	20.01.03.01 sugar transport	1.53E-02
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 3	20.01.23 allantoin and allantoate transport	2.36E-02
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 3	20.01.25 vitamine cofactor transport	3.56E-02
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 2	20.03 transport facilities	1.53E-02
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 3	20.09.18 cellular import	4.51E-05
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 4	20.09.18.07 non vesicular cellular import	1.43E-07
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 3	32.01.04 pH stress response	9.93E-03
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 2	32.05 disease virulence and defense	1.60E-05
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 3	32.05.03 defense related proteins	3.18E-02

NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 3	32.05.05 virulence disease factors	7.61E-03
NcA1 vs. young-PcW NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 1	01 METABOLISM	3.28E-02
NcA1 vs. young-PcW NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 4	01.01.09.04 metabolism of phenylalanine	4.76E-02
NcA1 vs. young-PcW NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 4	01.01.09.05 metabolism of tyrosine	1.94E-03
NcA1 vs. young-PcW NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 2	01.05 C compound and carbohydrate metabolism	3.55E-04
NcA1 vs. young-PcW NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 2	01.20 secondary metabolism	2.68E-03
NcA1 vs. young-PcW NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 3	01.20.17 metabolism of secondary products derived from primary amino acids	1.45E-02
NcA1 vs. young-PcW NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 4	01.20.17.09 metabolism of alkaloids	2.17E-02
NcA1 vs. young-PcW NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 4	01.20.35.01 metabolism of phenylpropanoids	4.76E-02
NcA1 vs. young-PcW NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 3	01.25.03 extracellular protein degradation	2.05E-02
NcA1 vs. young-PcW NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 1	02 ENERGY	3.28E-02
NcA1 vs. young-PcW NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 2	02.16 fermentation	1.19E-07
NcA1 vs. young-PcW NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 3	02.16.01 alcohol fermentation	1.45E-02
NcA1 vs. young-PcW NcA1 vs. old-	Before vs. After	UP	LEVEL 3	16.17.03 potassium binding	2.05E-02

PcW					
NcA1 vs. young-PcW NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 3	16.17.09 heavy metal binding Cu Fe Zn	3.69E-02
NcA1 vs. young-PcW NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 2	32.10 degradation modification of foreign exogenous compounds	3.47E-02
NcA1 vs. old-PcW	Before vs. After	DOWN	LEVEL 1	01 METABOLISM	1.06E-02
NcA1 vs. old-PcW	Before vs. After	DOWN	LEVEL 4	01.02.02.09 catabolism of nitrogenous compounds	4.01E-02
NcA1 vs. old-PcW	Before vs. After	DOWN	LEVEL 3	01.06.06 isoprenoid metabolism	4.89E-02
NcA1 vs. old-PcW	Before vs. After	DOWN	LEVEL 4	01.06.06.13 tetraterpenes carotinoids metabolism	3.37E-03
NcA1 vs. old-PcW	Before vs. After	DOWN	LEVEL 1	02 ENERGY	2.64E-02
NcA1 vs. old-PcW	Before vs. After	DOWN	LEVEL 2	02.10 tricarboxylic acid pathway citrate cycle Krebs cycle TCA cycle	2.46E-02
NcA1 vs. old-PcW	Before vs. After	DOWN	LEVEL 2	02.11 electron transport and membrane associated energy conservation	2.46E-02
NcA1 vs. old-PcW	Before vs. After	DOWN	LEVEL 3	02.11.05 accessory proteins of electron transport and membrane associated energy conservation	5.76E-03
NcA1 vs. old-PcW	Before vs. After	DOWN	LEVEL 3	16.21.05 FAD FMN binding	2.04E-02
NcA1 vs. old-PcW	Before vs. After	DOWN	LEVEL 3	16.21.08 Fe S binding	4.57E-03
NcA1 vs. old-PcW	Before vs. After	DOWN	LEVEL 3	20.01.15 electron transport	2.16E-02
NcA1 vs. old-PcW	Before vs. After	DOWN	LEVEL 3	32.07.01 detoxification involving cytochrome P450	4.89E-02

Supplemental Table 1. FunCat functional enrichment of shared and unique genes between NcA1 interacting with young and old PcW.
[1] Interaction with unique genes or interactions with shared genes.

[2] Comparisons observed: alone versus before, before versus after, alone versus after.
[3] Gene regulation being observed: upregulated or downregulated genes
[4] FunCat hierarchy level. The higher the level the more specific the FunCat category, e.g. “01 - Metabolism” is level one and “01.01.11.04.02 degradation of leucine” is level 5.
[5] FunCat term significantly enriched for genes shared between interactions or unique to interactions.
[6] Adjusted p-value of FunCat enrichment, the smaller the adjusted p-value the more significant.

Interactions[1]	Comparison[2]	Genes Up/Down Regulated[3]	FunCat Level[4]	FunCat Term[5]	P-Value[6]
NcA1 vs. old-PcW	Alone vs. Before	UP	LEVEL 1	01 METABOLISM	8.53E-04
NcA1 vs. old-PcW	Alone vs. Before	UP	LEVEL 3	01.01.11 metabolism of the pyruvate family alanine isoleucine leucine valine and D alanine	3.19E-02
NcA1 vs. old-PcW	Alone vs. Before	UP	LEVEL 4	01.01.11.02 metabolism of isoleucine	2.79E-02
NcA1 vs. old-PcW	Alone vs. Before	UP	LEVEL 5	01.01.11.02.01 biosynthesis of isoleucine	1.02E-02
NcA1 vs. old-PcW	Alone vs. Before	UP	LEVEL 4	01.01.11.03 metabolism of valine	2.79E-02
NcA1 vs. old-PcW	Alone vs. Before	UP	LEVEL 5	01.01.11.03.01 biosynthesis of valine	1.02E-02
NcA1 vs. old-PcW	Alone vs. Before	UP	LEVEL 4	01.01.11.04 metabolism of leucine	5.22E-04
NcA1 vs. old-PcW	Alone vs. Before	UP	LEVEL 5	01.01.11.04.01 biosynthesis of leucine	3.20E-05
NcA1 vs. old-PcW	Alone vs. Before	UP	LEVEL 5	01.01.11.04.02 degradation of leucine	1.02E-02
NcA1 vs. old-PcW	Alone vs. Before	UP	LEVEL 2	01.02 nitrogen sulfur and selenium metabolism	2.42E-02
NcA1 vs. old-PcW	Alone vs. Before	UP	LEVEL 3	01.02.02 nitrogen metabolism	4.21E-02
NcA1 vs. old-PcW	Alone vs. Before	UP	LEVEL 4	01.06.06.13 tetraterpenes carotinoids metabolism	1.50E-03

NcA1 vs. old-PcW	Alone vs. Before	UP	LEVEL 4	20.09.18.07 non vesicular cellular import	2.79E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 1	01 METABOLISM	4.86E-09
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 5	01.01.03.02.02 degradation of glutamate	4.72E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 5	01.01.06.04.02 degradation of threonine	3.99E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 5	01.01.09.02.02 degradation of serine	4.69E-03
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 2	01.05 C compound and carbohydrate metabolism	3.03E-09
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 2	01.20 secondary metabolism	4.64E-08
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	01.20.01 metabolism of primary metabolic sugar derivatives	2.79E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	01.20.05 metabolism of acetic acid derivatives	3.31E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	01.20.17 metabolism of secondary products derived from primary amino acids	1.87E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 4	01.20.17.09 metabolism of alkaloids	5.51E-04
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	02.16.01 alcohol fermentation	1.87E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 2	16.21 complex cofactor cosubstrate vitamine binding	3.55E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	16.21.07 NAD NADP binding	5.69E-03
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 6	20.01.01.01.01.01 siderophore iron transport	3.41E-04
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	20.01.03 C compound and carbohydrate transport	3.08E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	20.01.23 allantoin and allantoate transport	4.71E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	20.01.25 vitamine cofactor transport	3.08E-02

NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	20.09.18 cellular import	4.71E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 4	20.09.18.07 non vesicular cellular import	5.50E-04
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	32.05.05 virulence disease factors	4.90E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 3	01.06.06 isoprenoid metabolism	3.67E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Alone vs. Before	DOWN	LEVEL 4	01.06.06.13 tetraterpenes carotinoids metabolism	5.42E-05
NcA1 vs. young-PcW NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 2	01.05 C compound and carbohydrate metabolism	4.33E-02
NcA1 vs. young-PcW NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	01.05.03 polysaccharide metabolism	6.29E-04
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 1	01 METABOLISM	1.61E-03
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 5	01.01.03.02.02 degradation of glutamate	4.99E-03
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 5	01.01.05.01.02 degradation of polyamines	1.06E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	01.01.09 metabolism of the cysteine aromatic group	1.95E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd NcA1 vs. old-	Alone vs. Before	DOWN	LEVEL 4	01.01.09.04 metabolism of phenylalanine	1.39E-03

PcW					
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 5	01.01.09.04.01 biosynthesis of phenylalanine	4.99E-03
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 4	01.01.09.05 metabolism of tyrosine	1.39E-03
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 2	01.05 C compound and carbohydrate metabolism	1.38E-04
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	01.05.02 sugar glucoside polyol and carboxylate metabolism	3.34E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 4	01.05.02.07 sugar glucoside polyol and carboxylate catabolism	4.10E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	01.05.11 aromate metabolism	3.34E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 4	01.05.11.07 aromate catabolism	2.37E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 5	01.05.11.07.01 aerobic aromate catabolism	4.99E-03

NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 2	01.20 secondary metabolism	6.63E-05
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	01.20.17 metabolism of secondary products derived from primary amino acids	1.00E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	01.20.35 metabolism of secondary products derived from L phenylalanine and L tyrosine	4.42E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 4	01.20.35.01 metabolism of phenylpropanoids	4.10E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 1	02 ENERGY	6.10E-03
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 2	02.13 respiration	1.44E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	02.13.03 aerobic respiration	4.42E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 2	02.16 fermentation	2.65E-05
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd NcA1 vs. old-	Alone vs. Before	DOWN	LEVEL 3	02.16.03 lactate fermentation	1.00E-02

PcW					
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	16.17.03 potassium binding	1.88E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 2	16.21 complex cofactor cosubstrate vitamine binding	4.86E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	16.21.07 NAD NADP binding	1.00E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	20.01.15 electron transport	3.72E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	20.03.01 channel pore class transport	3.34E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 1	32 CELL RESCUE DEFENSE AND VIRULENCE	4.33E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	32.01.01 oxidative stress response	1.93E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	32.01.03 osmotic and salt stress response	1.88E-02

NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 2	32.05 disease virulence and defense	1.44E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	32.05.03 defense related proteins	1.88E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 2	32.07 detoxification	3.65E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	32.07.03 detoxification by modification	3.34E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	32.07.07 oxygen and radical detoxification	4.42E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd NcA1 vs. young-PcW NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	01.25.03 extracellular protein degradation	4.21E-02
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd NcA1 vs. young-PcW NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	20.03.25 ABC transporters	4.21E-02
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 1	01 METABOLISM	1.36E-11
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 5	01.01.03.02.02 degradation of glutamate	5.85E-04

NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 5	01.01.05.01.02 degradation of polyamines	1.16E-02
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 5	01.01.06.04.02 degradation of threonine	2.53E-02
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 3	01.01.09 metabolism of the cysteine aromatic group	8.98E-03
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 5	01.01.09.02.02 degradation of serine	3.49E-03
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 4	01.01.09.04 metabolism of phenylalanine	1.74E-03
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 5	01.01.09.04.01 biosynthesis of phenylalanine	3.49E-03
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 5	01.01.09.04.02 degradation of phenylalanine	2.53E-02
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 4	01.01.09.05 metabolism of tyrosine	1.84E-03
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 2	01.05 C compound and carbohydrate metabolism	3.52E-12
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 3	01.05.02 sugar glucoside polyol and carboxylate metabolism	2.68E-02
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 3	01.05.11 aromate metabolism	2.62E-02
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 4	01.05.11.07 aromate catabolism	3.49E-02
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 5	01.05.11.07.01 aerobic aromate catabolism	1.51E-02
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 2	01.06 lipid fatty acid and isoprenoid metabolism	2.10E-02
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 2	01.20 secondary metabolism	5.93E-12
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 3	01.20.01 metabolism of primary metabolic sugar derivatives	1.90E-02

NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 4	01.20.01.09 metabolism of aminoglycoside antibiotics	3.93E-02
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 3	01.20.05 metabolism of acetic acid derivatives	1.08E-02
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 3	01.20.17 metabolism of secondary products derived from primary amino acids	1.85E-04
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 4	01.20.17.09 metabolism of alkaloids	1.98E-04
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 3	01.20.29 metabolism of secondary products derived from L glutamic acid L proline and L ornithine	1.56E-02
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 2	02.16 fermentation	9.04E-05
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 3	02.16.01 alcohol fermentation	1.42E-02
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 3	02.16.03 lactate fermentation	1.75E-02
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 4	02.16.03.03 heterofermentative pathway and fermentaton of other saccharides	3.93E-02
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 2	16.21 complex cofactor cosubstrate vitamine binding	1.66E-03
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 3	16.21.07 NAD NADP binding	4.75E-05
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 6	20.01.01.01.01.01 siderophore iron transport	7.13E-04
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 3	20.01.03 C compound and carbohydrate transport	2.25E-03
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 3	20.01.23 allantoin and allantoate transport	5.20E-03
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 3	20.01.25 vitamine cofactor transport	1.08E-02
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 3	20.09.18 cellular import	1.42E-02
NcA1 vs. old-	Before vs. After	UP	LEVEL 4	20.09.18.07 non	1.88E-04

PcW				vesicular cellular import	
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 3	32.01.01 oxidative stress response	4.05E-02
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 3	32.01.04 pH stress response	1.56E-02
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 2	32.05 disease virulence and defense	1.92E-03
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 3	32.05.03 defense related proteins	1.27E-03
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 3	32.05.05 virulence disease factors	3.93E-02
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 2	32.07 detoxification	2.62E-02
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 3	32.07.03 detoxification by modification	1.42E-02
NcA1 vs. young-PcW NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 2	01.05 C compound and carbohydrate metabolism	4.96E-02
NcA1 vs. young-PcW NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 3	01.05.03 polysaccharide metabolism	1.29E-02
NcA1 vs. young-PcW NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 3	01.25.03 extracellular protein degradation	2.71E-02
NcA1 vs. young-PcW NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 3	20.03.25 ABC transporters	3.29E-02
NcA1 vs. old-PcW NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 3	02.16.01 alcohol fermentation	4.35E-02
NcA1 vs. old-PcW	Before vs. After	DOWN	LEVEL 1	01 METABOLISM	0.010550597
NcA1 vs. old-PcW	Before vs. After	DOWN	LEVEL 4	01.02.02.09 catabolism of nitrogenous compounds	0.040062129
NcA1 vs. old-PcW	Before vs. After	DOWN	LEVEL 3	01.06.06 isoprenoid metabolism	0.048901092
NcA1 vs. old-PcW	Before vs. After	DOWN	LEVEL 4	01.06.06.13 tetraterpenes carotinoids metabolism	0.00336843
NcA1 vs. old-	Before vs. After	DOWN	LEVEL 1	02 ENERGY	0.0263635

PcW					88
NcA1 vs. old-PcW	Before vs. After	DOWN	LEVEL 2	02.10 tricarboxylic acid pathway citrate cycle Krebs cycle TCA cycle	0.024636662
NcA1 vs. old-PcW	Before vs. After	DOWN	LEVEL 2	02.11 electron transport and membrane associated energy conservation	0.024636662
NcA1 vs. old-PcW	Before vs. After	DOWN	LEVEL 3	02.11.05 accessory proteins of electron transport and membrane associated energy conservation	0.005760009
NcA1 vs. old-PcW	Before vs. After	DOWN	LEVEL 3	16.21.05 FAD FMN binding	0.020447749
NcA1 vs. old-PcW	Before vs. After	DOWN	LEVEL 3	16.21.08 Fe S binding	0.004568
NcA1 vs. old-PcW	Before vs. After	DOWN	LEVEL 3	20.01.15 electron transport	0.021563396
NcA1 vs. old-PcW	Before vs. After	DOWN	LEVEL 3	32.07.01 detoxification involving cytochrome P450	0.048901092
NcA1 vs. old-PcW NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	DOWN	LEVEL 2	32.05 disease virulence and defense	0.005599325
NcA1 vs. NcA1 NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Alone vs. After	DOWN	LEVEL 4	32.05.01.03 chemical agent resistance	1.09E-02

Supplemental Table 2. Genes shared and unique to NcA1 interacting with young-PcW, old-PcW, and all *Neurospor*a (NcA1, NcA2, NcC, Nd).

[1] Interaction with unique genes or interactions with shared genes.

[2] Comparisons observed: alone versus before, before versus after, alone versus after.

[3] Gene regulation being observed: upregulated or downregulated genes

[4] FunCat hierarchy level. The higher the level the more specific the FunCat category, e.g. “01 - Metabolism” is level one and “01.01.11.04.02 degradation of leucine” is level 5.

[5] FunCat term significantly enriched for genes shared between interactions or unique to interactions.

[6] Adjusted p-value of FunCat enrichment, the smaller the adjusted p-value the more significant.

[1] Interaction with unique genes or interactions with shared genes.

[2] Comparisons observed: alone versus before, before versus after, alone versus after.
[3] Gene regulation being observed: upregulated or downregulated genes
[4] FunCat hierarchy level. The higher the level the more specific the FunCat category, e.g. “01 - Metabolism” is level one and “01.01.11.04.02 degradation of leucine” is level 5.
[5] FunCat term significantly enriched for genes shared between interactions or unique to interactions.
[6] Adjusted p-value of FunCat enrichment, the smaller the adjusted p-value the more significant.

Interactions[1]	Comparison[2]	Genes Up/Down Regulated[3]	FunCat Level[4]	FunCat Term[5]	P-Value[6]
NcA1 vs. old-PcW	Alone vs. Before	UP	LEVEL 1	01 METABOLISM	8.53E-04
NcA1 vs. old-PcW	Alone vs. Before	UP	LEVEL 3	01.01.11 metabolism of the pyruvate family alanine isoleucine leucine valine and D alanine	3.19E-02
NcA1 vs. old-PcW	Alone vs. Before	UP	LEVEL 4	01.01.11.02 metabolism of isoleucine	2.79E-02
NcA1 vs. old-PcW	Alone vs. Before	UP	LEVEL 5	01.01.11.02.01 biosynthesis of isoleucine	1.02E-02
NcA1 vs. old-PcW	Alone vs. Before	UP	LEVEL 4	01.01.11.03 metabolism of valine	2.79E-02
NcA1 vs. old-PcW	Alone vs. Before	UP	LEVEL 5	01.01.11.03.01 biosynthesis of valine	1.02E-02
NcA1 vs. old-PcW	Alone vs. Before	UP	LEVEL 4	01.01.11.04 metabolism of leucine	5.22E-04
NcA1 vs. old-PcW	Alone vs. Before	UP	LEVEL 5	01.01.11.04.01 biosynthesis of leucine	3.20E-05
NcA1 vs. old-PcW	Alone vs. Before	UP	LEVEL 5	01.01.11.04.02 degradation of leucine	1.02E-02
NcA1 vs. old-PcW	Alone vs. Before	UP	LEVEL 2	01.02 nitrogen sulfur and selenium metabolism	2.42E-02
NcA1 vs. old-PcW	Alone vs. Before	UP	LEVEL 3	01.02.02 nitrogen metabolism	4.21E-02
NcA1 vs. old-PcW	Alone vs. Before	UP	LEVEL 4	01.06.06.13 tetraterpenes carotinoids metabolism	1.50E-03
NcA1 vs. old-PcW	Alone vs. Before	UP	LEVEL 4	20.09.18.07 non vesicular cellular import	2.79E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 1	01 METABOLISM	1.36E-11
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 5	01.01.03.02.02 degradation of glutamate	5.85E-04
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 5	01.01.05.01.02 degradation of polyamines	1.16E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 5	01.01.06.04.02 degradation of threonine	2.53E-02
NcA1 vs. old-	Alone vs.	DOWN	LEVEL 3	01.01.09 metabolism of	8.98E-03

PcW	Before			the cysteine aromatic group	
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 5	01.01.09.02.02 degradation of serine	3.49E-03
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 4	01.01.09.04 metabolism of phenylalanine	1.74E-03
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 5	01.01.09.04.01 biosynthesis of phenylalanine	3.49E-03
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 5	01.01.09.04.02 degradation of phenylalanine	2.53E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 4	01.01.09.05 metabolism of tyrosine	1.84E-03
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 2	01.05 C compound and carbohydrate metabolism	3.52E-12
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	01.05.02 sugar glucoside polyol and carboxylate metabolism	2.68E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	01.05.11 aromate metabolism	2.62E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 4	01.05.11.07 aromate catabolism	3.49E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 5	01.05.11.07.01 aerobic aromate catabolism	1.51E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 2	01.06 lipid fatty acid and isoprenoid metabolism	2.10E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 2	01.20 secondary metabolism	5.93E-12
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	01.20.01 metabolism of primary metabolic sugar derivatives	1.90E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 4	01.20.01.09 metabolism of aminoglycoside antibiotics	3.93E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	01.20.05 metabolism of acetic acid derivatives	1.08E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	01.20.17 metabolism of secondary products derived from primary amino acids	1.85E-04
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 4	01.20.17.09 metabolism of alkaloids	1.98E-04
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	01.20.29 metabolism of secondary products derived from L glutamic acid L proline and L ornithine	1.56E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 2	02.16 fermentation	9.04E-05
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	02.16.01 alcohol fermentation	1.42E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	02.16.03 lactate fermentation	1.75E-02

NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 4	02.16.03.03 heterofermentative pathway and fermentaton of other saccharides	3.93E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 2	16.21 complex cofactor cosubstrate vitamine binding	1.66E-03
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	16.21.07 NAD NADP binding	4.75E-05
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 6	20.01.01.01.01.01 siderophore iron transport	7.13E-04
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	20.01.03 C compound and carbohydrate transport	2.25E-03
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	20.01.23 allantoin and allantoate transport	5.20E-03
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	20.01.25 vitamine cofactor transport	1.08E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	20.09.18 cellular import	1.42E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 4	20.09.18.07 non vesicular cellular import	1.88E-04
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	32.01.01 oxidative stress response	4.05E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	32.01.04 pH stress response	1.56E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 2	32.05 disease virulence and defense	1.92E-03
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	32.05.03 defense related proteins	1.27E-03
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	32.05.05 virulence disease factors	3.93E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 2	32.07 detoxification	2.62E-02
NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	32.07.03 detoxification by modification	1.42E-02
NcA1 vs. young-PcW NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 2	01.05 C compound and carbohydrate metabolism	4.96E-02
NcA1 vs. young-PcW NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	01.05.03 polysaccharide metabolism	1.29E-02
NcA1 vs. young-PcW NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	01.25.03 extracellular protein degradation	2.71E-02
NcA1 vs. young-PcW NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	20.03.25 ABC transporters	3.29E-02

NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd NcA1 vs. old-PcW	Alone vs. Before	DOWN	LEVEL 3	02.16.01 alcohol fermentation	4.35E-02
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 1	01 METABOLISM	4.34E-04
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 2	01.05 C compound and carbohydrate metabolism	1.86E-07
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 2	01.20 secondary metabolism	1.08E-04
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 2	20.03 transport facilities	8.79E-03
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 2	32.05 disease virulence and defense	2.03E-04
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 3	01.05.03 polysaccharide metabolism	4.32E-02
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 3	01.20.01 metabolism of primary metabolic sugar derivatives	1.04E-02
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 3	16.21.07 NAD NADP binding	4.32E-02
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 3	20.01.03 C compound and carbohydrate transport	9.07E-06
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 3	20.01.23 allantoin and allantoate transport	2.07E-02
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 3	20.01.25 vitamine cofactor transport	3.87E-02
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 3	20.09.18 cellular import	2.25E-05
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 3	32.01.04 pH stress response	1.10E-02
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 3	32.05.05 virulence disease factors	1.40E-02
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 4	20.01.03.01 sugar transport	1.27E-02
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 4	20.09.18.07 non vesicular cellular import	7.60E-08
NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 6	20.01.01.01.01.01 siderophore iron transport	1.34E-04
NcA1 vs. young-PcW NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 2	01.05 C compound and carbohydrate metabolism	2.84E-03
NcA1 vs. young-PcW NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 3	01.25.03 extracellular protein degradation	1.92E-02
NcA1 vs. young-PcW NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 2	02.16 fermentation	4.00E-05

NcA1 vs. young-PcW NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 3	02.16.01 alcohol fermentation	9.02E-03
NcA1 vs. young-PcW NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 3	02.16.03 lactate fermentation	4.29E-02
NcA1 vs. young-PcW NcA1 vs. old-PcW	Before vs. After	UP	LEVEL 3	16.17.09 heavy metal binding Cu Fe Zn	4.29E-02
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 2	01.07 metabolism of vitamins cofactors and prosthetic groups	1.82E-02
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 3	32.01.01 oxidative stress response	6.70E-03
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 3	32.07.07 oxygen and radical detoxification	3.94E-03
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 3	34.11.03 chemoperception and response	4.67E-02
NcA1 vs. old-PcW NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 2	01.05 C compound and carbohydrate metabolism	2.85E-02
NcA1 vs. old-PcW NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 3	01.05.06 C 2 compound and organic acid metabolism	5.94E-04
NcA1 vs. old-PcW NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 4	01.05.06.07 C 2 compound and organic acid catabolism	5.38E-04
NcA1 vs. old-PcW NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 2	02.13 respiration	2.85E-02
NcA1 vs. old-PcW NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 2	02.45 energy conversion and regeneration	8.45E-03

NcA1 vs. old-PcW NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 2	32.05 disease virulence and defense	8.45E-03
NcA1 vs. young-PcW NcA1 vs. old-PcW NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 2	01.01 amino acid metabolism	7.24E-04
NcA1 vs. young-PcW NcA1 vs. old-PcW NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 3	01.01.05 metabolism of urea cycle creatine and polyamines	2.52E-02
NcA1 vs. young-PcW NcA1 vs. old-PcW NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 3	01.01.09 metabolism of the cysteine aromatic group	2.52E-02
NcA1 vs. young-PcW NcA1 vs. old-PcW NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 4	01.01.09.04 metabolism of phenylalanine	8.19E-03
NcA1 vs. young-PcW NcA1 vs. old-PcW NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 4	01.01.09.05 metabolism of tyrosine	4.82E-04
NcA1 vs. young-PcW NcA1 vs. old-PcW NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 2	01.20 secondary metabolism	7.99E-04
NcA1 vs. young-PcW NcA1 vs. old-PcW NcA1 vs. NcA2 NcA1 vs. NcC	Before vs. After	UP	LEVEL 3	01.20.17 metabolism of secondary products derived from primary amino acids	2.52E-02

NcA1 vs. Nd					
NcA1 vs. young-PcW NcA1 vs. old-PcW NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 2	02.16 fermentation	5.78E-03
NcA1 vs. young-PcW NcA1 vs. old-PcW NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	UP	LEVEL 3	20.03.25 ABC transporters	2.52E-02
NcA1 vs. old-PcW	Before vs. After	Down	LEVEL 1	01 METABOLISM	1.06E-02
NcA1 vs. old-PcW	Before vs. After	Down	LEVEL 4	01.02.02.09 catabolism of nitrogenous compounds	4.01E-02
NcA1 vs. old-PcW	Before vs. After	Down	LEVEL 3	01.06.06 isoprenoid metabolism	4.89E-02
NcA1 vs. old-PcW	Before vs. After	Down	LEVEL 4	01.06.06.13 tetraterpenes carotinoids metabolism	3.37E-03
NcA1 vs. old-PcW	Before vs. After	Down	LEVEL 1	02 ENERGY	2.64E-02
NcA1 vs. old-PcW	Before vs. After	Down	LEVEL 2	02.10 tricarboxylic acid pathway citrate cycle Krebs cycle TCA cycle	2.46E-02
NcA1 vs. old-PcW	Before vs. After	Down	LEVEL 2	02.11 electron transport and membrane associated energy conservation	2.46E-02
NcA1 vs. old-PcW	Before vs. After	Down	LEVEL 3	02.11.05 accessory proteins of electron transport and membrane associated energy conservation	5.76E-03
NcA1 vs. old-PcW	Before vs. After	Down	LEVEL 3	16.21.05 FAD FMN binding	2.04E-02
NcA1 vs. old-PcW	Before vs. After	Down	LEVEL 3	16.21.08 Fe S binding	4.57E-03
NcA1 vs. old-PcW	Before vs. After	Down	LEVEL 3	20.01.15 electron transport	2.16E-02
NcA1 vs. old-PcW	Before vs. After	Down	LEVEL 3	32.07.01 detoxification involving cytochrome P450	4.89E-02

NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	DOWN	LEVEL 5	20.09.16.09.03 exocytosis	4.53E-02
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	Before vs. After	DOWN	LEVEL 4	30.01.09.11 polyphosphoinositol mediated signal transduction	2.61E-02

Supplemental Table 3. Genes shared and unique to NcA1 interacting with young-PcW, old-PcW, and all *Neurospor*a (NcA2, NcC, Nd).

[1] Interaction with unique genes or interactions with shared genes.

[2] Comparisons observed: alone versus before, before versus after, alone versus after.

[3] Gene regulation being observed: upregulated or downregulated genes

[4] FunCat hierarchy level. The higher the level the more specific the FunCat category, e.g. “01 - Metabolism” is level one and “01.01.11.04.02 degradation of leucine” is level 5.

[5] FunCat term significantly enriched for genes shared between interactions or unique to interactions.

[6] Adjusted p-value of FunCat enrichment, the smaller the adjusted p-value the more significant.

[1] Interaction with unique genes or interactions with shared genes.

[2] Comparisons observed: alone versus before, before versus after, alone versus after.

[3] Gene regulation being observed: upregulated or downregulated genes

[4] FunCat hierarchy level. The higher the level the more specific the FunCat category, e.g. “01 - Metabolism” is level one and “01.01.11.04.02 degradation of leucine” is level 5.

[5] FunCat term significantly enriched for genes shared between interactions or unique to interactions.

[6] Adjusted p-value of FunCat enrichment, the smaller the adjusted p-value the more significant.

Interaction[1]	Expression Pattern[2]	FunCat Level[3]	FunCat term[4]
NcA1 vs. young-PcW	DNN	LEVEL 3	01.05.03 polysaccharide metabolism
NcA1 vs. young-PcW	DNN	LEVEL 3	01.05.06 C 2 compound and organic acid metabolism
NcA1 vs. young-PcW	DNN	LEVEL 4	01.05.06.07 C 2 compound and organic acid catabolism
NcA1 vs. young-PcW	DNN	LEVEL 3	20.01.03 C compound and carbohydrate transport
NcA1 vs. young-PcW	DNN	LEVEL 3	20.01.27 drug toxin transport
NcA1 vs. young-PcW	DNN	LEVEL 3	20.03.25 ABC transporters
NcA1 vs. young-PcW	DUN	LEVEL 3	01.25.03 extracellular protein degradation

NcA1 vs. young-PcW	NNN	LEVEL 3	01.01.03 assimilation of ammonia metabolism of the glutamate group
NcA1 vs. young-PcW	NNN	LEVEL 5	01.01.03.02.02 degradation of glutamate
NcA1 vs. young-PcW	NNN	LEVEL 3	01.05.02 sugar glucoside polyol and carboxylate metabolism
NcA1 vs. young-PcW	NNN	LEVEL 2	01.20 secondary metabolism
NcA1 vs. young-PcW	NNN	LEVEL 2	02.01 glycolysis and gluconeogenesis
NcA1 vs. young-PcW	NNN	LEVEL 3	20.01.03 C compound and carbohydrate transport
NcA1 vs. young-PcW	NNN	LEVEL 3	20.01.13 lipid fatty acid transport
NcA1 vs. young-PcW	NNN	LEVEL 3	20.01.23 allantoin and allantoate transport
NcA1 vs. young-PcW	NNN	LEVEL 3	20.09.18 cellular import
NcA1 vs. young-PcW	NNN	LEVEL 4	20.09.18.07 non vesicular cellular import
NcA1 vs. young-PcW	NNN	LEVEL 3	32.01.01 oxidative stress response
NcA1 vs. young-PcW	NNN	LEVEL 2	32.05 disease virulence and defense
NcA1 vs. young-PcW	NNN	LEVEL 3	32.05.03 defense related proteins
NcA1 vs. young-PcW	NNN	LEVEL 3	32.05.05 virulence disease factors
NcA1 vs. young-PcW	NNN	LEVEL 2	32.07 detoxification
NcA1 vs. young-PcW	NUN	LEVEL 1	01 METABOLISM
NcA1 vs. young-PcW	NUN	LEVEL 2	01.01 amino acid metabolism
NcA1 vs. young-PcW	NUN	LEVEL 4	01.01.03.03 metabolism of proline
NcA1 vs. young-PcW	NUN	LEVEL 5	01.01.03.03.01 biosynthesis of proline
NcA1 vs. young-PcW	NUN	LEVEL 3	01.01.05 metabolism of urea cycle creatine and polyamines
NcA1 vs. young-PcW	NUN	LEVEL 4	01.01.05.01 metabolism of polyamines
NcA1 vs. young-PcW	NUN	LEVEL 3	01.01.09 metabolism of the cysteine aromatic group
NcA1 vs. young-PcW	NUN	LEVEL 4	01.01.09.04 metabolism of phenylalanine
NcA1 vs. young-PcW	NUN	LEVEL 4	01.01.09.05 metabolism of tyrosine
NcA1 vs. young-PcW	NUN	LEVEL 3	01.02.07 regulation of nitrogen sulfur and selenium metabolism
NcA1 vs. young-PcW	NUN	LEVEL 2	01.05 C compound and carbohydrate metabolism

NcA1 vs. young-PcW	NUN	LEVEL 3	01.05.06 C 2 compound and organic acid metabolism
NcA1 vs. young-PcW	NUN	LEVEL 4	01.05.06.07 C 2 compound and organic acid catabolism
NcA1 vs. young-PcW	NUN	LEVEL 3	01.05.11 aromate metabolism
NcA1 vs. young-PcW	NUN	LEVEL 4	01.05.11.07 aromate catabolism
NcA1 vs. young-PcW	NUN	LEVEL 5	01.05.11.07.01 aerobic aromate catabolism
NcA1 vs. young-PcW	NUN	LEVEL 2	01.20 secondary metabolism
NcA1 vs. young-PcW	NUN	LEVEL 3	01.20.17 metabolism of secondary products derived from primary amino acids
NcA1 vs. young-PcW	NUN	LEVEL 4	01.20.17.09 metabolism of alkaloids
NcA1 vs. young-PcW	NUN	LEVEL 1	02 ENERGY
NcA1 vs. young-PcW	NUN	LEVEL 2	02.13 respiration
NcA1 vs. young-PcW	NUN	LEVEL 3	02.13.03 aerobic respiration
NcA1 vs. young-PcW	NUN	LEVEL 2	02.16 fermentation
NcA1 vs. young-PcW	NUN	LEVEL 3	02.16.01 alcohol fermentation
NcA1 vs. young-PcW	NUN	LEVEL 3	02.16.03 lactate fermentation
NcA1 vs. young-PcW	NUN	LEVEL 2	16.17 metal binding
NcA1 vs. young-PcW	NUN	LEVEL 3	16.17.03 potassium binding
NcA1 vs. young-PcW	NUN	LEVEL 3	16.21.07 NAD NADP binding
NcA1 vs. young-PcW	NUN	LEVEL 3	20.03.01 channel pore class transport
NcA1 vs. young-PcW	NUN	LEVEL 4	20.03.01.01 ion channels
NcA1 vs. young-PcW	NUN	LEVEL 3	32.07.07 oxygen and radical detoxification
NcA1 vs. old-PcW	DNN	LEVEL 1	01 METABOLISM
NcA1 vs. old-PcW	DNN	LEVEL 2	01.06 lipid fatty acid and isoprenoid metabolism
NcA1 vs. old-PcW	DNN	LEVEL 4	01.06.06.05 sesquiterpenes metabolism
NcA1 vs. old-PcW	DNN	LEVEL 4	01.20.17.09 metabolism of alkaloids
NcA1 vs. old-PcW	DUN	LEVEL 2	01.01 amino acid metabolism
NcA1 vs. old-PcW	DUN	LEVEL 5	01.01.03.02.02 degradation of glutamate

NcA1 vs. old-PcW	DUN	LEVEL 3	01.01.05 metabolism of urea cycle creatine and polyamines
NcA1 vs. old-PcW	DUN	LEVEL 4	01.01.05.01 metabolism of polyamines
NcA1 vs. old-PcW	DUN	LEVEL 5	01.01.05.01.02 degradation of polyamines
NcA1 vs. old-PcW	DUN	LEVEL 6	01.01.06.06.01.01 diaminopimelic acid pathway
NcA1 vs. old-PcW	DUN	LEVEL 3	01.01.09 metabolism of the cysteine aromatic group
NcA1 vs. old-PcW	DUN	LEVEL 5	01.01.09.04.01 biosynthesis of phenylalanine
NcA1 vs. old-PcW	DUN	LEVEL 3	01.05.02 sugar glucoside polyol and carboxylate metabolism
NcA1 vs. old-PcW	DUN	LEVEL 3	01.05.03 polysaccharide metabolism
NcA1 vs. old-PcW	DUN	LEVEL 3	01.05.06 C 2 compound and organic acid metabolism
NcA1 vs. old-PcW	DUN	LEVEL 4	01.05.06.07 C 2 compound and organic acid catabolism
NcA1 vs. old-PcW	DUN	LEVEL 3	01.20.01 metabolism of primary metabolic sugar derivatives
NcA1 vs. old-PcW	DUN	LEVEL 4	01.20.01.09 metabolism of aminoglycoside antibiotics
NcA1 vs. old-PcW	DUN	LEVEL 3	01.20.05 metabolism of acetic acid derivatives
NcA1 vs. old-PcW	DUN	LEVEL 4	01.20.17.09 metabolism of alkaloids
NcA1 vs. old-PcW	DUN	LEVEL 3	01.20.29 metabolism of secondary products derived from L glutamic acid L proline and L ornithine
NcA1 vs. old-PcW	DUN	LEVEL 3	01.20.35 metabolism of secondary products derived from L phenylalanine and L tyrosine
NcA1 vs. old-PcW	DUN	LEVEL 4	01.20.35.01 metabolism of phenylpropanoids
NcA1 vs. old-PcW	DUN	LEVEL 3	02.16.03 lactate fermentation
NcA1 vs. old-PcW	DUN	LEVEL 4	02.16.03.03 heterofermentative pathway and fermentaton of other saccharides
NcA1 vs. old-PcW	DUN	LEVEL 2	16.21 complex cofactor cosubstrate vitamine binding
NcA1 vs. old-PcW	DUN	LEVEL 3	16.21.07 NAD NADP binding
NcA1 vs. old-PcW	DUN	LEVEL 2	20.01 transported compounds substrates
NcA1 vs. old-PcW	DUN	LEVEL 6	20.01.01.01.01.01 siderophore iron transport
NcA1 vs. old-PcW	DUN	LEVEL 3	20.01.03 C compound and carbohydrate transport
NcA1 vs. old-PcW	DUN	LEVEL 3	20.01.09 peptide transport
NcA1 vs. old-PcW	DUN	LEVEL 3	20.01.23 allantoin and allantoate transport

NcA1 vs. old-PcW	DUN	LEVEL 3	20.01.25 vitamine cofactor transport
NcA1 vs. old-PcW	DUN	LEVEL 2	20.03 transport facilities
NcA1 vs. old-PcW	DUN	LEVEL 3	20.09.18 cellular import
NcA1 vs. old-PcW	DUN	LEVEL 4	20.09.18.07 non vesicular cellular import
NcA1 vs. old-PcW	DUN	LEVEL 3	32.01.04 pH stress response
NcA1 vs. old-PcW	DUN	LEVEL 2	32.05 disease virulence and defense
NcA1 vs. old-PcW	DUN	LEVEL 3	32.05.01 resistance proteins
NcA1 vs. old-PcW	DUN	LEVEL 3	32.05.03 defense related proteins
NcA1 vs. old-PcW	DUN	LEVEL 3	32.07.03 detoxification by modification
NcA1 vs. old-PcW	NDN	LEVEL 4	01.05.02.04 sugar glucoside polyol and carboxylate anabolism
NcA1 vs. old-PcW	NDN	LEVEL 4	01.05.02.07 sugar glucoside polyol and carboxylate catabolism
NcA1 vs. old-PcW	NDN	LEVEL 3	01.05.06 C 2 compound and organic acid metabolism
NcA1 vs. old-PcW	NDN	LEVEL 4	01.05.06.07 C 2 compound and organic acid catabolism
NcA1 vs. old-PcW	NDN	LEVEL 1	02 ENERGY
NcA1 vs. old-PcW	NDN	LEVEL 2	02.10 tricarboxylic acid pathway citrate cycle Krebs cycle TCA cycle
NcA1 vs. old-PcW	NDN	LEVEL 2	02.11 electron transport and membrane associated energy conservation
NcA1 vs. old-PcW	NDN	LEVEL 3	02.11.05 accessory proteins of electron transport and membrane associated energy conservation
NcA1 vs. old-PcW	NDN	LEVEL 2	02.13 respiration
NcA1 vs. old-PcW	NDN	LEVEL 3	02.13.03 aerobic respiration
NcA1 vs. old-PcW	NDN	LEVEL 3	16.21.05 FAD FMN binding
NcA1 vs. old-PcW	NDN	LEVEL 3	16.21.08 Fe S binding
NcA1 vs. old-PcW	NDN	LEVEL 2	20.01 transported compounds substrates
NcA1 vs. old-PcW	NDN	LEVEL 3	20.01.15 electron transport
NcA1 vs. old-PcW	NDN	LEVEL 2	42.16 mitochondrion
NcA1 vs. old-PcW	NNN	LEVEL 6	01.05.11.07.01.03 meta cleavage
NcA1 vs. old-PcW	NNN	LEVEL 2	02.25 oxidation of fatty acids

NcA1 vs. old-PcW	NNN	LEVEL 1	30 CELLULAR COMMUNICATION SIGNAL TRANSDUCTION MECHANISM
NcA1 vs. old-PcW	NNN	LEVEL 1	40 CELL FATE
NcA1 vs. old-PcW	NNN	LEVEL 2	40.01 cell growth morphogenesis
NcA1 vs. old-PcW	NNN	LEVEL 2	42.01 cell wall
NcA1 vs. old-PcW	UDN	LEVEL 1	01 METABOLISM
NcA1 vs. old-PcW	UDN	LEVEL 4	01.06.06.13 tetraterpenes carotinoids metabolism
NcA1 vs. old-PcW	UNN	LEVEL 3	01.01.11 metabolism of the pyruvate family alanine isoleucine leucine valine and D alanine
NcA1 vs. old-PcW	UNN	LEVEL 4	01.01.11.04 metabolism of leucine
NcA1 vs. old-PcW	UNN	LEVEL 5	01.01.11.04.01 biosynthesis of leucine
NcA1 vs. old-PcW	UNN	LEVEL 4	20.09.18.07 non vesicular cellular import
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	DUN	LEVEL 3	01.06.06 isoprenoid metabolism
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	DUN	LEVEL 3	32.01.01 oxidative stress response
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	DUN	LEVEL 3	32.07.07 oxygen and radical detoxification
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	NDN	LEVEL 4	20.09.18.09 vesicular cellular import
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	NDN	LEVEL 5	20.09.18.09.01 endocytosis
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	NDN	LEVEL 5	30.01.05.05.01 small GTPase mediated signal transduction
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	NDN	LEVEL 4	43.01.03.05 budding cell polarity and filament formation
NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	UNN	LEVEL 3	11.02.01 rRNA synthesis
NcA1 vs. young-PcW NcA1 vs. old-PcW	DNN	LEVEL 2	01.05 C compound and carbohydrate metabolism
NcA1 vs. young-PcW NcA1 vs. old-	NNN	LEVEL 1	01 METABOLISM

PcW			
NcA1 vs. young-PcW NcA1 vs. old-PcW	NNN	LEVEL 2	01.05 C compound and carbohydrate metabolism
NcA1 vs. young-PcW NcA1 vs. old-PcW	NNN	LEVEL 3	01.05.03 polysaccharide metabolism
NcA1 vs. young-PcW NcA1 vs. old-PcW	NNN	LEVEL 2	01.06 lipid fatty acid and isoprenoid metabolism
NcA1 vs. young-PcW NcA1 vs. old-PcW	NNN	LEVEL 3	32.01.03 osmotic and salt stress response
NcA1 vs. old-PcW NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	DUN	LEVEL 1	01 METABOLISM
NcA1 vs. old-PcW NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	DUN	LEVEL 4	01.01.09.04 metabolism of phenylalanine
NcA1 vs. old-PcW NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	DUN	LEVEL 4	01.01.09.05 metabolism of tyrosine
NcA1 vs. old-PcW NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	DUN	LEVEL 2	01.05 C compound and carbohydrate metabolism
NcA1 vs. old-PcW NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	DUN	LEVEL 2	01.20 secondary metabolism
NcA1 vs. old-PcW NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	DUN	LEVEL 3	01.20.17 metabolism of secondary products derived from primary amino acids
NcA1 vs. old-PcW NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	DUN	LEVEL 2	02.16 fermentation

NcA1 vs. old-PcW NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	DUN	LEVEL 3	02.16.01 alcohol fermentation
NcA1 vs. old-PcW NcA1 vs. NcA2 NcA1 vs. NcC NcA1 vs. Nd	DUN	LEVEL 2	32.07 detoxification

Supplemental Table 4. FunCat functional enrichment categories shared and unique to expression patterns found in NcA1 interacting with young-PcW, old-PcW, and nonself *Neurospora* (NcA2, NcC, Nd).

[1] Interaction with unique genes or interactions with shared expression patterns and enriched FunCat terms.

[2] Expression pattern shared by interaction/s.

[3] FunCat hierarchy level. The higher the level the more specific the FunCat category, e.g. “01 - Metabolism” is level one and “01.01.09.04 metabolism of phenylalanine” is level 4.

[4] FunCat term significantly enriched (adjusted p-value<0.05) for genes shared between interactions or unique to interactions.